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Quinolone resistance acquisition and impact on virulence in *Salmonella enterica*: a cost-benefit matter

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"Quinolone resistance acquisition and impact on virulence in *Salmonella enterica*: a cost-benefit matter"

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Presentada per Clara Celia Ballesté Delpierre.

Sota la direcció del Dr. Jordi Vila Estapé i de la Dra. Anna Fàbrega Santamaria.

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CERTIFIQUEN:

Que el treball de recerca titulat "Quinolone resistance acquisition and impact on virulence in *Salmonella enterica*: a cost-benefit matter", presentat per Clara Celia Ballesté Delpierre, ha estat realitzat en el Laboratori de Microbiologia de l'Hospital Clínic de Barcelona, sota la seva direcció i compleix tots els requisits necessaris per la seva tramitació i posterior defensa davant del Tribunal corresponent.

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INTRODUCTION

I. INTRODUCTION

As suggested by the presence of bacteria in the organisms of annelids (earthworms) animals and bacteria have co-evolved for more than 800 million years [1]. In our case, the human body is inhabited by a diversity of microorganisms, representing a proportion of ten times more bacteria than human cells and constituting around 2% of the body mass. The distribution of bacterial species throughout the body is site-specific and is the result of the selection of co-evolving organisms due to the great genetic plasticity of these microorganisms. Bacteria have become specialized to survive and adapt to specific environmental conditions. Genetic modifications have driven changes in their biochemical activities leading to a particular physiological differentiation. These changes include gene gain, loss and acquisition of mutations that modify the biochemical properties of the translated proteins [2].

The study of the microbial community present in our organism, the so-called microbiota, and its complete genetic content, the microbiome, is currently a focus of intensive research due to its importance in relation to human health. A relevant example is the Human Microbiome Project (HMP), initiated in 2007. This five-year consortium of researchers led by the National Institutes of Health of different countries has been centered on mapping the microbial composition of healthy individuals through DNA sequencing of bacterial samples recovered from different sites of 242 healthy volunteers (mouth, nose, skin, lower intestine and vagina in women). The data obtained from this project has had outstanding relevance in characterizing the human microbiota and understanding its implications in human health [3].

As mentioned above, the human body is a huge reservoir of bacteria (**Figure 1**), being the gastrointestinal tract the site where the highest number of aerobic and anaerobic bacteria coexist and compose the intestinal microbiota, mainly colonizing the distal ileum and colon. Interaction between the gut and commensal bacteria is characterized by mutual benefits: the nutrient-rich environment of the gut together with a mild temperature constitutes optimal conditions for the microbiota while, in turn, humans obtain increased digestive capacity from commensal bacteria. These microorganisms provide essential nutrients, such

as vitamins, and energy from polysaccharides that humans are unable to digest due to the limited number of genes encoding for enzymes with a complex carbohydrate-metabolizing function [4–6].

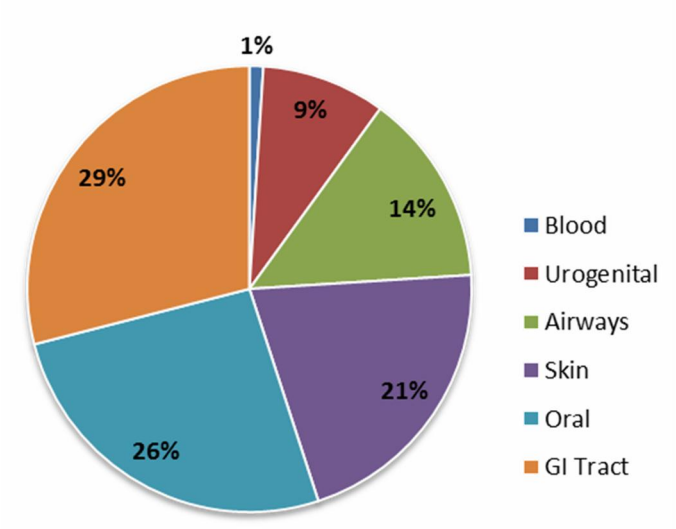

Figure 1. Bacterial distribution by body site. Adapted from [3].

The maintenance of intestinal homeostasis between commensal bacteria and the gut is achieved thanks to the innate mucosal immunity. Most of the bacteria that colonize the intestine have diminished virulence whereas pathogenic strains have exclusive virulence factors. These factors, named pathogen-associated molecular patterns (PAMPs), allow the attachment to and invasion of host cells through the recognition by specific receptors, termed pattern recognition receptors (PRRs). These receptors are present in the antigen-presenting cells such as macrophages and dendritic cells as well as in intestinal epithelial cells. Commensal bacteria also express PAMPs, although in a lesser proportion than pathogenic strains, and only interact with accessible PRRs in the apical membrane of epithelial cells. This results in a limited activation or even inhibition of the host immune response, as is the case of *Bacteroides* and *Lactobacillus* spp. In addition to these strategies, tolerance of the microbiota is also achieved due to the release of immunomodulatory cytokines by specific dendritic cells promoted by intestinal epithelial cells into the lamina propria, the component of the mucosa located beneath the intestinal epithelium [5]. In the case of virulent bacteria, the interaction between PAMPs and PRRs triggers the initiation of an innate immune response by inducing the expression of cytokines, the production of

antimicrobial peptides, phagocytic microbial killing and reactive oxygen species. Consequently, recruitment of acute inflammatory cells takes place to establish localized inflammation [7–9].

The equilibrium between the gut and the microbiota is sometimes disrupted when external pathogenic microorganisms reach the intestinal tract causing gastroenteritis (also called diarrheal disease). The major symptoms of gastroenteritis include diarrhea, nausea, vomiting, fever, and abdominal pain. Host-related factors such as age, commensal bacteria composition and nutritional status play important roles in disease burden, being children and elderly populations the most vulnerable groups [10]. According to the World Health Organization (WHO) over 1.7 billion cases of diarrheal disease are reported annually worldwide with an estimated 2.2 million deaths, hence being considered the second leading cause of death in children under five years old in developing countries [11].

Enteric bacteria account for 10-20% of the cases of gastroenteritis after viruses (70%) and followed by helminthes (<10%). Bacterial infections usually arise upon ingestion of contaminated food or water and by person-to-person transmission as a result of poor hygiene. Once bacteria reach the gut lumen they usually disrupt the intestinal barrier and invade the intestinal epithelium, thus triggering inflammation and other physiological responses eventually leading to diarrhea or disrupted absorptive function [10]. The most relevant bacterial pathogens responsible for causing gastroenteritis worldwide are Gram-negative bacteria *Campylobacter* spp., *Salmonella enterica*, Shiga toxin-producing *Escherichia coli* (STEC), *Shigella* spp., *Vibrio cholerae*, *Listeria* spp. and *Yersinia* spp.[12–15].

Interventions including improvement of sanitation conditions, safe drinking water and hand washing with soap in countries lacking these hygiene bases must be undertaken to reduce diarrheal disease. In contrast, in the developed world efforts should be focused on the food chain industry as transmission mostly occurs through commercial industrial production of meat, eggs, and processed food.

This PhD thesis is focused on the study of *S. enterica*, an important pathogen causing disease worldwide. It is one of the leading causes of gastroenteritis and is the first and the second most commonly reported bacterial pathogen in the United States and Europe, respectively [16,17]. In low-income countries, salmonellosis leading to invasive disease is also a prominent cause of bloodstream infections [18–20].

1. *Salmonella enterica*

The genus *Salmonella* is constituted by Gram-negative bacteria which belong to the Enterobacteriaceae family and are facultative intracellular anaerobes that do not form spores. *Salmonella*e are non-lactose and -sucrose fermenters but they can obtain energy from glucose. They are rod shaped and most are motile by peritrichous flagella, and vary in diameter from around 0.7 to 1.5 µm and are from 2 to 5 µm in length [21] (**Figure 2**).

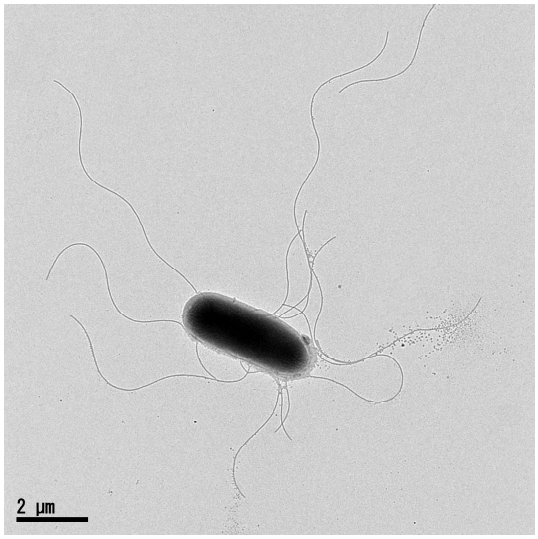

Figure 2. Image of *Salmonella* using electron microscopy. Adapted from (http://www.oist.jp)

The discovery of *Salmonella* is attributed to Theobald Smith, a pioneer epidemiologist, bacteriologist, and pathologist born in the United States in 1859 to German immigrants. He was the assistant of Daniel Elmer Salmon, an American veterinary pathologist at the Bureau of Animal Industry (BAI) in Washington DC (USA) when in 1884 he discovered a new species of bacteria: *Salmonella enterica* (at that time named *Salmonella choleraesuis*) while investigating the cause of hog cholera, a disease affecting mostly swines. Despite Smith being the discoverer of *Salmonella*, Daniel Elmer Salmon took credit for it and the newly discovered bacterium was named according to his name. However, later research revealed that they wrongly attributed *Salmonella* to be the cause of hog cholera and showed that the etiological agent of this infection was a virus, whereas the bacterium discovered was a secondary invader [22].

The taxonomy of the genus *Salmonella* has been extensively discussed and reconsidered over the years. The first description of a great number of serovars was carried out by White at the beginning of the 20th century followed by Kauffmann in the 60's based on serological analysis from which the Kauffmann-White scheme was established [23]. Based on this scheme, the WHO Collaborating Centre for Reference and Research on *Salmonella* at the Institut Pasteur (Paris, France) defines and updates the classification of this genus. According to the last revision, *Salmonella* consists of only two species, *S. bongori* and *S. enterica*, which is divided into six subspecies: I, *S. enterica* subsp. *enterica*; II, *S. enterica* subsp. *salamae*; IIIa, *S. enterica* subsp. *arizonae*; IIIb, *S. enterica* subsp. *diarizonae*; IV, *S. enterica* subsp. *houtenae* and V, *S. enterica* subsp. *indica.* Moreover, based on the presence of distinct surface antigens *Salmonella*e can be divided into 2,659 serovars, among which almost 60% belong to the *S. enterica* subsp. *enterica*, as seen in **Table 1** [24,25]. Each unique combination of the O (lipopolysaccharide [LPS]) antigens and H (flagellar) antigens (phase 1 and phase 2, if present) results in the designation of a new serovar (see Section 3.2.2) [25]. The nomenclature of the different serovars depends on the subspecies they are part of; serovars belonging to *S. enterica* subsp. *enterica* are designated by a name usually related to the geographical place where the serovar was first isolated whereas serovars belonging to other subspecies are designated by their antigenic formulae, following the subspecies name [26].

Table 1. Distribution of *Salmonella* serovars among each species and subspecies. Adapted from [24].

Salmonella		
S. enterica	subsp. *enterica*	1,586
	subsp. *salamae*	522
	subsp. *arizonae*	102
	subsp. *diarizonae*	338
	subsp. *houtenae*	76
	subsp. *indica*	13
S. bongori		22
Total		2,659

In terms of clinical impact the most relevant subspecies is *S. enterica* subsp*. enterica,* which is responsible for >95% of human infections, as well as also primarily infecting other warm-blooded animals. The other five subspecies as well as *S. bongori* primarily infect cold-blooded hosts [27].

2. Pathogenesis and virulence factors

Serovars belonging to *S. enterica* are classified into two distinct groups depending on the type of disease they cause. Serovars causing gastroenteritis are referred to as nontyphoidal *Salmonella*e (NTS), being the most commonly found *S. enterica* serovars Enteritidis (*S.* Enteritidis) and *S.* Typhimurium. On the contrary, *S.* Typhi and Paratyphi A cause a bloodstream infection known as typhoid fever and are called invasive *Salmonella*e. In addition to differences in disease outcome, another important feature that differentiates these two groups is the niche they occupy. NTS are host-generalist, able to infect and survive inside a wide number of animals whereas invasive *Salmonella* can only colonize and live inside the human body, being host-restricted [28].

Although *S. enterica* pathogens carry conserved virulence factors involved in the process of infection, host-restricted serovars have evolved strategies for persisting inside the host and evading the immune defenses [27]. One of the consequences of human-restriction of invasive *Salmonella*e is genome degradation due to the presence of a number of inactivated or disrupted genes (named pseudogenes). These pathogens present a reduction in the number of genes encoding for functions, somehow redundant, involved in adhesion to the intestinal epithelium among others, required for the gastrointestinal lifestyle of serovars causing gastroenteritis [28,29].

Upon ingestion of food or water containing *S. enterica*, bacteria arrive to the stomach and survive at the normally lethally low pH conditions. These pathogens are able to activate acid tolerance responses and acid resistance mechanisms to overcome the acid stress by maintaining an internal pH which allows cell viability [30]. Survival of the stomach passage permits bacteria to reach the intestinal lumen, where the first contact with the host takes place and the pathogenic process begins. The success of *S. enterica* infection lies in its virulence armamentarium. The most important virulence genes are located in highly conserved regions exclusively present in the genome of *Salmonella* named *Salmonella* Pathogenicity Islands (SPIs). The architecture of these regions indicates a different G+C content from the rest of the genome, and these fragments are mainly inserted in RNAt

genes. Moreover, they contain genes encoding for integrases and transposases flanked by direct repeats suggesting that they have been acquired by horizontal transfer [31].

To date, 21 SPIs have been identified in *S. enterica*. Eleven have been seen in both *S.* Typhimurium and *S.* Typhi genomes, 4 are specific to *S.* Typhi (SPI-7, 15, 17, and 18) and one to *S.* Typhimurium (SPI-14) [32,33]. In NTS, SPI-1 to SPI-5 have been identified as being clearly involved in virulence [34] and SPI-1 and SPI-2 have, by far, been the most deeply studied. Among the *S.* Typhi-specific SPIs, SPI-7 is particularly important as it contains the *viaB* locus, a 14-Kb DNA region which encodes genes for the biosynthesis of the virulence-associated (Vi) capsular polysaccharide. Furthermore, the *pil* operon, which encodes for the IVb fimbria, and the *sopE* gene, involved in the host invasion process, are also contained in SPI-7 (**Figure 3B**). This virulence factor seems to be responsible for the evasion of host immune response, and thus, lead to the development of slow intestinal inflammation (see Section 2.3.3). Moreover, it has been proposed as the cause of the long incubation periods that characterize typhoid fever [29]. A scheme of the most relevant SPIs involved in the pathogenesis process is represented in **Figure 3**.

Other relevant virulence genes involved in different stages of the infectious process encoded outside SPIs but still chromosomally-located are adhesins, flagella and biofilm-formation genes. In addition, the 8-kb region containing the *spvRABCD* locus present in a virulence plasmid, called pSLT, is only carried by some serovars such as *S.* Enteritidis, *S.* Typhimurium, *S.* Choleraesuis, and *S.* Dublin (see Section 2.3.2) [32,34].

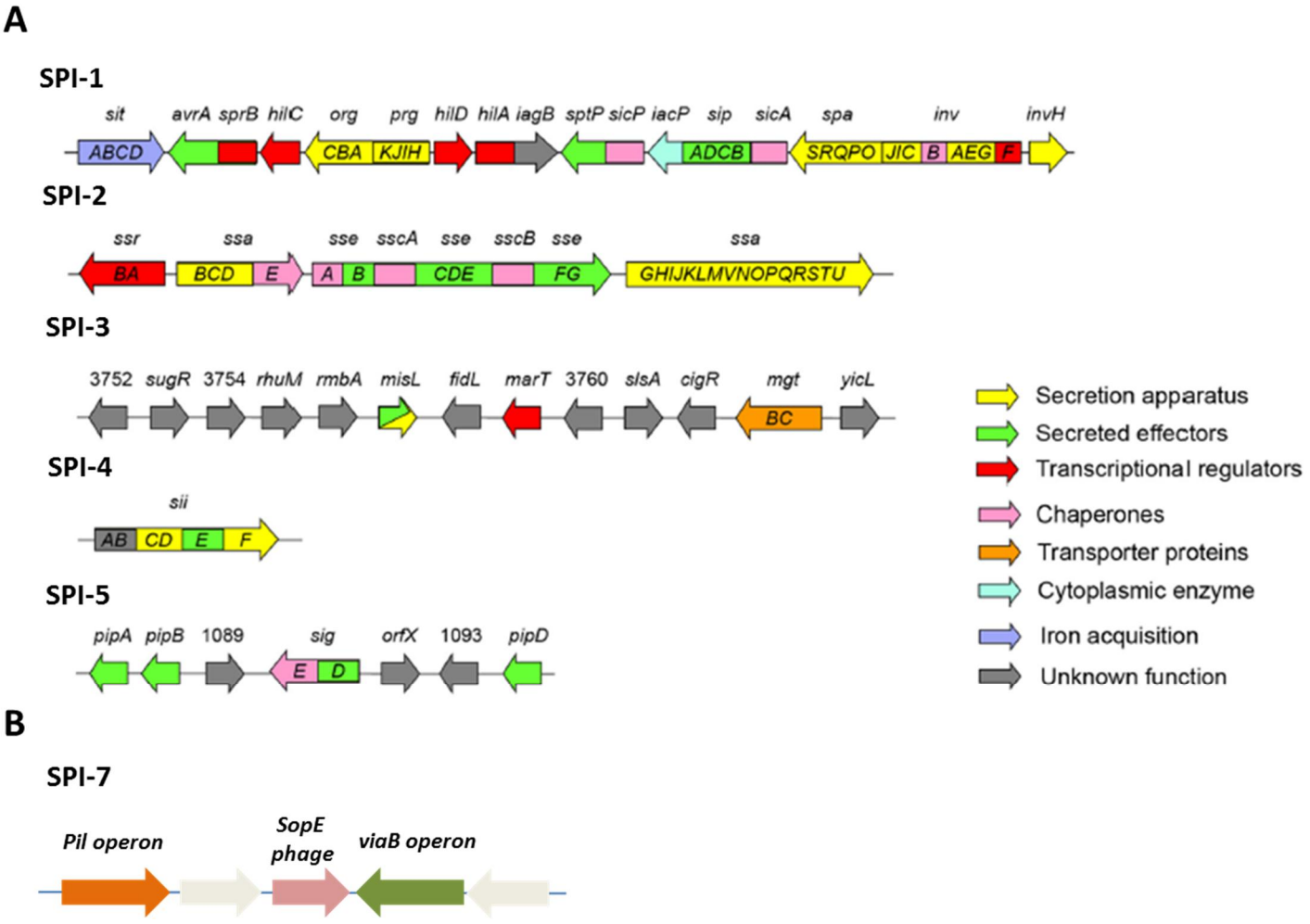

Figure 2. Schematic representation of the most significant SPIs of *S. enterica*. **A**) Genes contained in the five SPIs commonly found among serovars of *S. enterica* and their putative functions. Adapted from [34]. **B**) Genes encoded in the *S.*Typhi-specific SPI-7.

Most of the virulence factors required in the pathogenic process are exported through both the inner and outer membranes and injected inside the host cell from the bacterial cytoplasm through a highly specialized secretion apparatus called the type III secretion system (T3SS) conserved in many enteric pathogens (see **Figure 4**).

The architecture of this system consists of a needle emerging from the bacterial surface and connected to a base surrounded by an outer and an inner membrane ring. The internal structure forms an inner rod connecting the bacterial cytoplasm and the host cell membrane [35,36].

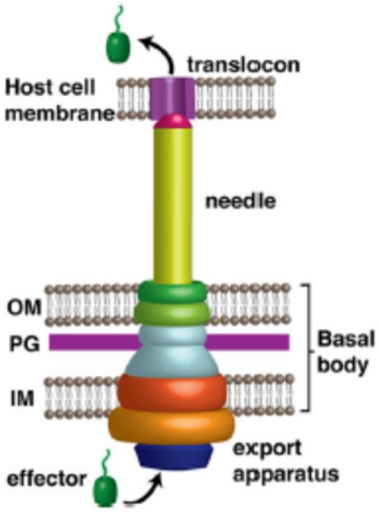

Figure 4. Standard structure of the type 3 secretion system of *Salmonella*. Adapted from [37].

Two T3SS encoded in SPI-1 (SPI-1 T3SS) and in SPI-2 (SPI-2 T3SS) have been described to be involved in the different steps of bacterial invasion by *Salmonella enterica.*

2.1 Approach to the intestinal epithelium

Once the stomach is crossed and the bacteria reach the intestinal lumen, motility is needed to increase the chances of the bacteria to encounter and adhere to the small bowel intestinal epithelial cells.

Motility occurs thanks to the expression of flagella, formed by three structural components: i) a basal body (anchored to the cell envelope); ii) a hook complex, which forms a molecular motor; and iii) a filament, composed of 11 protofilaments mainly integrated by flagellin monomers exported to the extracellular space through a flagellum-specific T3SS (**Figure 5**) [38][39]. Each flagellin monomer is composed of four globular domains (D0, D1, D2 and D3). In addition, to permit bacteria to move, flagellin is also recognized by the host immune system activating the recruitment of host inflammatory cells. Specifically, it has been demonstrated that core residues R90, L94 and Q97 belonging to the D1 domain are essential for protofilament assembly, motility and host recognition to induce bacterial clearance [38,40,41].

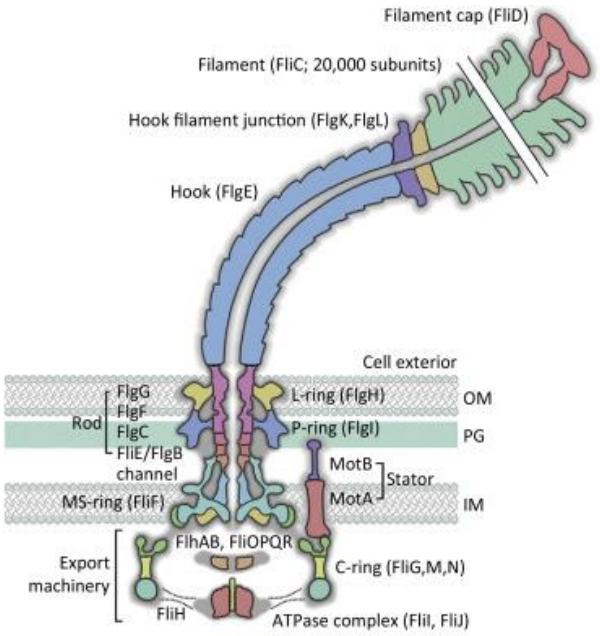

Figure 5. Schematic representation of the different parts constituting the flagella. Adapted from [42]

2.1.1 Flagellar regulatory pathway

Transcription of the flagellar system is complex and flagellum biogenesis is regulated at multiple levels, ranging from environmental conditions to the development of the flagellar structure.

The flagellar regulon is divided into three different transcriptional regulators called class I, II and III. The only class I promoter is sensitive to environmental conditions and cell state sensors. It drives transcription of the master regulators FlhC and FlhD, which in turn are transcriptional activators of the class II genes, involved in structure and assembly of the hook-basal body and the T3SS. The regulatory proteins FlgM and FliA (σ^{28}) are also encoded in the class II cluster. Thereafter, FlgM, which is normally bound to FliA preventing its activity, is secreted outside the cell allowing FliA-dependent transcription of the class III promoter once the hook-basal body is completed. Class III genes encode for proteins involved in the late assembly stage of flagellum formation, including hook-associated proteins, motor torque subunits MotA and MotB, chemotaxis proteins and the filament

subunits FliC and FljB, being the two latter compounds polymerized outside the cell to form the helical filament [41,43].

2.2 Attachment to intestinal cells

After reaching the intestinal lumen and by means of specific proteins called fimbriae, enteric pathogens adhere to receptors on the surface of M cells, specialized epithelial cells located on the intestinal lymphoid nodules called Peyer's Patches, prior to invading the intracellular space. Fimbriae are surface-exposed structures which, together with export, assembly and regulatory proteins, are typically encoded by a gene cluster. Different classes of fimbriae are expressed by *Salmonella* and they have the ability to bind different host tissues, from intestinal epithelial cells to specific immune cells and macrophages, thus determining their involvement in different stages of the pathogenic process [44].

Some of the fimbrial operons are conserved among *Salmonella* serovars while others are serovar-specific partly explaining the differences in pathogenesis. Eight putative fimbrial operons shared by both *S.* Typhimurium and *S.* Typhi have been detected by whole genome sequencing (*bcf, csg, fim, saf, stb, stc, std, sth*). Five serovar-specific fimbrial operons have been reported for *S.* Typhimurium (*lpf, stf, pef, sti and stj*) and *S.* Typhi (*sef, sta, ste, stg and tcf*) in addition to IVb fimbria uniquely expressed by the latter serovars [33].

2.2.1 Fimbriae classification

Fimbriae are classified into different families depending on their structure:

Chaperon/usher fimbriae. They are the most commonly found in *Salmonella* serovars (between 11 and 13 fimbrial operons have been reported) and each member is composed of structural (fimbriae) and assembly proteins (usher and chaperones) [45]. The type 1 pilus system belongs to this family and is encoded by the six-gene operon *fimAICDHF* encoding structural subunits, which include the chaperone FimC and the usher FimD, in addition to the three regulatory genes, *fimZ*, *fimY*, and *fimW*. Through the adhesive subunit FimH the resulting fimbria binds the host extracellular matrix component laminin mediating adhesion to eukaryotic cells [34,46,47].

Curli fibers. These are extracellular filaments highly conserved within related genera of Enterobacteriaceae. Genes encoding curli fimbriae are transcribed from the two operons *csgDEFG* and *csgAB.* CsgA is the major subunit and constitutes the structural fibrils by aggregation onto the membrane-associated minor subunit CsgB. Curli biogenesis is possible thanks to subunit secretion through CsgG, a pore-forming outer membrane lipoprotein. The master regulator responsible for induction of curli fimbriae production is CsgD. Curli fibrils have a dual role: they are involved in both cell adhesion by mediating attachment to the host extracellular matrix component fibronectin [34,47,48] and are also key factors in biofilm formation (see Section 2.4).

Type IV pili. These fimbriae are composed of fimbrial filaments assembled during transport to the bacterial surface through an outer membrane channel formed by secretin and are divided into types IVa and IVb. Type IVa fimbriae are distributed among all serovars whereas Type IVb is only present in *S.* Typhi and is encoded within the SPI-7. They mediate interaction with human monocytes and epithelial cells through the cystic fibrosis transmembrane conductance regulator receptor (CFTR) [49].

Other nonfimbrial adhesins such as BapA and SiiE (encoded in SPI-4), and the autotransporter MisL (encoded in SPI-3) which carries proteins across the outer membranes of Gram-negative bacteria and has been reported to bind to fibronectin, are also involved in the adhesion step [34].

2.3 Host-bacteria interaction

2.3.1 Invasion

Once bacteria are attached to specialized epithelial M cells through the above-mentioned mechanisms, assembly of the SPI-1 T3SS begins and translocation of the effector proteins into the host cell cytosol takes place [34]. The structural genes of SPI-1 T3SS are located on SPI-1 and include the *prgHIJK*, *spaMNOPQRS*, and *invABCEFGH* operons together with multiple regulatory and effector genes. First, inner ring assembly is conducted by the protein subunits PrgH and PrgK followed by the cytoplasmic export machinery formed by the InvA, InvC, SpaP, SpaQ, SpaR, and SpaS proteins. The outer ring structure is then built up in the

outer membrane, composed of InvG and InvH, and finally, connection between both rings is mediated by InvJ [36].

The translocated effector proteins cause cytoskeletal rearrangements which are important for bacterial engulfment and induce a pro-inflammatory response in the host cell which elicits mucosal inflammation. The SPI-1 effectors SipA, SipC and the inositol phosphatase SigD/SopB encoded in SPI-5 cause actin rearrangements resulting in the membrane ruffling needed for the formation of the *Salmonella*-containing vacuoles (SCVs), in which bacteria are able to survive (see Section 2.3.2). Additionally, SigD also contributes to the formation of intracellular vacuoles by sealing the invaginating regions of the plasma membrane. SopE and SopE2 act as guanidine exchange factors (GEFs) for the small Rho GTPases Cdc42 and Rac-1. Both proteins cause cytoskeletal rearrangements and induce production of pro-inflammatory cytokines (IL-8 and TNF-α) through activation of the nuclear transcriptional factor NF-κB [34,36,50]. This invasion step is represented in **Figure 6**.

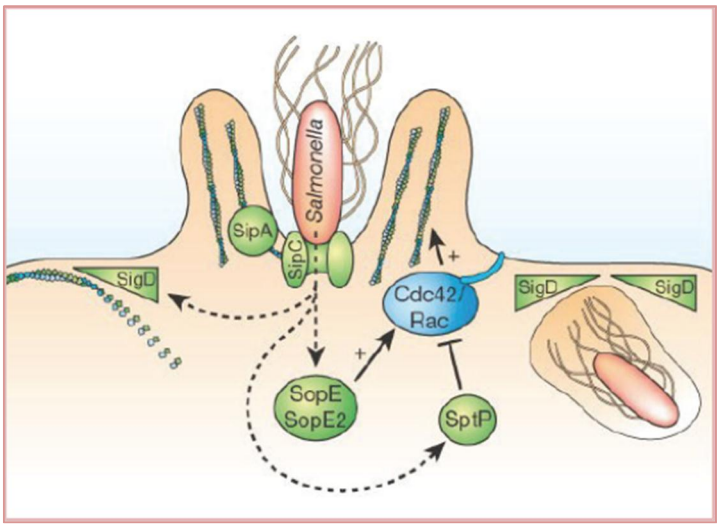

Figure 6. Entry of *S. enterica* into host cells is mediated by SPI-1 effectors translocated into the host cytosol through the SPI-1 T3SS. Adapted from [51].

2.3.2 Intracellular survival

Once inside M cells, bacteria cross the epithelial monolayer and transcytose to the basolateral membrane. In this process, bacteria survive inside the host cell cytosol inside SCVs, which are modified phagosomes unable to fuse with lysosomes. Then, *Salmonella* are translocated to the lamina propria where they are recognized by phagocytes and activated

through ligand-receptor interactions. Phagocytes include macrophages and dendritic cells, latter of which are present in the lymphoid follicles located beneath the intestinal barrier.

Upon engulfment of *Salmonella* by macrophages the bacteria are partly neutralized, but a number of them survive intracellularly inside SCVs. In these conditions, the host cell is unable to deliver microbicidal compounds to kill the engulfed bacteria and expression of the SPI-2 T3SS is stimulated. This secretion system is composed of a number of SPI-2 encoded genes including structural genes (*ssaG* through *ssaU*), effector genes (*sseABCDEF*), chaperones (*sscAB*), and secretion system regulatory genes (*ssrAB*) [34,36]. Some of the SPI-2 effector proteins translocated by the SPI-2 T3SS, which are relevant for survival and replication inside the host cell, include the SPI-2-encoded proteins SseJ, SseF and SseG as well as SifA, SifB, PipB and SopD, encoded elsewhere in the chromosome. They are all involved in the formation of *Salmonella*-induced filaments (SIFs), as a result of the fusion of SCV with other intracellular vesicles [36]. In general terms, translocation of SPI-2 effectors through SPI-2 T3SS mediates bacterial replication inside SCVs, as well as stabilization of the vacuolar membrane and induction of delayed host cell death, among other functions (see **Figure 7**) [35,52].

In relation to *S.* Typhi, controversial information is available regarding the role of SPI-2 T3S*S.* Whereas its role in survival and replication inside host cells has been described by Khan *et al.* [53] and is well accepted, a recent study has reported that SPI-2 T3SS defective mutants do not present survival and replication defects inside human macrophages and suggest that this secretion system might be required instead in the modulation of the immune response [54].

The plasmid-mediated virulence genes *spvB* and *spvC,* encoded in the *spvRABCD* locus, have also been reported to be translocated into the host cell through the SPI-2 T3SS in *S.* Typhimurium [45]. SpvB is an ADP-ribosylating toxin that contributes to actin depolymerization, and thus, has a cytotoxic effect, whereas SpvC is a phosphothreoninelyase implicated in reducing *Salmonella*-induced inflammation [55]. The transcriptional regulator *spvR*, encoded in the same locus as *spvB* and *spvD*, acts as the local activator of the *spv* locus [34].

Interestingly, it has also been demonstrated that inside dendritic cells, where *Salmonella* do not replicate, SPI-2 T3SS effector proteins induce migration and antigen presentation [56].

2.3.3 Immune system recognition

Inside SCVs, bacteria cross the epithelial monolayer and transcytose to the basolateral membrane, being released to the lamina propria where they are recognized by phagocytes and activated through ligand-receptor interactions. As mentioned previously, PAMPs (pathogen-associated molecular patterns) are recognized by PRRs (pattern-recognition receptors) expressed in the surface and/or inside host cells including macrophages, intestinal epithelial cells, dendritic cells and neutrophils [38,45,57].

The most important PAMPs are flagellin, particularly the FliC subunit highly expressed on the bacterial surface [58] and the lipopolysaccharide (LPS). Upon binding their specific PRRs, named Toll-like receptor (TLR) TLR-5 and TLR-4, respectively, they promote innate pathways of inflammation [34,38,57]. Stimulation of the TLRs induces activation of the nuclear transcriptional regulator NF-κB, thus triggering the production of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β, as represented in **Figure 7** [59].

In addition to TLR-5 activation, cytoplasmic flagellin also activates the protease factor Ipaf present in the mammalian cytosol. This leads to the proteolytic activation of IL-1β and IL-18 through caspase-1 and participates in lysis of the host cell [60]. The production of these cytokines stimulates the recruitment of immune cells, and thereby further amplifies the inflammatory response. Moreover, Tukel *et al.* also reported that the curli fimbria member CsgA was an agonist of TLR-2, thus contributing to host recognition of *Salmonella* [61].

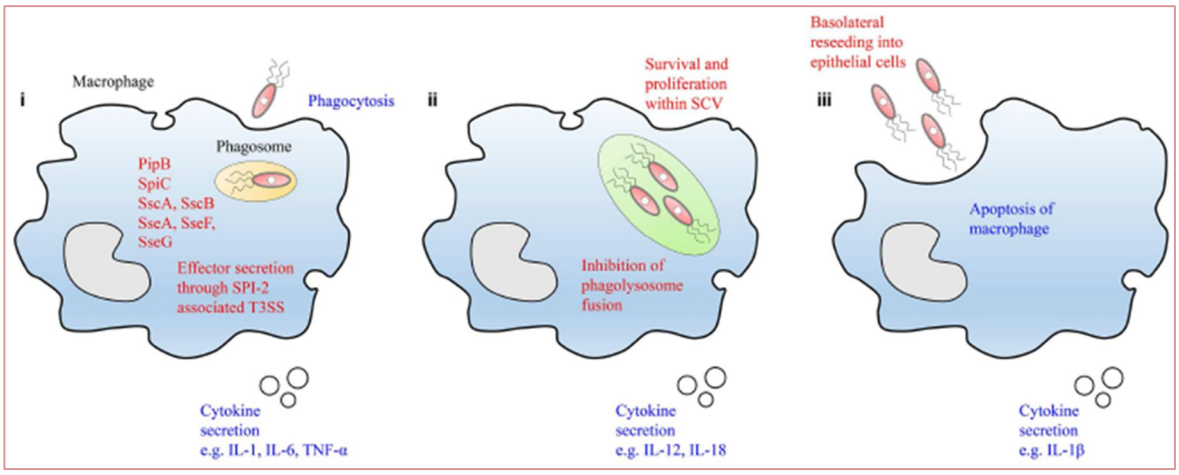

Figure 7. Schematic illustration of the different steps involved in the infection of macrophages by *Salmonella*. During this process, cytokines are secreted. **i)** Engulfment of bacteria and secretion of effector proteins through SPI-2 T3SS. **ii)** Survival and proliferation of *Salmonella* inside SCVs. **iii)** Release of bacteria and re-infection of host cells. Adapted from [62].

In the case of non-invasive *Salmonella*, recognition by host cells causes a rapid recruitment of neutrophils into the intestinal mucosa, thus causing inflammation and a self-limiting diarrhea in immunocompetent patients. However, there are important differences regarding the interaction of *S.*Typhi with the innate immune system which explains the particular pathogenesis of typhoid fever.

2.3.3.1 Host immune evasion

Only pathogens belonging to Typhi serovar are able to elude host immune response and disseminate throughout the body. The virulence factors involved in this process are encoded in SPI-7, only present in *S*. Typhi, specifically in the *viaB* locus that harbors genes for regulation (*tviA*), biosynthesis (*tviB*, *tviC,tviD* and *tviE*) and export (*vexA*, *vexB*, *vexC*, *vexD* and *vexE*) of the virulence-associated (Vi) capsular polysaccharide.

One of the mechanisms of immune evasion is directly mediated by the production of the Vi capsule that prevents LPS recognition through TLR-4 [32,63]. Another mechanism involves the regulatory protein TviA, the activator of capsule biosynthesis and negative regulator of the genes encoding flagella and SPI-1 T3SS. At high osmolarity conditions, such as those encountered in the intestinal lumen, this protein is repressed, and thus, the Vi capsule is not produced although the invasion machinery is activated. On the contrary, once bacteria reach

the lamina propria, osmolarity decreases and TviA is expressed, leading to aflagellated bacteria and hence preventing TLR-5 induction, which causes evasion of the cytokine-mediated inflammatory response and allows systemic dissemination [63,64].

2.3.3.2 Dissemination of invasive *Salmonella*

Due to the low immunogenic response triggered by host-restricted *Salmonella* serovars and invasive NTS (in this latter case due to the immunocompromised state of the patient) these bacteria are able to overtake the lamina propria by evading host immune response and reaching the bloodstream where bacteria are released causing systemic infection. Dissemination is led by *Salmonella*-infected dendritic cells and macrophages that are transported through the mesenteric lymph nodes to the liver, spleen and bone marrow where *S.* Typhi is able to survive and replicate [32,36,65].

2.4 Persistence

In harsh conditions, such as nutrient limitations, extreme temperatures and acidic pH or in the presence of antimicrobial agents, the strategy that *Salmonella*, among other bacteria, have developed to survive and persist is through biofilm production, which is considered an important virulence factor. Biofilm is mainly produced by the two tightly linked extracellular matrix components: cellulose and curli fimbriae. Proteins involved in cellulose biosynthesis are encoded in the *bcsABZC* and *bcsEFG* operons, whereas curli fimbriae genes are located within the two above-mentioned operons *csgBAC* and *csgDEFG* (see Section 2.2.1) [34]. In addition to these factors, under the control of CsgD as curli fimbriae, the cell surface protein BapA is secreted through a T1SS encoded by the *bapBCD* operon and has also been reported to be required for biofilm formation [66].

S. Typhi persisters are typically located in the gall bladder, where they arrive through the vasculature or the ducts that emanate from the liver. There is scientific evidence supporting the hypothesis that chronic infection caused by *S.* Typhi is due to the formation of biofilm in gallstones [67,68]. Crawford *et al.* have shown that O-antigen production, which requires the genes for assembly and extracellular translocation encoded in the *yihU-yshS* and *yihVW* operons, is needed for biofilm formation on cholesterol-coated surfaces (such as the main

component of gallstones) and that bile also induces O-antigen production independently of *csgD* [68].

2.5 Key regulation of the virulence factors

The different environmental conditions, such as oxygen availability and osmolarity, encountered by *Salmonella* along its passage throughout the body play an important role in regulating the transcription of the virulence factors required in each step of pathogenesis. These external factors are sensed by a number of bacterial receptors that target specific regulators which, in turn, activate or repress the different effectors. In this section, the major regulators that orchestrate the infectious process are detailed and a schematic representation of the regulation is shown in **Figure 8**.

The most important regulator involved in the invasion process is HilA, a transcriptional activator belonging to the OmpR/ToxR family and encoded in SPI-1. HilA activates the genes encoding the functional SPI-1 T3SS by direct binding to the promoter (*inv/spa* operons) or indirectly through activation of the SPI-1 encoded gene *invF* (*sic/sip* operons). The *sii* operon (SPI-4) and the *sigD* gene (SPI-5) are both involved in the invasion step and similarly activated by HilA [34,69]. However, the SPI-2 encoded genes are repressed by HilA but activated by the two-component regulatory system SsrA-SsrB, encoded in the same SPI. When the membrane-located sensor kinase SsrA is stimulated under specific environmental conditions, such as acidic pH and poor nutritional status of the lumen of SCV, it phosphorylates the regulator SsrB activating the transcription of genes expressing SPI-2 T3SS effectors encoded inside and outside SPI-2 [34,55].

Regulation of HilA is positively mediated by HilC and HilD, encoded in SPI-1, and RtsA encoded outside this island. These transcriptional regulators directly bind to the *hilA* promoter activating its expression. On the contrary, HilE, a regulator encoded outside SPI-1, exerts negative regulation of HilA through posttranscriptional interaction with HilD, a process that prevents HilD activation of the *hilA* gene. Moreover, HilD is also able to directly bind to the *ssrAB* promoter, inducing expression of the SPI-2 genes [34,50].

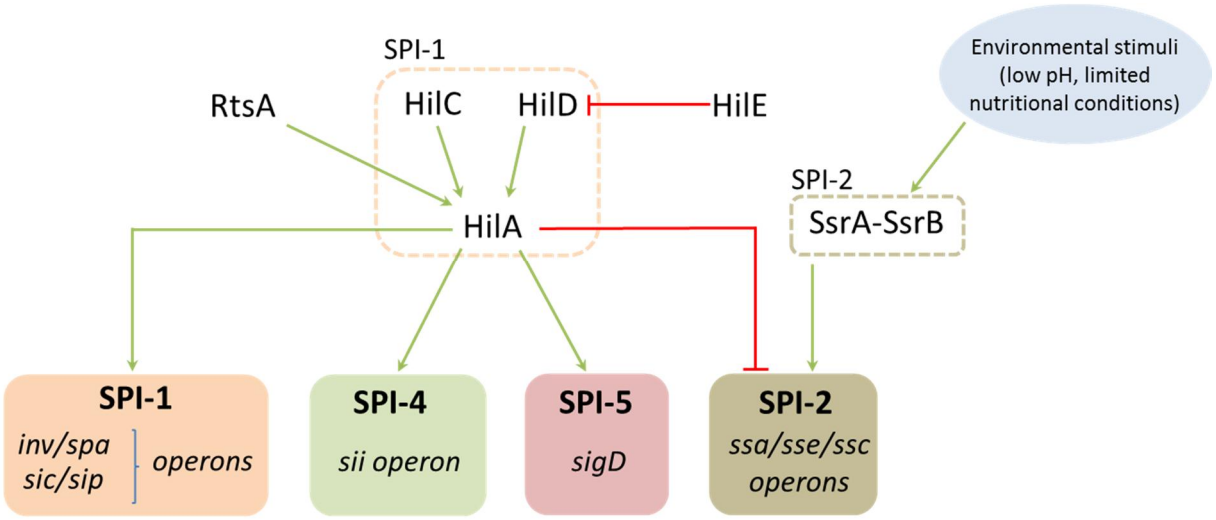

Figure 8. Simplification of the global transcription regulation of the most important SPIs.

3. Salmonellosis

3.1 Clinical manifestations

Evolution of the genus *Salmonella* has led to adaptation to a large number of niches, including reptiles, birds and mammals, resulting in a range of different syndromes depending on the infective serovar and the host. In the case of human infection, the clinical outcome elicited by *Salmonella* is classified into two groups depending on the pathogen: NTS serovars, mostly represented by *S.* Typhimurium and *S.* Enteritidis cause self-limiting gastroenteritis whereas *S.* Typhi and *S.* Paratyphi A cause a systemic disease called typhoid fever. In sub-Saharan Africa, invasive NTS (iNTS) also causes a systemic disease comparable to typhoid fever. However, other factors such as age, nutritional state and bacterial load also determine the severity of the disease [32,34,57,70].

3.1.1 Non-invasive salmonellosis

The first symptoms of human disease occur after a period ranging from hours to 2-3 days upon ingestion of food or water contaminated with NTS [57].

Abdominal pain and diarrhea are common symptoms as well as nausea and vomiting. Diarrhea occurs due to intestinal inflammation, mostly in the ileum, during bacterial invasion and translocation to the lamina propria [11,57]. The severity of the clinical manifestations depends on the age and immune system of the patient, being children and immunocompromised people those at increased risk of complications. In these populations the infectious disease lasts for a longer period of time and consists in an increased inflammatory response and bloody diarrhea [11]. Less commonly, extraintestinal infections such as bacteremia, urinary tract infection, or osteomyelitis can also affect these populations [21].

Infections confined to the intestinal tract are usually self-limiting and resolve spontaneously after 4-7 days; however, in the presence of complications antimicrobial therapy is required with fluoroquinolones as first-line treatment [57] (see Section 4).

3.1.2 Invasive salmonellosis

Typhoid or enteric fever is similarly transmitted by ingestion of contaminated water and less frequently by food with human fecal material. This infectious disease remains common in regions with poor standards of hygiene and sanitation conditions. The main representative pathogen is *S.* Typhi, although *S.* Paratyphi (A, B or C) has been reported to cause a similar syndrome [70].

Manifestations of the disease occur 1-2 weeks after infection and include malaise, abdominal pain and progressive increase of fever. Less frequent symptoms are nausea, anorexia and diarrhea, this latter trait being typically present in immunocompromised patients. Other symptoms such as hepatosplenomegaly and the appearance of rose-colored spots (maculopapular lesions of 2-4 mm in diameter) on the skin can also be seen in this syndrome [11,57,71].

Antimicrobial therapy is required for the treatment of typhoid fever with fluoroquinolones being the most commonly prescribed antibiotics, and it is effective in 95% of the cases. However, after recovering 2-5% of the individuals become asymptomatic carriers with the pathogen persisting in their bodies during months or years [71]. The site of bacterial persistence is usually the gall bladder, presumably in the gallstones [67], mesenteric lymph nodes and bone marrow [11,72,73]. An association has been established between the persistence of *S.* Typhi in the biliary tract and gallbladder carcinoma in endemic regions [74]. Chronically infected individuals continue to intermittently shed the bacteria in their feces through bile excretion, thus contributing to the transmission of the pathogen to healthy individuals. In addition, persistent bacteria can re-colonize the intestinal wall leading to the reappearance of typhoid fever [72].

Alternatively, infections caused by iNTS, predominantly due to *S.* Typhimurium and *S.* Enteritidis [75], affect mainly children with malnutrition, severe anemia, malaria or HIV as well as HIV-infected adults [76]. The clinical outcome is a febrile systemic illness that can be indistinguishable from malaria or pneumonia, especially in young children, and is rarely accompanied by diarrhea. In addition to bloodstream infection, bacteria can also reach the meninges causing meningitis. Different hypotheses related to a dysfunction in host

recognition of the bacteria have been suggested regarding the mechanisms by which iNTS are able to cause a typhoid-like disease. Nonetheless, to date this information still remains unclear [18].

3.2 Diagnostic methods

This section describes the most relevant techniques used in the clinical laboratory to identify and discriminate strains of *Salmonella* causing disease. They are classified as phenotypic methods since they are based on the expression of genes differentially present in these bacteria. However, differences between serovars, which are at a subspecies level, are not always evident due to an important degree of clonality.

3.2.1 Selective agar media

Clinical microbiology laboratories use selective agar media as a first screening to identify the causal pathogen in a clinical sample. MacConkey agar plates are generally used since this medium only allows the growth of Gram-negative enteric bacteria. Depending on the fermentation by-products produced by the genus of the Enterobacteriaceae family, the appearance of the colonies differs. Thus, colonies of non-lactose fermenting bacteria, such as *Salmonella*, appear to be colorless contrary to lactose-fermenters which grow as pink colonies.

Additional solid media are also used for more precise identification of the genus *Salmonella* based on the utilization of different sugar sources and hydrogen sulfide production, a particularity shared by the isolates belonging to this genus. In combination with fermentation of different sugar sources the production of this gas lead to the formation of colonies with black centers when culturing them in the following agar media: *Salmonella-Shigella* (SS) agar, xylose-lysine-deoxycholate (XLD) agar, Hektoen enteric (HE) agar, Kligler's iron agar (KIA) and triple sugar iron (TSI) agar [34]. Rambach agar is another chromogenic medium also used in some clinical laboratories for the identification of non-Typhi *Salmonella*. When cultured, colonies appear red due to the ability to form acid from propylene glycol, one of the components of this selective agar [77,78].

3.2.2 Slide agglutination serotyping

Antigenic detection by the serotyping method is the reference procedure for the identification of the different serovars of *Salmonella* in the clinical setting. It is based on the macroscopic observation of agglutination when a suspension of bacteria is mixed with specific O (LPS) and H (flagellar) antigens. A great variability of antigens has been identified, with 64 O and 114 H variants having been reported so far [79], and the differential reactivity establishes the basis of the *Salmonella* serotyping scheme, called the Kauffmann-White-Le Minor scheme [25].

The O-antigen is an important saccharidic component of the LPS, a bacterial structure located in the outer membrane of Gram-negative bacteria. It is the most abundant molecule on the bacterial surface and is particularly important for host recognition and activation of immune response. The O-antigen is extremely variable according to its nature, order and linkage of the different sugars within the polysaccharide, and several types may be expressed at the same time on the surface of a single cell [79,80].

The H-antigen is conferred by flagellin, the major external filament of *Salmonella*'s flagellum which is involved in motility and is a target of the host immune system. Contrary to the diversity of the O-antigen, *Salmonella* produces one of two possible forms of the antigenic flagellin at a time. Thus, isolates are termed diphasic depending on the H-antigen they produce: H1 and H2, also named phase I and phase II, respectively [79,81]. However, some clinically relevant serovars such as Typhi and Enteritidis are monophasic, meaning that they only produce one form of flagellin. Triphasic and quadriphasic subtypes also exist but are exceptional [82].

This phenotypic technique is appropriate for the identification of isolates at the serovar level but is useless to establish clonal relatedness for epidemiological purposes.

3.2.3 Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF-MS)

This technique is based on the recognition of characteristic patterns obtained from mass-spectrometry analysis of small proteins and peptides generated from whole cells, cell lysates, or crude bacterial extracts [83]. Despite being a simple, rapid and inexpensive method, controversial studies have been conducted validating the technique to different degrees of specification. Whereas Deickmann *et al.* validated the identification of the frequently isolated serovars Typhimurium, Enteritidis, Virchow, Infantis and Hadar [84], more recent studies have demonstrated that this technique was able to discriminate only at the genus [83] and species level [85]. These inconclusive results limit the MALDI-TOF-MS approach for the identification of the genus *Salmonella*.

3.3 Global incidence of salmonellosis

3.3.1 Non-invasive salmonellosis

Estimation of the global burden of nontyphoidal salmonellosis was reported by Majowicz *et al.* in 2010 based on the data available. According to this report, 93.8 million cases of gastroenteritis due to *Salmonella* infections occur annually, representing around 3% of the total annual number of diarrheal disease. Among them, 80.3 million cases are foodborne and an estimated 155,000 deaths occur annually due to NTS gastroenteritis [86].

However, in certain regions where there is a lack of data (Africa, Asia, Latin America and the Caribbean) the incidence of the gastrointestinal disease has been calculated based on a previous report published by Ekdhal *et al.* [87]. Over a period of 7 years (1997-2003) these authors collected clinical information from Swedish travellers returning to their country with travel-associated nontyphoidal salmonellosis. According to the interpretation of the data presented by Majowicz *et al.*, Asia is the continent with the highest incidence rates, particularly in regions of the East and South-East where 3,600 cases per 100,000 inhabitants were calculated. In Africa, the incidence rate ranges from 69-471 cases per 100,000 inhabitants, being East Africa the region most affected. In Latin America and the Caribbean, the average of illnesses correspond to 100 cases per 100,000 inhabitants [86].

In Europe reliable data regarding the incidence of disease caused by foodborne pathogens is available through the European Food Safety Authority (EFSA). According to the last report published by this institution together with the European Centre for Disease Prevention and Control (ECDC) in 2013 almost 82,700 cases of gastrointestinal infections were found to be caused by *Salmonella.* This foodborne bacterial pathogen is reportedly the second most common microorganism found in humans in Europe, with 20.4 cases per 100,000 inhabitants. Although a decrease of almost 8% was detected compared to the previous year, *Salmonella* was still the most frequently found causative agent in foodborne outbreaks, being responsible for 22.5% of all cases [17].

In the United States a lower incidence than that seen in the European region has been reported according to a study conducted by the Foodborne Diseases Active Surveillance Network (FoodNet) [16]. Regarding this report, 7,800 cases of nontyphoidal salmonellosis were registered in 2012 corresponding to an incidence of 16.42 cases per 100,000 inhabitants.

Regarding both the European and the North-American networks, *Salmonella* is the most predominant genus causing foodborne infections, and the most prevalent serovars are Enteritidis followed by Typhimurium [16,17].

3.3.2 Invasive salmonellosis

According to the last report published by the WHO in 2004, typhoid fever due to *S.* Typhi was estimated to cause 21 million illnesses and 200,000 deaths annually. Invasive infections due to Paratyphi serovars were estimated to cause 5.4 million illnesses worldwide [88]. Across the globe, Asia is the continent where the highest incidence rates of typhoid fever are detected, representing a major public health problem (**Figure 8**). The population most affected includes infants, children and adolescents from South-Central and South-East Asia [88,89]. In the case of Paratyphi A, a systematic review performed recently has shown that this pathogen is also commonly identified in Asia as a cause of enteric disease [90].

Contrarily, developed regions (e.g. North America and Europe, excluding Eastern European countries) show the lowest incidence rates of typhoid fever caused by *S.* Typhi (<10 cases per 100,000 habitants per year) [88,91], as indicated in **Figure 9**.

Little information is available regarding typhoid fever caused by *S.* Typhi in African countries, making it difficult to estimate the real incidence. Taking into account this limitation, a medium incidence has been calculated for this continent (10-100 cases per 100,000 inhabitants per year), being the same as that estimated for Latin America and the Caribbean [88].

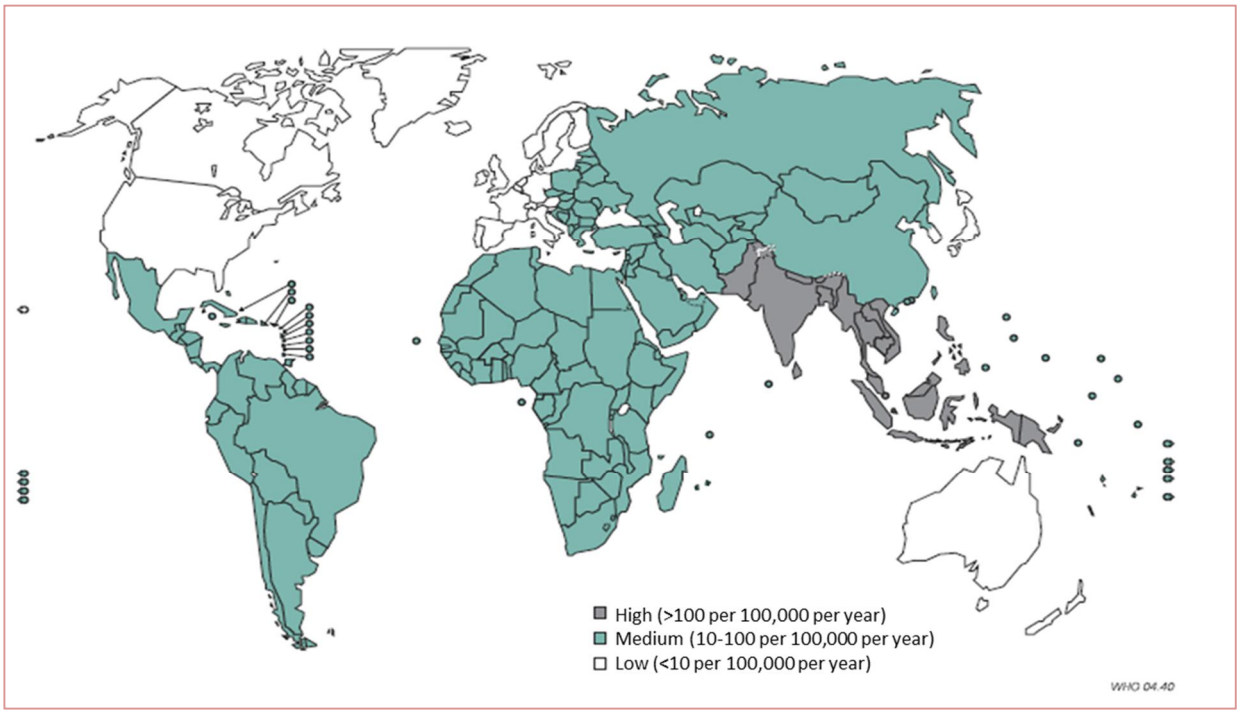

Figure 9. Global incidence of typhoid fever. Adapted from [88].

Concerning the geographical distribution of iNTS, it has been previously studied in two systematic reviews in which the predominant cause of bloodstream infection in Africa [20] and Asia [19] were determined. Results revealed that only 5% of the pathogens isolated and reported in Asia were iNTS *versus* 17% in Africa. In the latter continent, the estimated annual incidence of this invasive disease corresponds to 175–388 cases per 100,000 children below five years of age and 2,000–7,500 cases per 100,000 in HIV-infected adults [18]. Thus, invasive salmonellosis, particularly due to the serovars Enteritidis and Typhimurium,

represent a major cause of mortality and morbidity in children and immunocompromised adults in sub-Saharan Africa [20,52,75].

Among the different sequence types (STs) of *S.* Typhimurium reported to cause invasive disease, ST313 is the predominant type isolated in Africa and is uniquely present in this continent. However, ST19, the most prevalent sequence type elsewhere, also causes invasive salmonellosis despite affecting a reduced number of cases (around 8%) in the African setting [92]. Whole-genome sequencing of ST313 isolates has shown particular genome features, such as the presence of pseudogenes, a trait shared with the serovars Typhi or Paratyphi. These findings suggest a progressive adaptation of this particular ST to the human niche and the spread of this sequence type through person-to-person transmission [76].

3.4 Typing methods as essential tools for outbreak definition

Salmonella-related outbreaks are frequently reported, and many countries have national networks to control the spread of particular clones. However, commercial globalization consisting of international exchange of animals and foods together with the increase in the number of international travelers around the world has led to the establishment of a global network the mission of which is to facilitate the detection of international outbreaks in real-time. This network, called PulseNet International, is coordinated by the Center for Disease Control and Prevention of the United States of America (CDC) and is integrated by laboratories from all around the world that use standardized methods for the identification and characterization of foodborne pathogens in order to track their spread and to study the dynamics of important clones causing disease.

In this section, the most relevant typing methods are described. They mainly consist of genotypic techniques based on the identification of differences in the nucleotide sequence of the genome to type bacteria and identify outbreak-related strains. The objectivity of these techniques together with a high discriminatory power to differentiate between related strains of *Salmonella* makes them more reproducible and reliable than other phenotypic methods usually used in diagnosis (see Section 3.2).

3.4.1 Analysis of chromosomal DNA by digestion with low-frequency restriction enzymes and pulsed-field gel electrophoresis (PFGE)

This technique consists in comparing DNA fragments obtained from digestion of the genomes of interest with a rare cutting enzyme, of which XbaI, BlnI or SpeI are the most frequently used for *Salmonella* typing. After digestion, fragments are separated into an agarose gel subjected to specific electrophoretic conditions by alternating electric fields in order to allow separation of large fragments (ranging from 20 to 800-kb) [79,93]. The restriction band patterns obtained are then compared, and the degree of relatedness between strains during a disease outbreak is considered, mainly according to the Tenover criteria. These criteria are based on the assumption that a single genetic event in a restriction site, either a deletion or insertion, would result in up to three band differences. Depending on the number of band differences between isolates, they are defined as "indistinguishable" (no differences), "closely related" (2-3 band differences), "possibly related" (4–6 band differences) and ''different or unrelated'' (≥7 band differences) [94]. Complementation of the Tenover criteria with epidemiological factors is currently adopted to increase the accuracy in the interpretation of strain's relatedness [95].

At present, PFGE is considered the ''gold standard'' molecular typing method for bacteria as it is highly reproducible and allows good discriminatory power for the vast majority of strains [79,93,96]. Standardization of this technique, led by the above-mentioned PulseNet network allows inter-laboratory comparison of the PFGE patterns stored in a set of databases [97,98].

Despite the extensive use of this technique, its sensitivity does not allow highly clonal serovars such as Enteritidis [99] to be discriminated. In these cases, PFGE combining multiple-enzyme digestion has been shown to improve strain differentiation [100].

3.4.2 Multilocus sequence typing (MLST)

The basis of this technique lies in the sequence variability within particular genes to determine the relatedness of bacteria. The objective is to define a particular set of genes for sequencing analysis. The results show a specific combination of the nucleotide sequences which determine the ST of the strain. Housekeeping genes, which are involved in essential

cell functions, are the most frequently used for amplification and sequencing. Their distinctive feature lies in that they are present in all isolates of a particular species and are highly conserved despite having sufficient variability to discriminate between different alleles depending on the strain[101].

Several MLST schemes have been used to study the clonality of *S. enterica* isolates. These schemes involve sequencing of different sets of genes which are related to different sensitivities [79]. In the case of strains with more recent genetic divergence, multivirulence-loci sequence typing (MVLST), which consists in sequencing of virulence genes (that are under a greater selective pressure than housekeeping genes) has been shown to be more discriminatory [96]. However, despite not being the best option, the MLST scheme described by Kidgell *et al.* based on sequencing of the 7 housekeeping genes *aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA*, and *thrA* [102] is still the most commonly used. The MLST database with sequencing data of *S. enterica* is available online (http://mlst.warwick.ac.uk/mlst/dbs/Senterica) [79].

The unambiguity of the results is the major advantage of MLST but the elevated cost and time required are still important limitations.

3.4.3 Phage typing

This method is traditionally used for the surveillance and subtyping of pandemic clones and is based on the selective ability of bacteriophages to infect and lyse certain strains of *Salmonella* belonging to the same serovar. This ability depends on the molecular characteristics of the phage receptor in the bacterial surface and the phage itself [96]. The number and size of plaques produced upon the inoculation of different phage types are recorded to determine the ability to lyse the bacteria and designate them into a definitive phage type (DT) [103].

Although phage typing is a simple method with no specific laboratory requirements and it has shown to be useful for the description of pandemic clones of *Salmonella*, the number of phages available is limited, thus making many strains non typeable and being limited to a few distinguishable types. Moreover, the need for having well-maintained phage stocks and

trained personnel is the reason why this technique is usually performed by reference laboratories [96].

3.5 Non-human reservoir

Except for the human-restricted *Salmonella* Typhi and Paratyphi, another host-restricted pathogen is serovars Gallinarum, only found in poultry [27]. The rest of the *S. enterica* serovars are able to colonize other niches, ranging from the ubiquitous *S.* Typhimurium and Enteritidis found in almost all vertebrates, to other serovars adapted to certain hosts in which they cause different syndromes, as described in **Table 2**. For instance, *S.* Dublin commonly infect cattle triggering intestinal inflammatory disease, bacteremia and causing abortion; whereas serovars Choleraesuis is mostly found in pigs causing septicemia [57,104]. Interestingly, mice infected with serovar Typhimurium bacteria develop a systemic disease similar to human typhoid fever. For this reason, it is used as an animal model to understand the pathogenesis of human-restricted *S.* Typhi [57].

Table 2. Description of diseases and symptoms caused by different *S. enterica* serovars found in humans and higher vertebrates. Adapted from [104].

Host species	Disease	*S. enterica* serovar(s) most frequently encountered	Most susceptible age groups	Typical symptoms or sign(s) of disease
Humans	Salmonella enteritis	Typhimurium, Enteritidis	Children (< 4 yrs)	Diarrhea, dysentery, fever
	Typhoid fever	Typhi	Children and adults	Septicemia, fever
	Paratyphoid fever	Sendai; Patayphi A, B and C	Children and adults	Septicemia, fever
Cattle	Salmonellosis	Typhimurium	Calves (< 8 wk)	Diarrhea, dysentery, septicemia, fever
		Dublin	Calves and adult cattle	Diarrhea, dysentery, septicemia, abortion, fever
Poultry	Pullorum disease	Pullorum	Newly hatched birds	Diarrhea, septicemia
	Fowl typhoid	Gallinarum	Growing stock and adults	Diarrhea, comb discoloration, septicemia
	Avian paratyphoid	Enteritidis, Typhimurium	Newly hatched birds	Diarrhea, septicemia
Sheep	Salmonellosis	Abortusovis	Adult sheep	Septicemia, abortion, vaginal discharge
			Lambs	Diarrhea, dysentery, septicemia

		Typhimurium	Lambs	Diarrhea, dysentery, septicemia
Pigs	Pig paratyphoid	Choleraesuis	Weaned and adult pigs	Skin discoloration, septicemia, fever
	Salmonellosis	Typhimurium	Weaned pigs (< 4 mo)	Diarrhea
	Chronic paratyphoid	Typhisuis		Intermittent diarrhea
Horses	Salmonellosis	Abortusequi	Adult horses	Septicemia, abortion
			Foals	Diarrhea, septicemia
		Typhimurium	Foals	Diarrhea, septicemia
Wild rodents	Murine typhoid	Typhimurium, Enteritidis		Septicemia, fever

At a food safety level, ingestion of contaminated food is an important route of transmission for human salmonellosis. According to the EFSA, *Salmonella* is most frequently found in turkey meat (*S.* Derby, *S.* Typhimurium and *S.* Stanley), followed by poultry (*S.* Infantis and *S.* Enteritidis), pig and bovine meat (*S.* Typhimurium). Interestingly, although *Salmonella* is rarely found in table eggs, they are the major food vehicles associated with 44.9% of the outbreaks, followed by pig meat [105].

In addition to the animal reservoir, *Salmonella* can also colonize vegetables, seeds and raw fruits, entering the food chain and therefore causing disease in animals and humans through the ingestion of contaminated products [106–108]. The nationwide salmonellosis outbreak which occurred in the United States due to peanut derived-products contaminated with *S.* Typhimurium is a clear example of this transmission route. This outbreak affected 714 patients among which 9 people died and resulted in one of the largest recalls in this country, with an estimated loss of $1 billion [109].

4. Antimicrobial treatment

Non-invasive salmonellosis is usually self-limiting and rehydration is required in cases in which serious fluid loss and electrolyte imbalance occur. However, 155,000 deaths occur annually in immunocompromised patients showing signs of invasive disease and in children under 1 year of age; in this risk population, chemotherapy is needed [86]. This is also the

case for typhoid fever, for which the absence of antimicrobial treatment is linked to a mortality of 10-20% [72].

The treatment for typhoid fever and complicated NTS salmonellosis has been changing and adapting together with the spread of drug-resistant strains. Chloramphenicol was the drug of choice until the 1970s, but the emergence of plasmid-mediated resistance to this drug led to the introduction of trimethoprim/sulphamethoxazole and ampicillin as preferred treatments. However, in the 1980s ceftriaxone and ciprofloxacin started to be used as an alternative due to the spread of isolates harboring plasmids containing resistance genes for the previously used antibiotics. Currently, oral administration of the fluoroquinolones ciprofloxacin and ofloxacin are the drugs of choice showing better effectiveness and a lower price compared to ceftriaxone. Nonetheless, empirical treatment can vary depending on drug availability and limitations of economic resources in particular regions.

In the last decades the emergence of isolates showing increased levels of resistance to these compounds has led to the use of new-generation fluoroquinolones (e.g.: gatifloxacin, levofloxacin or moxifloxacin) and other drugs like azithromycin and the extended spectrum cephalosporins ceftriaxone and cefotaxime as alternatives [34,72,110,111].

However, re-emergence of susceptibility to the previously used antibiotics such as chloramphenicol and trimethoprim/sulphamethoxazole has been reported [112] and re-introduction of these drugs to treat typhoid fever has become an option; however, great concerns about the rapid emergence of resistance need to be considered.

The duration of the treatment depends on the severity of the disease and the background of the patient, ranging from 7-14 days to 2-6 weeks in patients with CD4 T-lymphocyte counts below 200 cells/mm^3 [111]. However, co-infection with tuberculosis in HIV-positive patients is frequent and should be taken into account, as fluoroquinolones are also used for the treatment of this disease and thus, the selection of resistant strains can arise more easily [18].

4.1 Quinolones

The quinolone antibiotic family is the origin of fluoroquinolones, which are broad-spectrum antibacterial agents with excellent oral absorption properties and systemic distribution. For these reasons, they are widely used in the management of a variety of bacterial infections. A large number of antibiotics belonging to this class have been developed. Each compound shows diverse chemical features which allow different clinical uses depending on their particular pharmacokinetic and pharmacodynamic properties. Some examples of the currently approved drugs and indications are listed in **Table 3**.

Nalidixic acid is considered to be the first quinolone and the origin of the following generations of this antimicrobial class. It was accidentally discovered by Lesher and co-workers as a by-product in the synthesis of the antimalarial agent chloroquine and was introduced for clinical use in 1962 for the treatment of Gram-negative urinary tract infections in humans and animals [113]. Its limited usefulness was due to high protein binding (around 90%) and a shorthalf-life; thus, modifications of the initial structure were introduced to improve the antimicrobial properties and pharmacokinetic profile of this drug [114,115]. The chemical structure of quinolones consists of heterocycles with a bicyclic core structure (**Figure 10**). The carboxylic acid group at position 3 and the carbonyl at position 4 seem to be essential for the activity of the compound [115], and the introduction of modifications at position 7 play a relevant role in broadening the spectrum of activity. Initially, the addition of a piperazinyl side chain at this latter position, giving rise to pipemidic acid, improved the activity against Gram-negative bacteria (e.g. *Pseudomonas spp*.) thanks to the increased ability of quinolones to penetrate the bacterial cell wall. The introduction of a fluorine atom at position 6 was seen to confer activity against Gram-positive bacteria, obtaining the first fluoroquinolone, named flumequine, patented in 1973 [113,114,116].

Table 3. List of approved clinical uses of the most representative fluoroquinolones. Adapted from [113].

Agent	Approved indication
Ciprofloxacin	Acute uncomplicated cystitis in females (oral use only)
	Urinary tract infections
	Chronic bacterial prostatitis
	Uncomplicated cervical and urethral gonorrhea
	Skin and skin-structure infections
	Bone and joint infections
	Infectious diarrhea (oral use only)
	Typhoid fever (oral use only)
	Complicated intra-abdominal infections, in combination with metronidazole
	Acute sinusitis
	Lower respiratory tract infections
	Nosocomial pneumonia (iv use only)
	Empirical therapy for patients with febrile neutropenia, in comb. with piperacillin sodium (iv use only)
	Inhalational anthrax (after exposure)
	Complicated urinary tract infections and pyelonephritis in pediatric patients (1-17 years old)
Levofloxacin	Uncomplicated urinary tract infections (mild to moderate)
	Complicated urinary tract infections (mild to moderate)
	Acute pyelonephritis (mild to moderate)
	Chronic bacterial prostatitis
	Uncomplicated skin and skin-structure infections (mild to moderate)
	Complicated skin and skin-structure infections
	Acute maxillary sinusitis
	Acute bacterial exacerbation of chronic bronchitis
	Community-acquired pneumonia[a]
	Nosocomial pneumonia
Moxifloxacin	Acute bacterial sinusitis
	Acute bacterial exacerbation of chronic bronchitis
	Community-acquired pneumonia[a]
	Uncomplicated skin and skin-structure infections
Gatifloxacin	Uncomplicated urinary tract infections
	Complicated urinary tract infections
	Pyelonephritis
	Uncomplicated urethral and cervical gonorrhea
	Acute uncomplicated gonococcal rectal infections in women
	Uncomplicated skin and skin-structure infections
	Acute sinusitis
	Acute bacterial exacerbation of chronic bronchitis
	Community-acquired pneumonia[a]
Gemifloxacin	Acute bacterial exacerbation of chronic bronchitis
	Community-acquired pneumonia (mild to moderate)[a]

[a] Includes pneumonia due to multidrug-resistant *Streptococcus pneumoniae*

Subsequently, the second generation of fluoroquinolones emerged by combining both features in positions 6 (fluorine atom) and 7 (piperazinyl group) resulting in norfloxacin, which was patented in 1978. This drug presents broad spectrum activity against Gram-negative and some Gram-positive bacteria but cannot be used for systemic infections due to its poor pharmacokinetic properties. From this point, improvements were rapidly made in norfloxacin and new compounds were synthesized including **ciprofloxacin**, discovered in 1981, ofloxacin, its L-isomer levofloxacin, and others such as enoxacin, fleroxacin and pefloxacin. Structural modifications included the introduction of a cyclopropyl side chain at position 1, as in the case of ciprofloxacin, or a bridging ring between the N-1 and position 8, in ofloxacin and levofloxacin. These compounds are characterized to be well absorbed by the gastrointestinal tract and active against Gram-positive and Gram-negative microorganisms. A third generation of fluoroquinolones was then developed with a more potent bactericidal effect and a greater potency against Gram-positive bacteria, particularly pneumococci, including gatifloxacin, grepafloxacin, sparfloxacin, temafloxacin, tosufloxacin and pazufloxacin. Thereafter, an additional group of compounds with potent activity against anaerobes and increased activity against pneumococci constituted the fourth generation of fluoroquinolones comprising moxifloxacin, trovafloxacin, clinafloxacin, sitafloxacin and gemifloxacin. This last group was characterized by the addition of a substituent at position 8 as is the case of a methoxy group in moxifloxacin [113–115].

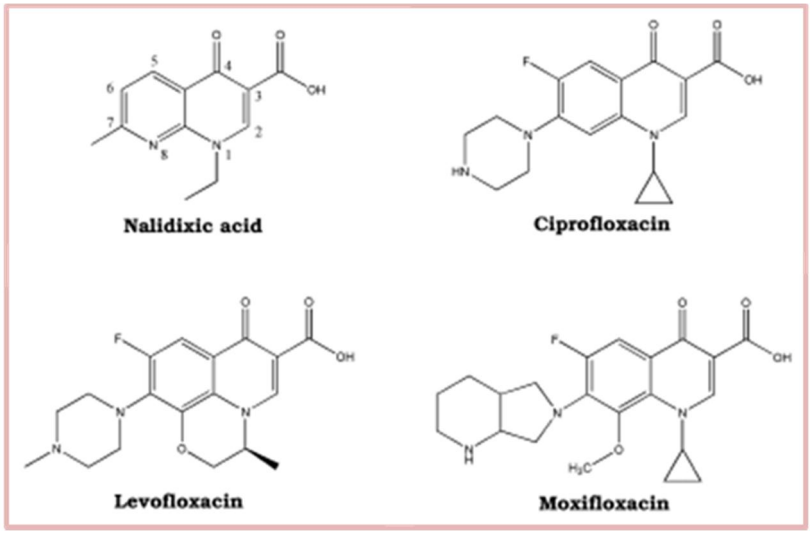

Figure 10. Chemical structure of representative (fluoro)quinolones. Adapted from [115].

4.1.1 Mechanism of action

Quinolones target two enzymes belonging to the type II topoisomerase class called DNA gyrase and topoisomerase IV. In general terms, the antibiotic impedes bacterial DNA replication, transcription and chromosomal segregation. Thus, the consequence of quinolone action is the interception of cellular division and bacterial cell death as a final result.

4.1.1.1 Target genes: DNA gyrase and topoisomerase IV

In the replication and transcription processes helicases unwind the DNA double helix creating an excess of supercoiling. Type II topoisomerases allow the relaxation of this supercoiled DNA by breaking both strands of the DNA chain, crossing them over, and then religating them [114,115]. Although both target enzymes are composed of two A and two B subunits forming a highly homologous (almost 40% amino acid homology) heterotetramer (A2B2), they do have some unique functions. A subunits are involved in DNA breakage and relinking by forming a covalent bond between an active site tyrosine (residues 122 in the A subunit of the DNA gyrase and residue 120 in topoisomerase IV) and the DNA phosphate backbone [117]; while B subunits present ATPase activity. The processes in which DNA gyrase and topoisomerase IV are involved are illustrated in **Figure 11**.

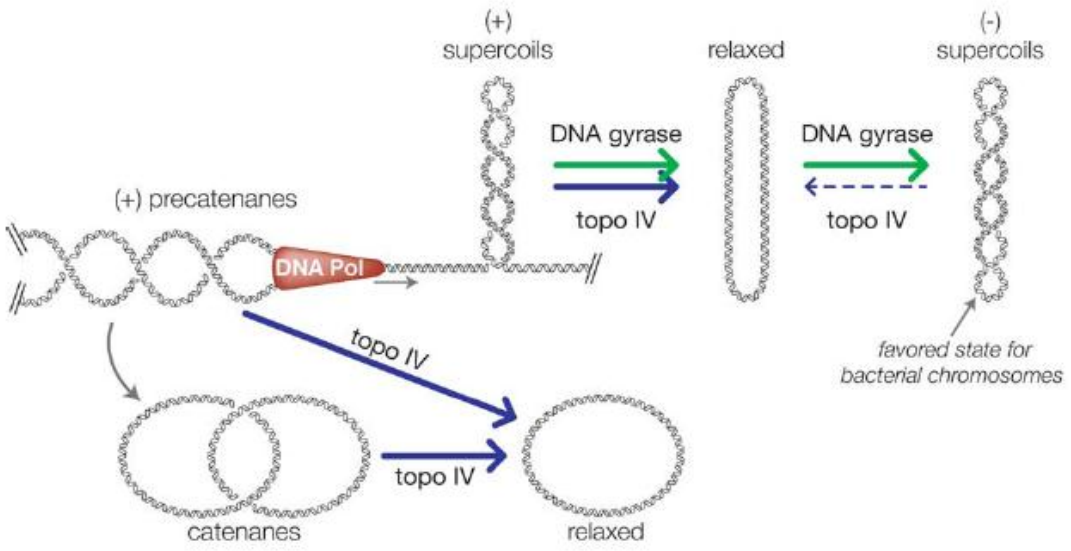

Figure 11. Activities of the DNA gyrase and topoisomerase IV. Adapted from [118].

The **DNA gyrase** is an essential enzyme only present in bacteria, the A and B subunits of which are encoded in the *gyrA* and *gyrB* genes, respectively. This enzyme hydrolyzes ATP to introduce negative supercoils needed for chromosome condensation into DNA and relieve the torsional stress that occurs in the transcription and replication complexes.The A subunits of the DNA gyrase act by generating a pair of single-stranded cleavages of the first DNA segment (called G or gate). These two DNA ends, separated by 4-bp, form a transient gate or gap through which the second DNA segment (T or transfer), captured when the B subunits bind ATP and dimerize, is then passed. In this process, negative supercoiling is generated, with the C-terminus of the GyrA subunit being responsible for this unique activity of the DNA gyrase [115,118].

In the case of **topoisomerase IV**, the A and B subunits are encoded in the *parC* and *parE* genes, respectively. This enzyme also catalyzes relaxation of the supercoiled DNA, but it is not able to introduce negative supercoils, like the DNA gyrase, as mentioned above. Moreover, although the cleavage and resealing mechanism of the DNA segments is similar for both enzymes, the DNA gyrase wraps the DNA around itself, while topoisomerase IV does not [115,117,118]. In addition to this function, topoisomerase IV is also required for its decatenating (unlinking) activity during the terminal stage of DNA replication, segregating newly replicated daughter chromosomes and plasmids [115,118,119]. However, some bacteria species such as *Mycobacterium* spp., *Campylobacter* spp., *Corynebacterium* spp. and *Helicobacterpylori* lack topoisomerase IV, and it has been demonstrated that in these microorganisms the DNA gyrase adopts decatenase activity [120].

4.1.1.2 Drug-target interaction

The mechanism by which interaction between quinolones and the target enzymes occurs has been reported to be through the formation of a stable drug/enzyme/DNA complex. This interaction causes the inhibition of topoisomerase activity as well as poisoning of the cell [115,121].

Recently, crystallography has been used to elucidate the molecular mechanism responsible for the interaction between these antibiotics and the target enzymes, proposing different models. It has been reported that the C-7 ring of quinolones interacts with amino

acids in GyrB/ParC [122,123]. Moreover, some evidence support the idea that the carboxyl end of fluoroquinolones forms a stabilizing magnesium-water bridge with the GyrA residues 83 and 87, which are involved in conferring resistance to quinolones when substituted. This model also places the C-7 ring of the quinolone near the GyrA residue 81 [123]. Several computational-based approaches have also been conducted involving different parts of the enzymes [124–126]. These studies highlight the importance of particular residues in the binding of the quinolone to the DNA gyrase such as GyrA residue 87, as mentioned above, and residue 121 located near the active-site tyrosine of GyrA [127] (see Section 5.3.1.1).

5. Antimicrobial resistance

Development of multidrug resistance (MDR), defined as resistance to three or more antibiotic classes [128], which include chloramphenicol, ampicillin and trimethoprim/sulphamethoxazole in the case of *Salmonella* [129], threatens the efficacy of antimicrobial therapy to treat salmonellosis. Prolonged use of fluoroquinolones has led to an increase in the incidence of nalidixic acid-resistant strains with a decreased ciprofloxacin susceptibility (DCS) phenotype due to mutations in the target genes (see Section 5.3.1.1), resulting in the need to adapt antibiotic treatment to the current situation.

Moreover, one of the major routes of transmission of antimicrobial resistance is also through food of animal origin. Antibiotics are widely used in food animals for different purposes: to treat clinical disease, to prevent and control common disease events, and to enhance animal growth. Antibiotic-resistant pathogens can therefore develop and propagate in the livestock entering the food supply and finally cause human disease [130]. Although a number of antimicrobial drugs have veterinary-restricted use, others such as tetracycline, ampicillin, sulfonamides and quinolones are commonly used in both animal and clinical settings, thus causing public health issues. Accordingly, high levels of resistance to these antibiotics among *Salmonella* strains isolated from meat of different origins, including broilers, turkeys and pigs, have been detected in the last report from the EFSA [105]. Furthermore, MDR clones including isolates with the particular phenotype of nalidixic acid resistance and DCS have spread worldwide in the last years. It is reasonable to believe that

these clones will rapidly replace their susceptible counterparts reducing the therapeutic options to expensive drugs such as carbapenems or tygecycline [111,131].

5.1 Global incidence of antimicrobial resistance

5.1.1 Nontyphoidal *Salmonella* serovars

Antimicrobial resistance has been detected globally over the years in NTS isolates causing salmonellosis. Surveillance data collected from different countries around the world during the period of 1990-2000 highlight this situation, with the overall rate of antimicrobial resistance reaching 70% in some countries; however, these rates vary depending on the serovars involved [132]. According to the EFSA, MDR *Salmonella* isolates corresponded to 31.8% of the clinical isolates recovered in Europe in 2013, showing the highest rates of resistance to ampicillin, sulfonamides and tetracyclines in infections acquired within the European Union [105]. Of great concern are the MDR *S.* Typhimurium phage types 104 (DT104) which emerged in the 1990s and are resistant to ampicillin, chloramphenicol, streptomycin, sulfonamide, and tetracycline and continue to be the cause of outbreaks in different parts of the world [132–135]. An increase in nalidixic acid resistance together with DCS has also been described in different studies [136]; however resistance to ciprofloxacin remains globally low [137].

Regarding iNTS, mostly detected in the African continent (as described in Section 3.1.2), the circulation and spread of *S.* Typhimurium ST313 resistant to chloramphenicol, trimethoprim/sulphametoxazole and ampicillin as well as to additional drugs, is an important concern as therapeutic options are limited [111,138]. An interesting study conducted in Malawi during a 7-year period (1998-2004) reported that >90% of all the *S.* Typhimurium isolates were resistant to ampicillin and trimethoprim/sulfamethoxazole and were susceptible to ciprofloxacin, third-generation cephalosporins and chloramphenicol [75]. However, a sudden acquisition of resistance to the latter antibiotic occurred during the 2001–2002 period, replacing the chloramphenicol-susceptible ST313 clone with a resistant one showing distinct antibiotic resistance loci, as demonstrated in a subsequent study [76].

5.1.2 *Salmonella* Typhi

In areas where typhoid fever is endemic, antibiotic resistance in *S.* Typhi is commonly reported, being Asia the continent in which the highest rates of resistance are detected. **Figure 12** shows the distribution of antimicrobial resistant isolates across the globe according to the data available from the different countries.

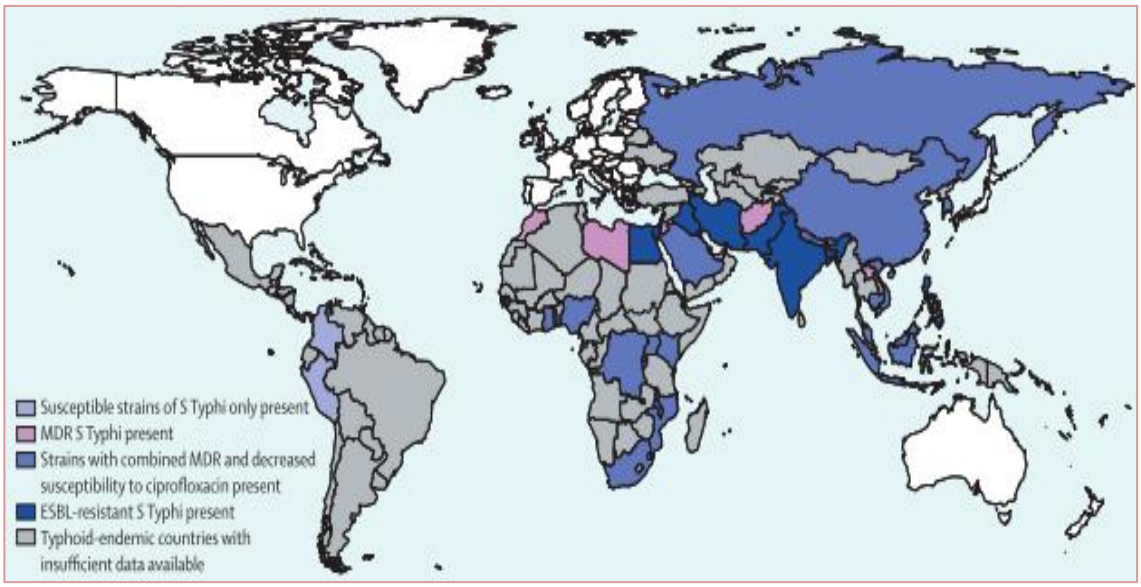

Figure 12. Worldwide distribution of antimicrobial drug resistance in *S.* Typhi isolates. Adapted from [131].

Due to the low burden of typhoid fever in high-income countries, few cases are reported and the majority result from travel-related infections. According to the CDC, in the one-year period from 1996-1997, 81% of the typhoid fever cases reported in the United States were acquired abroad and already accounted for a 9% of nalidixic acid resistance [139]. In 2008 a retrospective study conducted in the same country by Crump *et al.* showed a 19% increase in the number of cases reported with DCS in the period 1999-2002 (from 19% in 1999 to 38% in 2002), 7% being MDR [140]. In Europe, a steady increase has also been described in isolates with DCS, with rates of 26% in 2001 and almost 30% of MDR isolates; however, no isolate was resistant to cefotaxime in this study [141].

Data from Russia is scarce, however, a study conducted during the period 2005-2011 indicated full susceptibility to cephalosporins in contrast with high rates of nalidixic acid resistance [131].

Even though few data are available from Central Asia [131], in the Asian continent an increase in the incidence rate of nalidixic acid resistance concomitantly with DCS has been reported since the late 1990s due to the use of quinolones as first line treatment. In India, in 2001 almost 57% of the isolates showed DCS whereas in 2003 this proportion was almost 90% [142]. Similarly, studies conducted in Bangladesh and Nepal in 2003-2004 reported nalidixic acid resistance in 73% and 75% of the strains, respectively, and all showed DCS [143,144]. However, full resistance to ciprofloxacin has appeared more recently. It was firstly reported in India in 2006 by Gaind *et al.* who described 3 isolates exhibiting an MIC of ciprofloxacin ≥32 mg/L together with resistance to tetracycline and sulphamethoxazole due to the presence of a plasmid harboring resistance determinant genes [142]. In the last decade, several reports from different Asian countries have been published in which ciprofloxacin resistant strains were recovered from patients with typhoid fever, ranging from 8% in isolates from Vietnam to almost 40% in those from Bangladesh [145–148].

MDR has also been reported in several studies in which a progressive increase in the rate of incidence has been described along the years [142,143,145,146,149–151]. For example, a study conducted in India reported 25% of MDR strains in 2013 compared to 13.6% in 2009 [145], while another report in which isolates were collected in 2003-2004 in Bangladesh reported 50-54% of MDR strains [143]. More recently, in a prospective study conducted in Cambodia from 2007 to 2011, Vlieghe *et al.* reported an alarming 75% of MDR isolates [149]. Interestingly, 5% were also resistant to azithromycin suggesting the imminent rise of pan-resistant isolates on the Asian continent, and therefore, all around the world. Although different clones have been identified as being resistant to different antibiotics [145], global expansion of MDR isolates has mostly been linked to the dissemination of a predominant clone, the haplotype H58, most likely originating in India. The authors characterized a number of antimicrobial resistance genes either contained in a plasmid or integrated in the chromosome (see Section 5.2) [146,150,152,153].

Contrarily to Asia and the Indian subcontinent, where extensive information is available about MDR *S.* Typhi, outbreaks in sub-Saharan Africa are rarely documented and data regarding the incidence of antimicrobial resistance is scarce. However, the information available indicates a global increase in the number of MDR isolates as well as a rise in the combined nalidixic resistance and DCS phenotype on the African continent, particularly since the beginning of the 2000s [131,154–156]. Nonetheless, the rates of resistance vary among the countries; while in Zambia only 4.3% of the isolates reported in a recent study were nalidixic acid-resistant with DCS [154], this rate achieved 64% in Kenya [155] and 87.5% in Uganda [157]. According to MDR, the rates were more homogeneous with percentages greater than 75% registered in different countries in the last years [131,154–156].

Although a diversity of MDR clones belonging to different lineages are circulating in different areas of Africa [156], haplotype H58 is now also considered the predominant clone responsible for MDR dissemination on this continent [131,152,153].

5.2 Dissemination of multidrug resistant isolates

As seen in the previous section, the different serovars of *S. enterica* causing disease are constantly adapting and acquiring specific determinants in order to resist to the antimicrobial drugs used against these pathogens. In general terms, the two major strategies of the bacteria to become resistant to antibiotics consist in: a) modification of the target of the antibiotic in order to reduce the affinity between the drug and the target, or b) a decrease in the active intracellular concentration of the antibiotic that will reach the target.

Dissemination of antimicrobial resistance mediated by mutations in the target genes is usually due to the expansion of a particular clone carrying these modifications, usually located in the chromosome. On the contrary, antibiotic resistance genes located in transferable elements (e.g. transposons and plasmids) can be horizontally transferred [158]. Most of the resistance genes are located in specific structures that include an integrase-coding gene (*intI*) and a recombination site (*attI* loci) needed for the incorporation of the resistance-gene cassette. Over 100 integron types have been identified to date and each class has been characterized as having particular gene structures [159]. Integrons found in Enterobacteriaceae belong to class 1 and usually contain the *qacEΔ1* gene, encoding an

incomplete version of an efflux pump which confers resistance to quaternary ammonium compounds, and the *sul1* gene, encoding resistance to sulfonamides. Once inserted in integrons, resistance determinants become transmissible to plasmids or to the chromosome when incorporated into DNA segments flanked by terminal inverted repeats carrying a transposition region called transposons [160–162].

In the clinical setting combination of such mechanisms in a particular clone, which contains chromosome-mediated resistant determinants and acquires a plasmid with additional resistance genes, also occurs. These particular situations have been associated with outbreaks caused by strains resistant to almost all antibiotics and thus, causing important public health concerns. The most relevant MDR clones circulating among *S. enterica* include:

DT104. In this clone resistance genes are located in the chromosomal *Salmonella* genomic island-1 (SGI1), a 43-kb region containing a gene cluster encoding resistance to streptomycin (*aadA2*), chloramphenicol *(floR)*, tetracycline [*tet(G)*], ampicillin ($bla_{\text{PSE-1}}$), and sulfamethoxazole *(sul1)* [133].

Invasive MDR ST313. Resistance determinants in this MDR *S.*Typhimurium have been reported to be inserted in a Tn21-like transposon located in a plasmid, named pSLT-BT for its relatedness to the virulence-associated pSLT plasmid [18,76,92]. These genes include resistance to streptomycin (*strAB and aadA1),* chloramphenicol (*cat*), β-lactams ($bla_{TEM\text{-}50}$) and trimethoprim/sulfamethoxazole (combination of *dhfrI* and *sul1)* [76].

Haplotype H58. Genome sequencing of more than 1,800 *S.* Typhi isolated from patients in Asia and Africa has defined haplotype H58 as the dominant MDR clone. Up to seven resistance genes have been detected, all embedded in the Tn2670-like transposon. This structure is mostly located in the IncHI1-PST6 plasmid but is also detected in the chromosome in 26% of the isolates. Resistance genes found in the Tn2670-like transposon confer resistance to streptomycin (*strAB*), ampicillin ($bla_{TEM\text{-}1}$), chloramphenicol (*catA1*) and trimethoprim/sulfamethoxazole (*dfrA7*, *sul1* and *sul2*). A few isolates have also been shown to harbor the azithromycin resistance genes *ereA*, *msrA* and *msrD*. Quinolone and fluoroquinolone resistance has been reported to be chromosome-mediated and conferred

by mutations in the target genes. However, in some cases resistance was combined with the presence of the plasmid-mediated gene *qnrS1* [152].

5.3 Mechanisms of quinolone resistance

Quinolone resistance acquisition can either be mediated by impaired interaction between the antibiotic and the target or by a decrease in the active concentration of the drug inside the cell, thus reducing the antibacterial effect (see **Figure 13**). Usually, a combination of these mechanisms is responsible for fluoroquinolone resistance. In this section, quinolone resistance mechanisms and regulation of these systems in *S. enterica* are described.

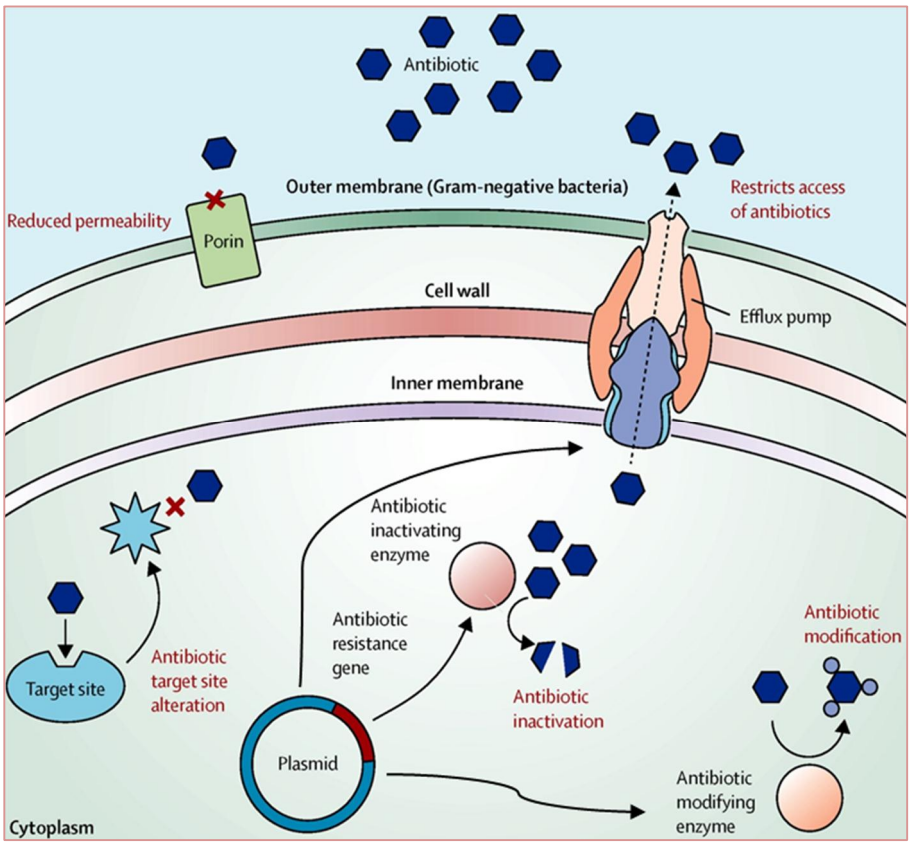

Figure 13. Schematic representation of the main antibiotic resistant mechanisms in Gram-negative bacteria. Adapted from [163].

5.3.1 Chromosome-mediated resistance

5.3.1.1 Mutations in the target genes

As a result of antibiotic pressure due to treatment with (fluoro)quinolones, a sequential acquisition of mutations occurs leading to amino acid changes in specific positions of the so-called quinolone resistance-determining regions (QRDRs) characterized in the DNA gyrase and topoisomerase IV.

The relevance of these target gene mutations has been well characterized in *E. coli*, although information from *Salmonella* isolates also support this situation. First, a single amino acid change in the QRDR of GyrA (residues comprising Ala67 to Gln106), mainly Ser83Leu and Asp87Asn, confers resistance to nalidixic acid (MIC >32 mg/L) and a low level of ciprofloxacin resistance (MIC: 0.25–1 mg/L), known as the DCS (decreased ciprofloxacin susceptibility) phenotype. However, other substitutions are also found at these codons, including Tyr, Phe or Ala at codon 83 and Val, Tyr and Gly at position 87 [70,115,164]. Moreover, it has been demonstrated that different amino acid substitutions at the same codon differentially affect quinolone-target binding and therefore confer a different pattern of quinolone susceptibility. Additionally, it has also been evidenced that selection of a particular amino acid substitution is influenced by the fluoroquinolone used; for example, ciprofloxacin tends to more easily select Asp87Gly whereas enrofloxacin prioritizes the selection of Ser83Phe substitutions [164,165]. Secondly, the further acquisition of an additional mutation in the *parC* gene, either in the amino acid codon Ser80 or in Glu84, confers a greater but still moderate level of ciprofloxacin resistance (MIC: 1–4 mg/L). Subsequently, a third amino acid substitution in GyrA results in a high level of ciprofloxacin resistance (8–64 mg/L), and finally, a fourth change, corresponding to a second mutation in *parC,* is responsible for full resistance to ciprofloxacin (MIC >128 mg/L) [115].

Globally, most of the clinical studies reporting nalidixic acid resistance and a DCS phenotype, mainly among *S.* Typhi, show a single mutation in *gyrA*, being amino acid changes at residue 83 the most commonly found [111,140,144,149–151,155,166], although substitutions in Asp87 have also been described [147]. Despite being much less reported, a combination of mutations in *gyrA* affecting the above-mentioned positions, and *parC,* mainly

in Ser80, have also been found [142,148]. Interestingly, clinical isolates of *S.* Typhi recovered from 2003 to 2007 in South Africa showed amino acid substitutions in ParC at positions Thr57, Thr66 and Ser80, showing that the additional mutation at position 57 was not associated with an increase in resistance to ciprofloxacin [167]. A recent study reported by Chiou *et al.* has shown that resistance to ciprofloxacin in *S.* Typhi is conferred by two amino acid changes in GyrA (residues 83 and 87) and an additional change in ParC (residue 92) together with the presence of the plasmid-mediated *qnrS* determinant and efflux-related factors [146]. Although at lower proportion, amino acid changes in the B subunits of the type II topoisomerases GyrB and ParE have also been described in *S. enterica*. In GyrB, codon Ser464 is considered a target position for mutation, whereas in ParE, Ser458Pro seems to be the most usual [164,168–170]. However, substitutions in other positions have also been described in *S. enterica* and *E. coli* clinical isolates [171,172], and mutations outside these QRDR regions have also been suggested to contribute to fluoroquinolone resistance, although this has not been yet proven [147].

5.3.1.2 Alteration of the intracellular accumulation of the drug

Another strategy developed by bacteria, which is of great concern in *S. enterica,* is to fight against the action of antibiotics through alteration of the outer membrane permeability by: i) an increase in the extrusion of the drug through overexpression of efflux pumps, and ii) a decrease in the entrance of the antimicrobial by limiting the expression of porins [115,173,174].

5.3.1.2.1 Efflux pumps

Efflux transporters are energy-dependent systems expressed in both Gram-negative and Gram-positive bacteria that are used in normal conditions by the bacteria for different purposes such as to maintain homeostasis and expel intracellular metabolites in order to detoxify the cell. Additionally, these systems also extrude antimicrobial agents [175]. Multidrug efflux pumps export a broad range of structural and chemically different antibacterial drugs, being macrolides, tetracyclines and quinolones the most commonly extruded [176]. The important involvement of active efflux in quinolone resistance acquisition has been demonstrated in Enterobacteriaceae showing that inhibition of efflux

reduces the MIC levels of this antimicrobial family independently of the presence of QRDR mutations [177].

Five families of multidrug efflux systems have been described to date: the ATP binding cassette (ABC) family, the major facilitator superfamily (MFS), the resistance-nodulation-division (RND) family, the multidrug and toxic compound extrusion (MATE) family and the small multidrug resistance (SMR) family. The major efflux system involved in quinolone resistance in clinical isolates of Enterobacteriaceae is the AcrAB/TolC system, a three-component structure embedded in the bacterial envelope that belongs to the RND family [115,172,178]. Other efflux systems such as EmrAB (MFS family) and AcrEF (RND family), as well as the pump protein AcrD (RND family) have previously been reported to be related to antimicrobial resistance, including fluoroquinolone resistance, although this has not been seen in the clinical setting [115,179–181].

The AcrAB/TolC system is composed of AcrB, the inner membrane protein which acts as a proton-motive-force-dependent transporter and contains 12 transmembrane segments; TolC, the functional outer membrane protein and AcrA, a periplasmic lipoprotein with an elongated shape which coordinates the outer and inner components allowing the drug efflux through the membranes directly to the extracellular milieu [174]. A representation of AcrAB/TolC is shown in **Figure 14**.

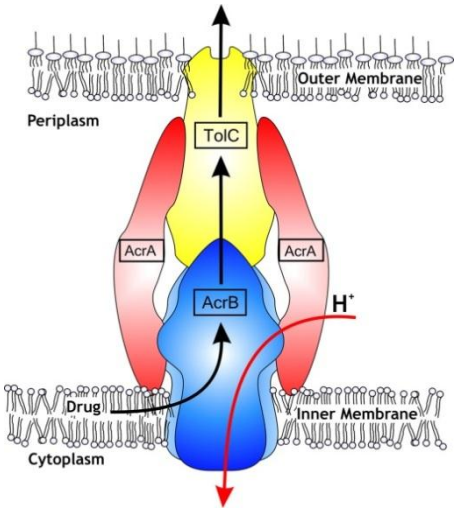

Figure 14. Representation of the AcrAB/TolC efflux system. Adapted from [182].

Regulation of the expression of this efflux system has been well-established for Enterobateriaceae and is mediated by different routes, as shown in **Figure 15**.

The local repressor AcrR, the gene of which is encoded upstream of the *acrAB* operon and transcribed in the opposite direction, controls the expression of the AcrAB pump. Besides, its expression is positively regulated by the transcriptional activators MarA, SoxS and RamA (this latter absent in *E. coli*), which belong to the XlyS/AraC family. These three homologous activators bind to an asymmetric and degenerated 20-bp sequence, usually designated marbox, located upstream of each member of the regulon, such as the *acrAB* and *tolC* operons [115,174,183,184].

In turn, expression of the global activators MarA, SoxS and RamA is controlled by their local regulators encoded in the same regulatory region: *marR*, located in the *marRAB* operon, represses *marA*; *soxR* is encoded in the *soxRS* region and activates *soxS*; and *ramR* is encoded upstream of the *ramA* gene and causes its repression [115].

In addition to the marbox located upstream of the efflux gene operons, as mentioned before, the *marRAB* operon also contains a marbox in its operator region, called *marO*. Therefore, the activators MarA, SoxS and RamA also bind to this region enhancing the expression of the efflux pump through overexpression of MarA (see **Figure 15**) [185,186].

Fluoroquinolone resistance and the MDR phenotype have been linked to mutations in different regulators, resulting in the overexpression of AcrAB/TolC. These mutations disrupt the above-mentioned repressors, and thus, cause a constitutive overexpression of the transcriptional activators leading to increased expression of the efflux system. In the case of the activator *soxR,* the effect of the mutation leading to quinolone resistance is inverse: in normal conditions oxidation or nytrosilation of the SoxR protein is needed for its activation, and thus, for triggering transcription of the *soxS* gene. Mutations in *soxR* confer a constitutive activation of this gene independently of the chemical modifications [186,187].

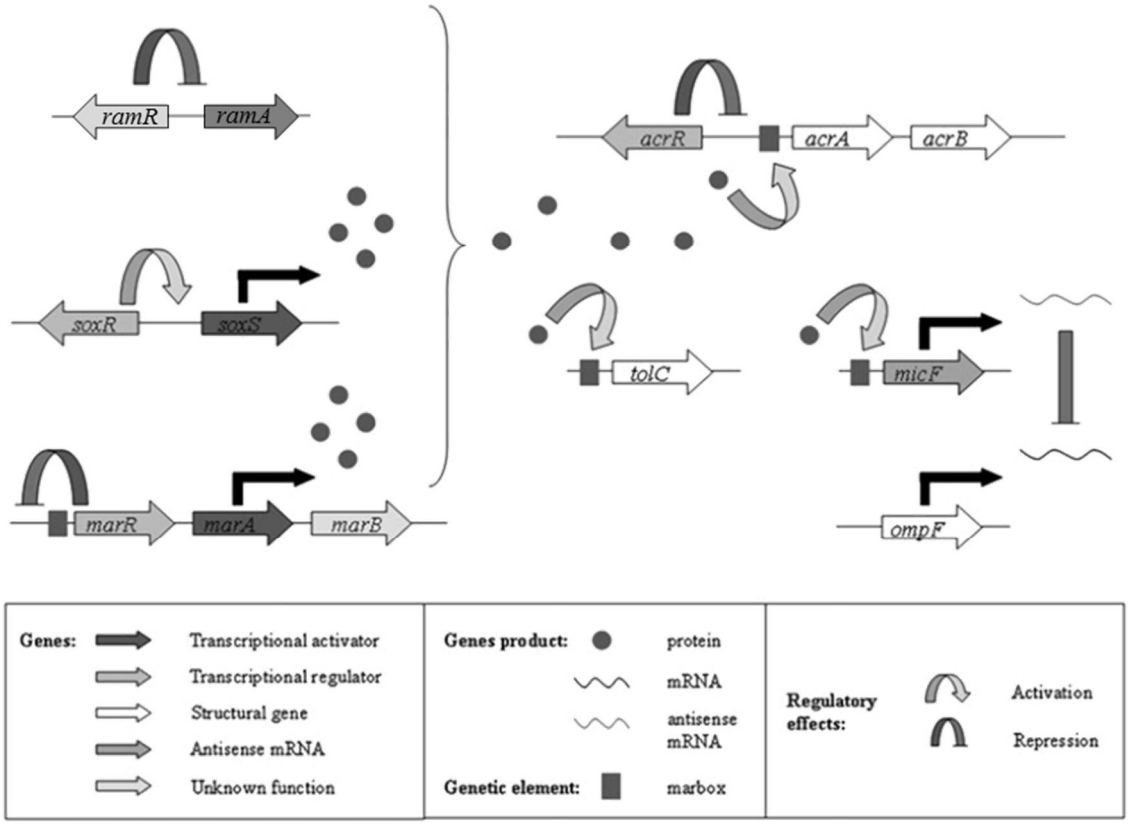

Figure 15. Transcriptional regulation of the major efflux system involved in quinolone-resistance AcrAB/TolC as well as the porin OmpF. Adapted from [115,188].

In *S. enterica*, mutations in the repressor *ramR* [186,188,189] and in the *ramA* promoter [115] have been described to be linked to quinolone resistance as well as to mutations in the activator *soxR* [186,187,190]. Additionally, overexpression of *marA* has been reported [179]; however, mutations in *marR* have not yet been demonstrated in this pathogen, although they have in the case of *E. coli* [191]. Moreover, mutations in the local repressor *acrR* related to fluoroquinolone resistance have also been reported in Enterobacteriaceae clinical isolates [192–194], but in the case of *S. enterica* this relation has only been demonstrated in *in vitro* mutants [195].

5.3.1.2.2 Porins

Porins are proteins anchored in the outer membrane of Gram-negative bacteria that form channels for passive diffusion and allow low molecular-weight solutes to cross the outer membrane and reach the periplasm. Some porins are substrate-specific whereas others

allow the penetration of different compounds, such as certain antibiotics like fluoroquinolones [196]. A decrease in the expression of these bacterial components reduces the internal accumulation of quinolones thus resulting in a reduced concentration of the drug. The major outer membrane proteins found in Enterobacteriaceae, the reduced expression of which is associated with fluoroquinolone resistance, are OmpC and OmpF [115,178,197]. However, porin loss is a minor contributor to the acquisition of quinolone resistance and is not always found in resistant isolates [172,174].

Transcriptional expression of *ompC* and *ompF* is regulated by the two-component EnvZ-OmpR regulatory system activated in response to extracellular osmolarity. In addition, these genes are also post-transcriptionally regulated by the small antisense RNA molecules *micC* and *micF* which downregulate OmpC and OmpF, respectively, by sequence complementation with their mRNA. Besides, the transcriptional homologous activators MarA, SoxS and RamA can also bind to the marbox located in the promoter region of *micF* upregulating its transcription, and therefore decreasing the expression of OmpF (see **Figure 15**) [115,174]. Thus, mutations in these global regulators that lead to an overexpression of the efflux system AcrAB/TolC, as described in the previous section, are also related to a decreased expression of porins and in both situations result in reduced accumulation of fluoroquinolones inside the cell [198].

5.3.2 Plasmid-mediated resistance

Quinolone resistance acquisition can also occur by horizontal gene transfer; however, isolates carrying plasmid-mediated quinolone resistance (PMQR) show a moderate reduction in fluoroquinolone susceptibility and have a minor contribution to nalidixic acid resistance [115].

To date, four different transferable determinants conferring quinolone resistance have been described: the *qnr* genes, the modified aminoglycoside acetyl transferase gene (aac(6')-1b-cr), a quinolone efflux pump (QepA) and the multidrug resistance pump OqxAB [199,200].

Plasmid-mediated quinolone resistance was first described in 1998 in *Klebsiella pneumoniae* by Martínez-Martínez *et al.* [201]. It was then demonstrated that quinolone

resistance was due to the *qnrA* gene encoding for a 218-residue protein with a tandemly repeated unit of five amino acids belonging to the pentapeptide repeat family of proteins. Variants of *qnrA,* differing in >35% in its sequence, have been reported, including *qnrS*, *qnrB, qnrC*, *qnrD* and *qnrVC*, this latter being found in *Vibrio cholerae.* In addition, allelic variants have been reported in each family ranging from 5 alleles for *qnrVC* to a diversity of 71 for *qnrB* [200].

The mechanism of action of Qnr is by protecting the target DNA gyrase and topoisomerase IV from the action of fluoroquinolones. A structural study has recently proposed that the Qnr protein destabilizes the complex formed between the enzyme, the DNA and the drug, allowing the DNA to religate and restore topoisomerase activity [202]. Local and general factors such as the type of promoter, environmental conditions and DNA damage caused by fluoroquinolones, have been reported to influence the expression of Qnr. In spite of its low contribution to quinolone resistance, it is thought that bacterial populations presenting this phenotype facilitate the selection of mutations causing high levels of resistance [115]. However, in general terms, the *qnr* genes are found in plasmids together with other resistance cassettes, thus *qnr*-positive clinical isolates are mostly multidrug resistant [200].

Similarly to *qnr* genes, the modified aminoglycoside acetyltransferase *aac(6')-1b-cr* gene is mainly found as part of an integron in MDR plasmids, although both can be integrated in the chromosome. This gene codifies for a variant of the Aac (6')-Ib enzyme, containing two amino acid changes that are needed for the acetylation of quinolones [203].

In addition to the above-mentioned determinants which act at the level of the drug and targets, two plasmid–mediated efflux pumps have also been described for the first time in *E. coli*. QepA belongs to the MFS superfamily and is responsible for the decrease in susceptibility of hydrophilic fluoroquinolones, such as ciprofloxacin and norfloxacin [204]. It is often located in a 10-kb region flanked by two copies of the insertion sequence IS*26* in plasmids encoding for other resistance genes, such as aminoglycoside ribosomal methylase [115,200]. The other efflux pump is OqxAB, which is encoded by the *oqxAB* operon and belongs to the RND family as the major pump AcrAB. The selection of this drug-resistance

determinant is likely due to the use of the growth promoter olaquindox in animal husbandry, mostly in pigs, and presents cross-resistance to chloramphenicol, trimethoprim, and quinolones such as ciprofloxacin, flumequin, norfloxacin, and nalidixic acid [205]. It is mainly located in a mobile genetic element, being the levels of expression much higher (>80%) than when found in the chromosome [152].

6. Preventive measures

Salmonellosis is a preventable disease but international traveling and exchange of animals and food products have facilitated the global dissemination of pathogens causing disease, and thus, the rapid spread of MDR strains. Greater efforts should be made at different levels in order to decrease the burden of these infectious diseases. Control and prevention of *Salmonella* infection requires the implementation of measures and the creation of challenges focused on: i) the implementation of health education programs and improvement of water quality in places with poor sanitation conditions, ii) promoting and implementing effective food safety interventions on a global scale, iii) inclusion of the current vaccine against typhoid fever in the routine immunization in endemic areas, and iv) improvement of the currently licensed vaccines against *S.* Typhi and the development of vaccines against iNTS [206,207].

Currently, no vaccine is available against NTS serovars causing enteric disease whereas two vaccines are commercially accessible against *S.*Typhi: **Ty21a** and **Vi polysaccharide**.

Ty21a is a live attenuated oral vaccine derived from the Ty2 reference strain treated with the mutagenic agent nitrosoguanidine, which lacks uridine diphosphate (UDP)-galactose-4-epimerase activity (*galE*) and the Vi antigen, although other mutations are responsible for this phenotype [207]. This vaccine has shown to be safe and to have low rates of adverse events. It is available as enteric coated capsules and as a liquid suspension, and both formulas are administered as 1 oral dose every other day. The liquid formulation is licensed for use in children >2 years old and capsules may be used in children over 5 years of age [206–208]. The oral Ty21a vaccine also strongly stimulates cell-mediated immunity through CD4+ helper T-cells and CD8+ cytotoxic-T-cells response and has been reported to confer 53-

78% protection against confirmed typhoid fever [206,207]. This vaccine was first licensed in Europe in 1983 and in 1989 in the USA and is now available in 56 countries in Asia, Africa, Europe and America [207], with every country having its own administration schedule. Its use has been mainlyrestricted to protect travelers and not to control endemic typhoid fever in endemic countries.

The other currently licensed vaccine is the **injectable Vi polysaccharide vaccine** which was developed in the 1980s and consists in the chemically purified Vi antigen of the Ty2 strain using the same technique as that used for the preparation of the meningococcal PS vaccine. It was first licensed in the USA in 1994 and is administered subcutaneously or intramuscularly as a single dose of 25 μg of the purified antigen. To maintain protection, re-vaccination every 3 years is recommended since the Vi polysaccharide stimulates the production of antibodies depleting the pool of memory B cells without generating new ones. The impact and effectiveness of this vaccine has been proven in different vaccination programs and efficacy studies have demonstrated around 70% of protection in immunized people [206].

In order to overcome the lack of immunogenicity in children under the age of 2 reported with the currently available vaccines, the development of conjugated Vi vaccines has been stimulated. Several Vi polysaccharide–protein conjugate vaccine candidates are now under development (or are nationally licensed but not for the international market), and it is foreseen that they will be available in the future for infant immunization [89,207–209]. Among them, the purified Vi polysaccharide conjugated to tetanus toxoid is only licensed in India after demonstrating safety and immunogenicity in a multisite clinical trial in this country, but data of efficacy is lacking as randomized controlled field trials have not yet been performed [208].

WORK JUSTIFICATION

II. WORK JUSTIFICATION

Infections due to *Salmonella enterica* are of great concern worldwide as they represent an important cause of morbidity and mortality.

Resistance to antibiotics used to treat salmonellosis has emerged along the years, and thus, the treatment of choice has been changing in order to adapt to the new features of the circulating pathogens causing disease. Currently, fluoroquinolones, mainly represented by ciprofloxacin and ofloxacin, are widely used to treat this kind of infections although other antimicrobial classes as well as new generations of fluoroquinolones are sometimes required when treatment failure occurs. This situation is mainly due to the rise in the number of isolates showing nalidixic acid resistance associated with a decrease in the susceptibility to fluoroquinolones (e.g. ciprofloxacin). Moreover, the spread of multidrug resistant isolates carrying several resistance plasmid or chromosomally-located determinants also explains, in part, the decrease in the efficacy of the current treatment and represents an important issue.

Despite this trend, a low frequency of fluoroquinolone-resistant *Salmonella enterica* isolates are still reported in the literature, a fact that has been the object of attention in our research group. In order to explain the current scene, we have hypothesized a presumable link between quinolone-resistance acquisition and the decrease in the virulence features among this species.

This PhD thesis addresses the context of *Salmonella* from different perspectives, including an epidemiological approach as well as *in vitro* models in order to understand the biology of this pathogen and its relation with quinolone resistance. The objectives established for the accomplishment of this research work are described in the next chapter.

OBJECTIVES

III. OBJECTIVES

In order to develop our hypothesis, the following specific objectives were defined:

1. Evaluation of the clonal relatedness of *S.* Enteritidis and *S.* Typhimurium clinical isolates using two different typing techniques.

2. Analysis of the outer-membrane subproteome of *S.* Typhimurium SL1344.

3. Investigation of the molecular mechanisms of quinolone resistance and their regulation.

4. Assessment of virulence-related properties in clinical and *in vitro*-selected mutants of *Salmonella enterica* isolates presenting different degrees of susceptibility/resistance to quinolones.

5. Identification of novel genes potentially involved in quinolone resistance and/or virulence in *S.* Typhimurium.

6. Genome comparison of *S.* Typhimurium isolates causing invasive *versus* non-invasive salmonellosis from different geographical areas.

Objectives 1 and **3** are accomplished in **Paper I** whereas **Objective 2** is fulfilled in **Paper II**. **Objectives 3** is achieved in **Paper V** as well as in **Papers III** and **IV**, and these latter two papers also contributed to the fulfillment of **Objective 4**. **Objective 5** is completed in the **Manuscript**; and **Additional Results** addresses **Objectives 4** and **6**.

RESULTS

IV. RESULTS

Taking into account the main objectives achieved in the Papers, Manuscript and Additional Results presented in this PhD thesis, the results have been classified into these five different sections:

1. Molecular characterization of quinolone resistance mechanisms and regulation in *Salmonella enterica.*

Paper I

Molecular study of quinolone resistance mechanisms and clonal relationship of *Salmonella enterica* clinical isolates.

Clara Ballesté-Delpierre, Mar Solé, Òscar Domènech, Jordi Borrell, Jordi Vila and Anna Fàbrega

International Journal of Antimicrobial Agents 43 (2014) 121– 125

The number of *Salmonella enterica* strains resistant to nalidixic acid has steadily increased in the last years. The aim of this study was to perform an in depth study of the molecular mechanisms responsible for quinolone resistance in a collection of *S. enterica* clinical isolates recovered from the period 2007-2008 at the Hospital Clínic (Barcelona, Spain). This collection included 38 *S. enterica* clinical isolates (19 *S. enterica* serovar Typhimurium and 19 *S. enterica* serovar Enteritidis), among which 42% showed nalidixic acid resistance (MIC ≥32 µg/mL) and ciprofloxacin susceptibility (MIC ≤0.75 µg/mL). Previous results showed that all the quinolone-resistant isolates belonged to the Enteritidis serovar. Resistant strains were screened for mutations in the quinolone resistance-determining regions (QRDRs) of the target genes *gyrA*, *gyrB* and *parC* as well as in the plasmid-mediated quinolone resistance genes *qnr, aac(6')-Ib-cr, qepA* and *oqxAB*. All harbored a single amino acid substitution in GyrA: D87Y in all cases except one, affecting position 83, S83F. Moreover, the MIC of nalidixic acid decreased from 4- to 64-fold when assessed in the presence of the efflux pump

inhibitor phenyl-arginine-β-naphthylamide (PAβN), suggesting the contribution of efflux in the acquisition of quinolone resistance.

In the present study, mutations in the QRDR of *parE* as well as in the regulators of the major efflux pump AcrAB (*acrR*, *marRAB*, *soxRS* and *ramR*) were screened. Gene expression of the efflux pump components *acrB*, *tolC*, *acrF* and *emrB* was also assessed by real-time PCR. Additionally, intracellular accumulation of nalidixic acid and ciprofloxacin was determined by means of a fluorescence method.

A second part of this study was dedicated to performing an epidemiological study using two different typing tools: MLST (multilocus sequence typing) and PFGE (pulsed-field gel electrophoresis). For this latter technique, two different restriction enzymes (XbaI and BlnI) were used for *S*. Enteritidis whereas only XbaI was used for *S.* Typhimurium. The objective was to evaluate the discriminatory power of these two techniques to differentiate clones of *S*. Typhimurium and *S*. Enteritidis.

Concerning the study of the mechanisms of resistance to quinolones, no mutations were detected in *parE*, whereas two amino acid substitutions were found in MarR (I84L) and AcrR (N214T) in one strain each of two susceptible strains, suggesting their role as polymorphisms. Moreover, efflux-related genes *acrB*, *tolC*, *acrF* and *emrB* were not differentially expressed between nalidixic acid-resistant and -susceptible strains. Intracellular accumulation of nalidixic acid could not be evaluated due to the low fluorescence properties of this drug, and no differences were seen between nalidixic acid-resistant and –susceptible strains when this assay was performed with ciprofloxacin. This was likely due to the low levels of resistance to this antimicrobial agent.

Altogether, the lack of contribution of the known resistance mechanisms related to antibiotic extrusion found in this study suggests the presence of novel efflux systems and/or regulatory genes as yet undiscovered.

Regarding the results obtained from the epidemiological approach conducted in the second part of this work, *S*. Enteritidis isolates showed a more important clonal relatedness than *S*. Typhimurium isolates. All *S.* Enteritidis isolates belonged to ST11, except two which

were not associated with any characterized ST (sequence type); a similar pattern was seen among all *S*. Enteritidis isolates by PFGE. In contrast, MLST was able to distinguish 3 different STs among *S.* Typhimurium strains: ST19 was the predominant ST (n=12), followed by ST34 (n=5) and ST334 (n=1); only one isolate could not be categorized as any known ST. The PFGE technique classified the strains into 12 different pulsotypes, demonstrating a greater discriminatory power than MLST for this serovars.

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Molecular study of quinolone resistance mechanisms and clonal relationship of *Salmonella enterica* clinical isolates

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ABSTRACT

In the last few years, the number of *Salmonella enterica* strains resistant to nalidixic acid has steadily increased. In a previous study, the quinolone susceptibility phenotype and genotype of 38 *S. enterica* clinical isolates (19 *S. enterica* serovar Typhimurium and 19 *S. enterica* serovar Enteritidis) were determined. Forty-two percent of the isolates showed nalidixic acid resistance associated with a mutation in *gyrA* together with putative overexpression of efflux pump(s). In this study, mutations in the quinolone resistance-determining region (QRDR) of *parE* and the regulators of AcrAB (*acrR*, *marRAB*, *soxRS* and *ramR*) were analysed. Intracellular accumulation of ciprofloxacin and nalidixic acid was determined. Gene expression of the efflux pump components *acrB*, *tolC*, *acrF* and *emrB* was also assessed. In addition, an epidemiological study of the isolates by multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE) was performed. No mutations were detected in *parE*, whereas two amino acid substitutions were found in two susceptible strains in MarR (I84L) and AcrR (N214T) in one strain each, although both were suggested to be polymorphisms. No changes in the gene expression of *acrB*, *tolC*, *acrF* and *emrB* were detected between nalidixic-acid-resistant and -susceptible strains. Intracellular accumulation was not useful to reveal differences. Epidemiological analysis showed an important clonal relatedness among the *S.* Enteritidis isolates, whereas major divergence was seen for *S.* Typhimurium. Altogether, these results suggest the presence of previously undiscovered drug efflux pump(s) and confirm the high clonality of *S.* Enteritidis and the genetic divergence of *S.* Typhimurium.

1. Introduction

Salmonellosis is one of the major causes of food-borne gastroenteritis in humans worldwide. Currently, among the high diversity of *Salmonella enterica* serovars, *S. enterica* serovar Typhimurium and *S. enterica* serovar Enteritidis are the two major aetiological agents. Although antimicrobial resistance in *S.* Enteritidis is reported to be lower than in *S.* Typhimurium [1], in the last few years the prevalence of multidrug-resistant *S.* Enteritidis isolates has increased. Of special concern is the increase in resistance to nalidixic acid and the decrease in susceptibility to fluoroquinolones such as ciprofloxacin, which is one of the first-choice agents to treat salmonellosis.

Quinolone resistance in Enterobacteriaceae is mainly due to point mutations in the quinolone resistance-determining regions (QRDRs) of the target genes that encode both the A and B subunits of the two type II topoisomerases, namely DNA gyrase (*gyrA* and *gyrB*) and DNA topoisomerase IV (*parC* and *parE*). Amino acid substitutions are mostly located in two particular positions in GyrA (S83 and D87) and in ParC (S80 and E84). However, mutations in *gyrB* and *parE* have also been described [2].

Resistance to quinolones is also due to changes in membrane permeability causing a reduction in concentration of these drugs in the cytoplasm. This phenotype can be achieved by a decrease in the expression of porins or by overexpressing efflux systems. The major efflux system in *Salmonella* is AcrAB-TolC, a tripartite multidrug efflux system belonging to the resistance nodulation-division (RND) family [3]. Other efflux systems such as EmrAB, which belongs to the major facilitator superfamily (MFS), and AcrEF, an RND member, have previously been reported to show increased expression levels in fluoroquinolone-resistant mutants [4].

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Table 1
List of primers used in this study.

Gene	Primers (5′–3′)	Annealing temperature (°C)	Reference
For sequencing			
Quinolone resistance-determining region (QRDR)			
parE	SparE.1 CCTGCGGCCCGGCGTTGCCGGGG	62	[8]
	SparE.2 CGCCCGCCTTCTCTTCTTCCGTCAGCGCG		
Regulatory genes			
soxRS	Ssox.1 GGCACTTTGCGAAGGCGTTACCA	54	[8]
	Ssox.2 GGGATAGAGCGAAAGACAA		
marRAB	Smar.1 AGCGGCGGACTTGTCATAGC	58	[8]
	Smar.2 ACGGTGGTTAGCGGATTGGC		
acrR–acrA	Sacr.1 CAGTGGTTCCGTTTTTAGTG	58	[8]
	Sacr.2 ACAGAATAGCGACACAGAAA		
ramR	SramR.1 CGTGTCGATAACCTGAGCGG	62	[15]
	SramR.2 AAGGCAGTTCCAGCGCAAAG		
For real-time PCR			
acrB	AcrB_RT_F TTTTGCAGGGCGCGGTCAGAATAC	60	[5]
	AcrB_RT_R TGCGGTGCCCAGCTCAACGAT		
tolC	TolC_RT_F GTGACCGCCCGCAACAAC	60	This study
	TolC_RT_R ATTCAGCGTCGGCAGGTGAC		
16S	16S_RT_F GCGGCAGGCCTAACACAT	60	[7]
	16S_RT_R GCAAGAGGCCCGAACGTC		
acrF	SacrF.RT.1 TACCCAGGACGACATCTCTGA	60	This study
	SacrF.RT.2 CACACCATTCAGACGGCTGAT		
emrB	SemrB_RT.F CCGTCGTCCTGATGACGTTA	60	This study
	SemrB_RT.R CCGTTCGGTATGCGTTTCAC		

Expression of AcrAB is controlled by AcrR, the local repressor of AcrAB, and the global regulators MarA (*marRAB*), SoxS (*soxRS*) and RamA. Mutations in *marR*, *soxR* and *ramR* lead to an increase in the expression of the transcriptional activators MarA, SoxS and RamA, respectively, which in turn interact with the *acrAB* promoter, thereby increasing the amount of AcrAB produced and effectively enhancing efflux. Otherwise, mutations in the local repressor *acrR* impair its repressive function and hence AcrAB can be overproduced [5].

Quinolone resistance in *Salmonella* can also be plasmid-mediated through the transmission of several determinants: Qnr, a pentapeptide repeat protein family that protects the target topoisomerases from quinolone inhibition; the Aac(6′)-Ib-cr protein, which acetylases fluoroquinolones; and two plasmid-encoded efflux pumps, QepA (MFS-type) and OqxAB (RND family) [2].

In this study, mutations in the QRDR of *parE* and in the regulators of AcrAB (*acrR*, *marRAB*, *soxRS* and *ramR*) were analysed, as was the expression of some efflux pumps components (acrB, tolC, acrF and emrB), quinolone accumulation and clonal relatedness of in quinolone-resistant and -susceptible *Salmonella* enterica clinical isolates.

2. Materials and methods

2.1. Bacterial strains

Forty-one *S. enterica* clinical strains were isolated during the period 2007–2008 in the Department of Clinical Microbiology of the Hospital Clínic, Barcelona (Spain). Both chromosomal (mutations in the QRDRs of the *gyrA*, *gyrB* and *parC* genes) and plasmid-mediated quinolone resistance mechanisms [presence of *qnr*, *aac(6′)-Ib-cr* and *qepA* genes] were initially characterised in a previous study [6]. Among these, 19 *S.* Typhimurium and 19 *S.* Enteritidis were recovered for further study.

2.2. Screening for mutations in the quinolone resistance-determining region of parE and the multidrug resistance regulatory genes (acrR, marRAB, soxRS and ramR) and promoter regions

Amplification of the QRDR of *parE* and the regulatory genes *acrR*, *marRAB*, *soxRS* and *ramR* including their promoter regions was carried out by PCR. PCR products were purified with a Gel Extraction Kit (Omega Bio-tek, Norcross, GA) and were sent for sequencing at Macrogen Inc. (Amsterdam, The Netherlands). Screening for mutations was done using BioEdit® software (Ibis Biosciences, Carlsbad, CA) through alignment with the corresponding template sequences, obtained from the genome of *S.* Enteritidis (RefSeq NC_003197.1) and *S.* Typhimurium (RefSeq NC_011294.1). The primers used for PCR amplification and sequencing are listed in Table 1.

2.3. RNA extraction and DNase treatment

Five *S.* Enteritidis isolates, comprising three nalidixic-acid-resistant isolates (44819, 20055 and 12345) and two nalidixic-acid-susceptible isolates (22601 and 35397) were selected for RNA extraction, as well as one *S.* Typhimurium strain that overexpresses AcrB (50–64) [8] to ensure the validity of the results.

RNA extraction was carried out as previously described [8]. Briefly, mid-logarithmic phase cultures [optical density at 600 nm (OD_{600}) = 0.4–0.6] were treated with RNAprotect™ Bacteria Reagent (QIAGEN, Hilden, Germany) and were harvested by centrifugation. Pellets were re-suspended in a mixture of TE [Tris–ethylene diamine tetra-acetic acid (EDTA)] and lysozyme and RNA extraction was achieved using an RNeasy® Mini Kit (QIAGEN). All of the procedures were performed according to the manufacturer's recommendations.

To remove contamination by genomic DNA (gDNA), all samples were treated with a DNA-free DNase Kit (Ambion, Austin, TX) and PCR was conducted to confirm the loss of gDNA. Quantification of the decontaminated RNA was accomplished with an ND1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Five independent RNA extractions of each isolate were performed.

2.4. cDNA synthesis and real-time PCR

Retro-transcription of 500 ng of each RNA sample was performed according to the manufacturer's instructions (Takara, Dalian, China). Then, the cDNA template was diluted 1/5 to a final amount of ca. 40 ng/μL.

The genes selected for gene expression analysis were a*crB*, *tolC*, *acrF* and *emrB*, and the 16S gene was used as an endogenous control.

The primers used (Table 1) were designed with Primer Express® software (Applied Biosystems, Foster City, CA) and their efficiency was evaluated by triplicate standard curves tested in three different assays as well as a melting curve to confirm the absence of primer–dimer formation.

Real-time PCR was performed in a StepOne™ Real-Time PCR System (Applied Biosystems) using a SYBR® Premix Ex *Taq* (Tli RNase H Plus Kit) (Takara) and the Universal Thermal Cycling conditions, which included 2 min at 50 °C (UNG activation), 10 min at 95 °C (enzyme activation) followed by 40 cycles of 95 °C for 15 s (denaturation) and 60 °C (anneal/extension) for 1 min. At the end of each PCR reaction, a melting curve was also performed. Primers were used at a concentration of 3 μM and the cDNA template was diluted 1/5 in the mix reaction. Relative gene expression was evaluated using the $2^{-\Delta\Delta CT}$ method to calculate fold induction of expression of the target gene. Normalisation of the transcriptional levels was done comparing expression of the 16S gene as an endogenous control.

2.5. Intracellular accumulation

Quinolone accumulation (ciprofloxacin and nalidixic acid) was performed as previously described by Mortimer and Piddock [9]. Briefly, bacteria were grown to late-log phase at 37 °C (OD_{600} = 0.8–0.9) and a pellet was obtained and washed in 50 mM sodium phosphate buffer (PBS) at pH 7.0. A final concentration of 10 μg/mL for ciprofloxacin and 200 μg/mL for nalidixic acid was added and aliquots of 500 μL were taken at different time intervals for 10 min. After 5 min, 100 μM of carbonyl cyanide *m*-chlorophenylhydrazone, an energy uncoupler, was added to the reaction. Every aliquot was diluted in 1 mL of ice-cold PBS, centrifuged and lysated for 15 h in 0.1 M glycine hydrochloride (pH 3.0). After centrifugation, the supernatant was measured in an SLM AMINCO 8100 spectrofluorometer (Spectronic Unicam, Cambridge, UK).

The concentration of the drug was calculated by comparison with a standard curve in 0.1 M glycine hydrochloride (pH 3.0).

2.6. Multilocus sequence typing (MLST)

Amplification of internal fragments of the seven housekeeping genes (*aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA* and *thrA*) was performed by PCR and was confirmed in 1% agarose gel. The corresponding bands were recovered and sequenced at Macrogen Inc. (Seoul, South Korea), as described above. Screening for mutations was also done using BioEdit® software. For every isolate, and according to the allelic definition of each of the seven sequences, a sequence type (ST) was assigned using the MLST Databases at the Environmental Research Institute (ERI), University College Cork (http://mlst.ucc.ie/mlst/dbs/Senterica).

2.7. Pulsed-field gel electrophoresis (PFGE)

Macrorestriction of total DNA from the isolates was adapted from the previously described protocol [10] using a CHEF DR III system (Bio-Rad Laboratories, Richmond, CA). Briefly, overnight cultures were adjusted to 1.5 McFarland standard. Then, 1 mL was pelleted and re-suspended in 120 μL of the suspension buffer [CSB; 100 mM Tris–HCl (pH 8.0), 10 mM EDTA] and was mixed with an equal volume of a 2% InCert® agarose (Lonza, Rockland, ME) prepared in CSB with 1% sodium dodecyl sulphate. Cell lysis was then performed by incubating the samples during 120 min at 55 °C with a mixture of 0.5 M EDTA, 1% *N*-lauroylsarcosine and 400 μg/mL proteinase K. Then, samples were digested by overnight incubation at 37 °C with 30 U per plug of the restriction endonuclease *Bln*I (Takara) for *S.* Enteritidis and with *Xba*I (New England BioLabs, Ipswich, MA) at a concentration of 40 U per plug for both *S.* Enteritidis and *S.* Typhimurium. The running conditions consisted of 6 V with pulse times of 2.2–50 s and linear ramping at 14 °C for 19.5 h.

3. Results and discussion

3.1. Quinolone resistance and mutations in the target genes

In a previous study, the quinolone resistance mechanisms of 38 *S. enterica* clinical isolates (19 *S.* Typhimurium and 19 *S.* Enteritidis) were initially studied [6]. Of 38 strains, 16 (42.1%), corresponding only to *S.* Enteritidis isolates, were resistant to nalidixic acid [assuming a minimum inhibitory concentration (MIC) ≥ 32 μg/mL for resistance versus MIC ≤ 12 μg/mL for susceptibility], whereas all strains were susceptible to ciprofloxacin (MIC ≤ 0.75 μg/mL). All *S.* Typhimurium isolates were susceptible to nalidixic acid. These results differ from the global situation in which the proportion of nalidixic-acid-resistant *S.* Enteritidis isolates remains lower than that reported for *S.* Typhimurium, as previously described [1]. Nonetheless, our findings support the current scenario observed in Europe in which resistance to nalidixic acid among *Salmonella* increased from 0.8% in 1995 to 8.5% in 2000 [11], together with a decrease in susceptibility to ciprofloxacin, as reported in a Danish study. As has been stated, overuse of quinolones in human medicine and the veterinary setting has contributed to the increase and spread of resistant isolates [12].

All of the resistant strains showed the same amino acid substitution in the QRDR of GyrA (D87Y), except one strain with S83F. No changes were found either in GyrB or in ParC. In the present study, no changes were observed in the QRDR of *parE* in any of the 38 *Salmonella* isolates, thereby assigning the substitution D87Y in GyrA as the most prevalent. Further studies support *gyrA* mutations as the most frequently detected at position D87 in addition to S83, leading to different amino acid substitutions. However, the prevalence of the replacing amino acid can vary depending on the study [13].

3.2. Contribution of efflux pumps and their regulators

In this collection and as reported previously [6], in the presence of the efflux pump inhibitor phenyl-arginine-β-naphthylamide, the MIC of the nalidixic-acid-resistant strains decreased 4–64-fold, whereas a decrease of 1.5–10.6-fold was observed for the susceptible strains. These differences in the MICs suggested a major contribution of efflux pump(s) in the nalidixic-acid-resistant strains.

Thus, intracellular accumulation of ciprofloxacin and nalidixic acid was assessed in four nalidixic-acid-resistant *S.* Enteritidis strains (29860, 44819, 20055 and 12345) compared with two susceptible strains (22601 and 35397). For ciprofloxacin, no differences were detected likely due to the low levels of resistance to this antimicrobial agent. Although the intracellular accumulation of nalidixic acid was assessed, the results obtained were not conclusive owing to the low fluorescence properties (data not shown).

Accordingly, we next searched for mutations in the AcrAB regulators. Only two point mutations leading to uncharacterised amino acid changes were detected in two different strains of *S.* Typhimurium (strains 7660 and 39645) in the regulatory proteins MarR (I84L) and AcrR (N214T), respectively. As these changes were found in isolates susceptible to nalidixic acid (MICs of 6 μg/mL and 8 μg/mL, respectively), we assumed that they play a role as polymorphisms. Other similar situations have also been observed in a previous study, in which several changes in MarR (S33N, A533E, G1033S and Y1373H) were not linked to a loss of repressor activity

Table 2
Characterisation of the molecular mechanisms of resistance among the collection of *Salmonella enterica* serovar Enteritidis and *S. enterica* serovar Typhimurium isolates and relative gene expression of efflux pumps.

Salmonella serovar	MIC of nalidixic acid	MIC decrease with PAβN	QRDR mutation(s) in GyrA	Regulatory gene mutations	Ranges of relative gene expression levels[a]			
					tolC	*acrB*	*acrF*	*emrB*
S. Enteritidis ($n = 19$)	R ($n = 16$), 64–512 μg/mL	4–64×	D87Y ($n = 15$), S83F ($n = 1$)	–	1.07–1.36	1.04–1.13	1.41–1.71	1.45–2.2
	S ($n = 3$), 8 μg/mL	2.6–5.3×	–		1–1.28	1–1.17	1–1.68	1–1.33
S. Typhimurium ($n = 19$)	S ($n = 19$), 4–12 μg/mL	1.5–10.6×	–	MarR (I84L) ($n = 1$), AcrR (N214T) ($n = 1$)				

MIC, minimum inhibitory concentration; PAβN, phenyl-arginine-β-naphthylamide (an efflux pump inhibitor); QRDR, quinolone resistance-determining region; R, resistant; S, susceptible.
[a] Values obtained from the resistant isolates 44819, 20055 and 12345 and the susceptible isolates 22601 and 35397.

[14]. As far as we know, the polymorphism detected in AcrR is the first reported to date. Unfortunately, additional mutations were not detected either in the regulatory genes or in their promoter regions. The absence of such mutations supports the idea of previous studies demonstrating that the inactivation of *marR*, *marA*, *soxR* or *soxS* does not affect the susceptibility of the strain [15,16].

Based on these results, gene expression analysis was performed for *acrB* and *tolC*, as well as for *acrF* and *emrB*, two other efflux pumps that have been shown to be overexpressed in quinolone-resistant mutants [4]. Surprisingly, no differences were found in the expression of any of these genes in three nalidixic-acid-resistant compared with two susceptible isolates (Table 2). The absence of a significant increase in the expression of any of the tested efflux components infers the existence of other resistance mechanisms related to antibiotic extrusion such as novel efflux systems and/or regulatory genes undiscovered until now.

3.3. Epidemiology

To evaluate the genetic relationship among the isolates belonging to *S.* Enteritidis and *S.* Typhimurium serovars, all of the strains were studied by MLST and PFGE, the most frequently used techniques. All *S.* Enteritidis were typified by MLST as ST11, the most commonly found ST in the Enteritidis serovar (http://mlst.ucc.ie/mlst/), except two, which showed the same pattern but did not have an associated ST. Moreover, even though samples were digested separately with the endonucleases *Bln*I and *Xba*I in order to increase the discriminatory power, the PFGE technique revealed the same pulsotype for all of the strains. Owing to the high clonality of *S.* Enteritidis isolates, new methodologies are emerging with high discriminatory power to differentiate homogenous populations, such as the use of PFGE with several restriction enzymes [17] and the genotyping technique of multilocus variable number tandem repeats (MLVA) [18].

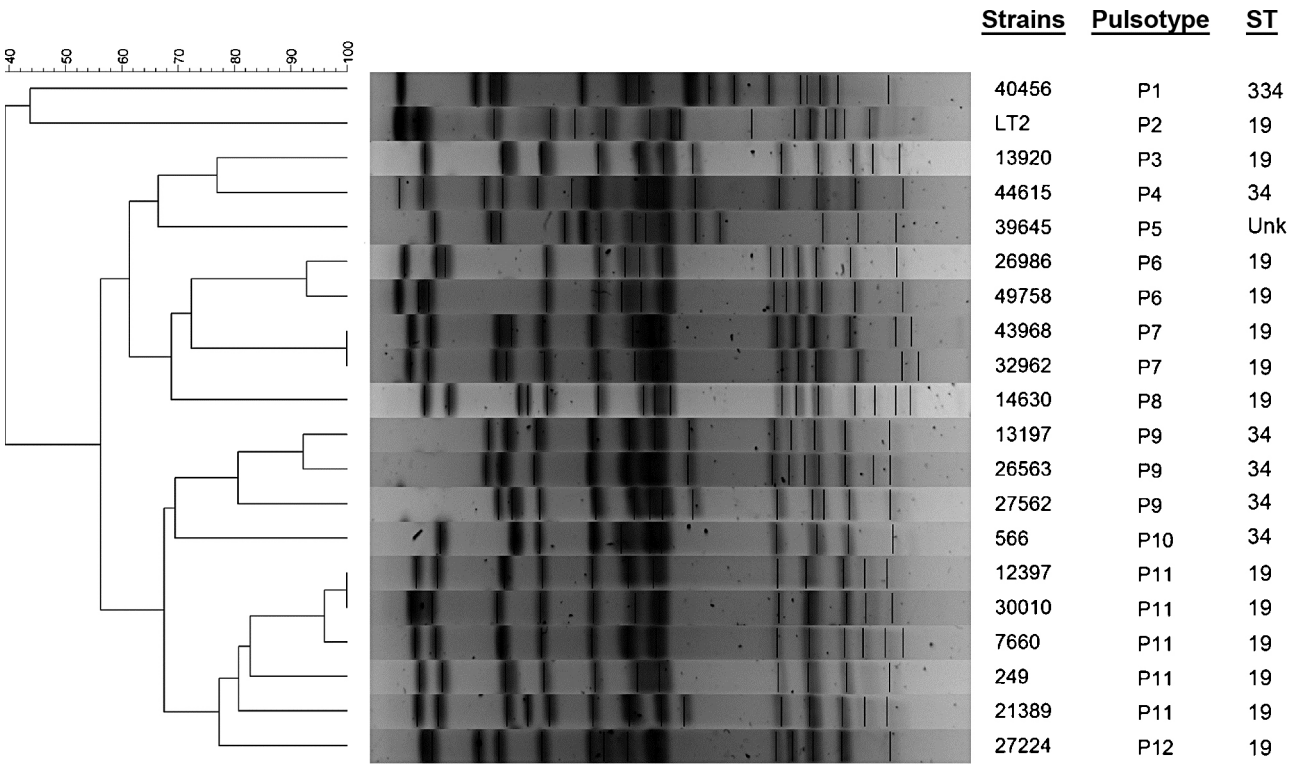

Fig. 1. Correlation between multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE) among *Salmonella enterica* serovar Typhimurium isolates. LT2 was used as reference strain. Unk, unknown sequence type.

Among the clinical isolates of *S.* Typhimurium, the MLST study identified 12 strains as ST19, which is the predominant *S.* Typhimurium genotype in the MLST database, 5 as ST34, 1 as ST334 and 1 with no associated ST, whereas PFGE allowed the classification of the strains into 12 different pulsotypes with ≥80% similarity. The predominant pulsotype was 11 and included five strains identified as ST19. Pulsotype 9 was the next most prevalent and included three strains belonging to ST34. In contrast with *S.* Enteritidis, this serovar was more clonally divergent as shown by both of the techniques used in this epidemiological study (Fig. 1). Accordingly, PFGE was revealed to be more discriminatory than MLST, suggesting that this methodology could be applied as a useful tool to elucidate the genetic divergence of *S.* Typhimurium isolates.

The high percentage of *S.* Enteritidis resistant to nalidixic acid found in the present study is in agreement with previous reports and implies that great efforts should be made and actions should be taken in the food industry and in the clinical setting to further limit the use of fluoroquinolones in order to stop this global trend of resistance. Otherwise, the dissemination of a particular clone could occur, as appears to be the case in this study, in which the amino acid substitution D87Y in GyrA predominates. Moreover, the lack of increased expression of any of the efflux system components tested among the resistant strains in the present study makes investigation in this field necessary in order to increase the knowledge of the causes of quinolone resistance and anticipate the emergence of *Salmonella* strains with novel resistance mechanisms.

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Paper V

Differential impact of *ramRA* mutations on both *ramA* transcription and decreased antimicrobial susceptibility

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The incidence of *S.* Typhimurium clinical isolates resistant to the commonly used antimicrobial therapy is currently on the rise. For this reason, research leading to understanding how pathogens become resistant is a matter of great interest. The aim of this study was to investigate the mechanisms involved particularly in the initial stages of acquisition of resistance to different antimicrobials including quinolones and cephalosporins, currently used to treat salmonellosis.

Two clinical isolates (59-wt and 60-wt) recovered at the Hospital Clínic (Barcelona, Spain) were used to generate *in vitro* mutants by exposure to increasing concentrations of ciprofloxacin. Three mutants were selected from 59-wt, named 59-mut1, 59-mut2 and 59-mut3; and two were obtained from the 60-wt isolate (60-mut1 and 60-mut1). A previous study included in this thesis (Paper III) [189] already characterized the 59-wt strain as well as other mutants selected, including 59-64, showing an MDR phenotype.

Antibiotic susceptibility to ciprofloxacin, norfloxacin, nalidixic acid, chloramphenicol, tetracycline, erythromycin, amoxicillin, ceftriaxone and cefoxitin was tested. All strains showed increased MIC values to all the antibiotics tested (>2.3-fold) compared to their parental strain except for kanamycin, the levels of which remained low (MIC: 1-1.5 μg/mL).

Sequencing of the QRDR of the four target genes (*gyrA*, *gyrB*, *parC* and *parE*) revealed an amino acid change in GyrB (E466D) in the 59-mut1, 59-mut2 and 59-mut3 strains, and the acquisition of an additional change in GyrA (S83Y) and ParC (S80R) in the last 59-mut3 mutant. Although 59-mut1 and 59-mut2 had the same QRDR profile, the latter strain showed higher MIC values (except for amoxicillin and chloramphenicol, which already

showed maximum Etest values in 59-wt). This suggests the effect of increased efflux in the decreased antibiotic susceptibility phenotype. Likewise, the derivative mutants of the 60-wt isolate, which did not present any mutation in the QRDR, also showed the same QRDR profile but different MIC values, implying the contribution of enhanced efflux mechanism in the more resistant strain.

To validate this hypothesis, the major efflux system AcrAB/TolC was investigated. Gene expression of the efflux components *acrB* and *tolC* was evaluated by RT-PCR. The strain 59-wt was previously reported to harbor a truncated AcrB protein [189], thus, this gene was not tested in this strain or its mutants. In the case of 60-mut1 and 60-mut2, both showed overexpression of *acrB* (5.2- and 9.5-fold, respectively) and *tolC* (>2.3-fold). For this latter gene, transcription levels of the 59-mut2 strain increased 5.4-fold compared to the wild-type isolate. The porin-encoding gene *ompF* was also tested and showed a trend to decreased expression in all strains. In order to evaluate if the levels of alternative efflux components were affected, *acrF* and *emrB* were evaluated. Only a slight decrease was seen for *emrB* in all the mutants (≥-2-fold) whereas 59-mut2 and 60-mut2 clearly overexpressed *acrF*, showing a 6- and 4.9-fold increase, respectively, compared to their original strains. According to these results, the contribution of AcrAB/TolC was seen in both 60-mut1 and 60-mut2, together with the effect of the AcrEF system only in the case of 60-mut2. The sole role of this latter efflux system was only seen in 59-mut2, although the contribution of other non-tested efflux systems cannot be excluded.

Transcription levels of the MDR regulatory genes (*ramA*, *marA*, *soxS* and *acrR*) were evaluated reflecting the overall overexpression of all the genes except *acrR*, the results of which were not conclusive. In the case of *soxS*, a clear overexpression of >4-fold was reported for 59-64 and 60-mut2. Regarding *marA*, the highest levels were seen in the 59-mut2, 59-64 and 60-mut2 mutants (3.6- to 4.3-fold), but the most important transcription levels were reported in *ramA* as all mutants revealed >13-fold increased expression levels compared to their parental strains. The highest levels were seen in 59-mut2 and 60-mut2 with 66-fold and 74.2-fold, respectively. Interpretation of these results suggests that both the high overexpression of *ramA* and the high values of *soxS* promote the transcription of *marA*. This is likely possible through binding of RamA and SoxS to the conserved sequenced

marbox located in the *marRAB* promoter. When screening for mutations was carried out in the nucleotide sequence of the MDR regulatory genes no changes were seen in *soxRS*, *marRAB* and *acrR*; however, several changes affecting the *ramRA* region were found. Deletions of 44 and 6 nucleotides within the *ramR* gene were reported in strains 59-mut1 and 59-mut3, respectively, whereas a single amino acid substitution (Q19P) was seen in 60-mut1. Interestingly, in the two strains with the highest levels of *ramA* expression (59-mut2 and 60-mut2), 6- and 16-nucleotide deletions, respectively, were reported in the *ramA* promoter. Moreover, in the same region, a single nucleotide change was reported in 59-64 (t158a). Regarding these findings, large deletions within the *ramA* promoter affect the transcription of this regulatory gene to a higher extent than changes in *ramR*. In contrast, we have no explanation for the increased expression levels of *soxS* in this study, as no mutations were found in the *soxRS* operon. Besides, the absence of contribution of AcrEF and EmrAB in the 59-wt derivative mutants and the overexpression of *tolC* in these strains suggest the presence of efflux components which remain undiscovered.

Differential impact of *ramRA* mutations on both *ramA* transcription and decreased antimicrobial susceptibility

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Running title: *ramRA* mutations and impact on MDR

Key words: *Salmonella*, *acrB*, *tolC*, *acrF*, MDR

SYNOPSIS

Objectives: This study has focused on analysing the heterogeneity of mutations occurring in the regulators of MDR in *Salmonella* Typhimurium. Moreover, the impact of such mutations on impairing the transcription of *ramA*, *acrB*, *tolC* and *acrF* was also assessed as was the impact on the MDR phenotype.

Methods: Strains were selected under increasing ciprofloxacin concentrations. Etest and broth microdilution tests were used to determine the MICs of several unrelated compounds. Screening of mutations in the quinolone target genes and the MDR regulators was performed. RT-PCR analysis was used to detect the levels of expression of *acrB*, *tolC*, *ompF*, *acrF*, *emrB*, *acrR*, *ramA*, *soxS* and *marA*.

Results: All mutant strains showed increased MICs of most of the antimicrobials tested, with the exception of kanamycin. The positive identification of mutations in the quinolone target genes did not occur in all mutants, which all harboured mutations in the *ramRA* regulatory region. All mutants overexpressed *ramA*, *tolC* and *acrB* (when active) whereas differential results were seen for the remaining genes.

Conclusions: Mutations in the *ramRA* region predominate in *Salmonella* in terms of MDR. There is heterogeneity in the type of mutations, with deletions affecting the RamR binding sites having a higher impact on *ramA* expression and the MDR phenotype.

INTRODUCTION

The ever increasing levels of resistance to antimicrobial compounds are of great concern, particularly for pathogens of clinical relevance. *Salmonella enterica* serovar Typhimurium is a worldwide-distributed pathogen which typically causes gastroenteritis in humans.[1] Fluoroquinolones and cephalosporins are the current first-line treatments, however, recent data reveal high percentages of resistance to such compounds, as nalidixic acid (61.9%) and cefepime, cefotaxime and ceftazidime (90%).[2]

Quinolone resistance has been widely studied in Enterobacteriaceae, particularly in *Escherichia coli* and *S. enterica*.[3] In *E. coli* the mechanism which largely contributes to resistance and/or decreased susceptibility to quinolones is the acquisition of mutations located in the genes encoding the two quinolone targets: DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*).[4,5] These mutations are usually acquired in the quinolone resistance-determining regions (QRDRs) detected in each of the target genes.[3] On the other hand, increased drug extrusion by means of the overexpression of AcrAB-TolC, the main efflux pump described in Enterobacteriaceae,[3] is also of great concern since it confers cross-resistance to several unrelated compounds, including antimicrobial drugs.[6,7] To a lesser extent, other efflux systems, such as AcrEF and EmrAB, have been reported to participate in the extrusion of antimicrobial compounds.[8,9] In *Salmonella* increased efflux has been described as the primary mechanism in quinolone resistance acquisition.[10] Alternatively, decreased production of the OmpF porin has at times been related to the MDR phenotype[11,12] despite controversial data suggesting no clear role in *S. enterica*.[13]

Several regulators have been reported to influence the expression of the *acrAB* operon in *Salmonella*. AcrR is the local repressor encoded upstream of the *acrAB* genes

and mutations within its coding sequence have been associated with increased expression of the pump.[14] In addition, three homologous transcriptional activators, RamA, SoxS and MarA, have been reported to increase not only *acrB* but also *tolC* expression levels. Each of these activators has its own regulator: RamR, SoxR and MarR, respectively.[3] In terms of the MDR phenotype, the clinical relevance of mutations located in the genes encoding for these latter regulators has been clearly shown for RamR,[15,16] while there have been few reports for mutations located in the *soxRS* region.[11,17] Concerning MarA, even when its overexpression has been detected in MDR *S. enterica* strains,[8,18] the putative responsible mutations in the *marRAB* region have not been mapped. Naturally-occurring mutations in this region have been widely reported *in E. coli*,[19,20] whereas, to our knowledge, such mutations in *S. enterica* have only been reported in a single study, associating it with high MarA overexpression and an MDR phenotype.[12]

The aim of this study was to determine the mechanisms involved in increasing the MICs of different antimicrobial agents in a collection of *S.* Typhimurium mutants selected *in vitro*, particularly at the initial stages of acquisition of resistance. The mechanisms studied included target gene mutations and the expression of several genes involved in decreasing the intracellular concentration of the drug. Moreover, and as a novel approach, we also assessed the role and heterogeneity of *ramRA* mutations and their impact on increasing the expression of *ramA* and the MDR phenotype.

MATERIALS AND METHODS

Bacterial strains and selection of resistant mutants

Two *S.* Typhimurium clinical isolates, strains 59-wt and 60-wt, were recovered from independent stool samples in the Department of Clinical Microbiology at the Hospital

Clinic of Barcelona, Spain. Strain 59-wt has previously been characterised as have its derivative mutants displaying increasing ciprofloxacin MICs, including the highly resistant mutant 59-64.[21] As indicated, the clinical isolate 59-wt was grown at 37ºC on MacConkey agar plates in the presence of ciprofloxacin (Fluka) in a multi-step selection process with doubling concentrations of the drug.[21] Single colonies were selected at different steps and previously characterised. In the present study we characterised additional colonies selected during the process (59-mut1, 59-mut2 and 59-mut3) to assess the occurrence of heterogeneity in the mechanisms of resistance. Likewise, strain 60-wt was similarly treated and exposed to increasing ciprofloxacin concentrations and two different mutants were selected (60-mut1 and 60-mut2).

Susceptibility testing

The MICs of several quinolones and unrelated antimicrobial compounds were determined by Etest (AB Biodisk) according to the manufacturerôs recommendations and interpreted according to CLSI guidelines.[22] The broth microdilution method was used to evaluate the MICs of ciprofloxacin, norfloxacin and nalidixic acid when maximum Etest values were reached. The compounds tested were: ciprofloxacin, norfloxacin, nalidixic acid, chloramphenicol, tetracycline, erythromycin, amoxicillin, ceftriaxone and cefoxitin.

Detection of mutations within the QRDRs and regulatory loci

Mutations acquired in the QRDRs of the *gyrA*, *gyrB*, *parC*, and *parE* genes, as well as in the MDR regulatory loci *soxRS*, *marRAB*, *acrR* and *ramR* were screened by PCR amplification as previously described.[23] Amplicons were purified and sent to Beckman Coulter Genomics (Essex, UK) for sequencing reactions. Detection of mutations was

carried out using the BioEdit® software (Ibis Biosciences, Carlsbad, CA) by comparison with the genome of *S.* Typhimurium LT2 as the reference strain (RefSeq NC_003197.1).

RNA extraction and real time PCR

Bacterial pellets were obtained as previously described.[23] Briefly, strains were grown in LB at 37ºC with shaking to reach the exponential phase (OD_{600}=0.6). Four mL of bacterial cells were treated with 8 mL of RNA Protect Bacteria Reagent (Qiagen) and subsequently incubated with TE buffer supplemented with lysozyme. RNA extractions were obtained using the Maxwell ® 16 Research Instrument (Promega) and the Maxwell® 16 LEV Blood DNA kit (Promega) following the manufacturerôs recommendations. Five independent RNA extractions were made.

The genes *acrB*, *tolC*, *ompF*, *acrF*, *emrB*, *ramA*, *marA*, *soxS* and *acrR* were tested for RT-PCR analysis following previously described conditions.[24] The 16S rRNA gene was used as an internal control for normalisation, and susceptible strains 59-wt and 60-wt were the reference strains for their respective derived mutants. The $2^{-\Delta\Delta CT}$ method was used for relative gene expression calculations.[25] Five independent assays were performed and each RNA sample was tested in triplicate. The primers used are reported in Table 1. Mean values and standard deviation are detailed in Table S1.

RESULTS AND DISCUSSION

Quinolone resistance and the MDR phenotype

Three and two derivative mutants were selected from the quinolone-susceptible clinical isolates 59-wt and 60-wt, respectively. Susceptibility testing to several unrelated compounds was used to determine the acquisition of the quinolone resistance and MDR

147 phenotypes (Table 2). The term MDR has been defined as resistance to one agent in
148 three or more antimicrobial categories,[26] or to four or more antimicrobials in the
149 particular case of nontyphoidal *Salmonella*.[27] Nonetheless, in the present study we used
150 this term when increased MICs to more than 4 antimicrobial compounds were seen even
151 though the resistance breakpoints were not reached. Strain 59-64, already characterised
152 in a previous study,[21] was also included in the present work for comparison with the
153 mutants. The results showed that in comparison with their wild-type strain, all selected
154 mutants had increased MICs (1.5- to >8-fold) to all the drugs tested, except for
155 kanamycin, for which no increase was recorded. Only 59-wt derivative mutants showed
156 the acquisition of QRDR mutations (Table 3). Strains 59-mut1 and 59-mut2 showed a
157 similar genetic background in terms of target gene mutations. However, higher MIC
158 values were seen for 59-mut2 concerning all the drugs (except for amoxicillin and
159 chloramphenicol, which had already shown maximum E-test values in 59-wt, and
160 tetracycline). Likewise, on comparing strains 60-mut1 and 60-mut2 a similar conclusion
161 was obtained, with higher MIC results seen for 60-mut2 despite having background
162 similarity. In accordance with the fact that strains 59-mut3 and 59-64 were selected at
163 higher ciprofloxacin concentrations, these strains showed the highest MICs, mostly
164 concerning quinolones, being maximal for strain 59-64.

165 Taking into account the increased MICs of most of these compounds in all the
166 mutants, and the fact that increased efflux (i.e. by means of increased AcrAB) confers a
167 cross-resistance phenotype,[6] enhanced extrusion activity was the most likely mechanism
168 underlying this phenotype. Moreover, the results obtained from 60-wt and its derivative
169 mutants strengthen the idea that efflux is selected at primary stages of the process of
170 quinolone resistance acquisition as previously suggested,[10,23] and this mechanism is
171 selected even before target gene mutations.

Expression of structural genes involved in MDR

Gene expression analysis was performed to determine the expression patterns of genes related to bacterial efflux and permeability. The results were interpreted after comparison of the expression levels of each clinical isolate with their respective mutant derivatives. The genes studied were *acrB*, *tolC*, *ompF*, *acrF* and *emrB* (Figure 1)(Table S1). Overexpression of the AcrAB-TolC efflux pump has been reported as the most relevant mechanism in terms of efflux.[3] In the present study *acrB* was only analysed in 60-wt and its derivates, which all overexpressed this gene (5.2- to 9.5-fold), since it was reported that 59-wt has a mutation inactivating the *acrAB* operon.[21] The *tolC* gene was found to be consistently overexpressed in all mutants (>2.3-fold), particularly for strains 59-mut2 and 60-mut2 (5.4- and 6.2-fold, respectively). On the contrary, *ompF* always showed decreased expression with the strongest results being seen in strains 59-64 (-3.3-fold) and 60-mut2 (-2.4-fold).

Next, we assessed other efflux-related genes, such as *acrF* and *emrB*, (Figure 1)(Table S1) which may play a secondary role in antibiotic resistance.[8,9] Our results showed that only two strains clearly overexpressed *acrF* [59-mut2 (6-fold) and 60-mut2 (4.9-fold)] whereas *emrB* showed slightly decreased expression in all mutants (-1.2- to -1.9-fold). Thus, we can only suggest a role in increasing the MICs mentioned for the AcrEF efflux system in these two particular mutants, one of which is also an AcrAB-overproducer (60-mut2).

Expression of the MDR regulators: the key role of ramA

In addition to the analysis of these structural genes, we also studied the levels of expression of the AcrAB regulators: *acrR*, *ramA*, *soxS* and *marA* (Figure 1)(Table S1). We could not find a clear interpretation for *acrR* expression. In contrast, *ramA* was

197 overexpressed in all the mutants thereby suggesting this regulator as the cause of the
198 increased MICs. Similar results highlighting the greater importance and prevalence of
199 increased RamA over that of the other regulators have also been reported.[28,29] Maximal
200 *ramA* expression levels were seen for 59-mut2 and 60-mut2 (66- and 74.2-fold,
201 respectively) above the levels detected for the remaining mutants (13.4- to 19.6-fold). In
202 line with these results, these two strains also showed higher MICs and *acrB* and *tolC*
203 expression values in comparison with their closely related mutants 59-mut1 and 60-
204 mut1, respectively. In addition, as mentioned above, 59-mut2 and 60-mut2 were also
205 reported to clearly overexpress *acrF*. This latter association between high *ramA*
206 expression (>60-fold in the present study) and *tolC* and *acrF* overexpression agrees
207 with a previously reported study.[30]

208 The *soxS* expression values detected in the present study were <2-fold higher in
209 most of the mutants *versus* the expression levels seen in the two clinical isolates (Figure
210 1). Only two mutants, strains 59-64 and 60-mut2 showed an overexpression of >4-fold.
211 Unfortunately, it was not possible to consistently associate this trait with higher
212 expression values of *ramA* or *acrF* in both mutants. Nonetheless, these two strains did
213 show the minimum levels of *ompF* expression (-3.3- and -2.2-fold, respectively).
214 Similarly, *marA* transcription also showed Ò2-fold increased expression in three mutant
215 strains: 59-mut1, 59-mut3 and 60-mut1. On the contrary, the highest levels were seen in
216 59-mut2 (4.3-fold), 59-64 (3.7-fold) and 60-mut2 (3.6-fold).

217 To understand our results it is worth mentioning that the RamA binding sites
218 have already been reported in *Salmonella* concerning the *acrAB* and *tolC* promoters.[31]
219 The 20-bp sequences recognised by this regulator resemble those initially reported to be
220 present in all members of the *marA*/*soxS*/*rob* regulon in *E. coli*.[32] It has been described
221 that most of the residues of the two helix-turn-helix motifs (important for DNA

sequence recognition) of MarA from *E. coli* are conserved in RamA from *Salmonella enterica* serovar Paratyphi B.[33] Moreover, it has previously been reported that the *marRAB* promotor contains its own marbox sequence.[32] In agreement with this, RamA from *S.* Paratyphi B has been shown to bind the MarA operator of *E.coli*.[33] Thus, the binding sites characterised for MarA and SoxS in *E. coli*, equally termed marbox or soxbox, are similar to the already mentioned rambox in *Salmonella*.[30,31] Therefore, increased levels of RamA (>60-fold) and/or SoxS (>4-fold) could bind to the rambox/marbox located in the *marRAB* promoter and activate *marA* transcription, hence explaining the increased levels of *marA* expression observed for strains 59-mut2 [RamA-overproducer (>60-fold)], 59-64 [SoxS-overproducer (>4-fold)] and 60-mut2 [RamA-overproducer (>60-fold) and SoxS-overproducer (>4-fold)]. Nonetheless, lower *ramA* overexpression values (13- to 20-fold) would not have the same effect, thereby reinforcing the idea of an activator concentration-dependent response.[30,34]

Unravelling the mutations leading to the MDR phenotype

In order to determine the mutations underlying the resistance phenotypes, sequencing and detection of mutations was performed in all the strains for all known regulators of MDR (*acrRA*, *ramRA*, *soxRS*, *marRAB* and *acrSE*). The results revealed the acquisition of mutations in the *ramRA* loci for all mutants (Table 3). Mutations were located within the *ramR* coding sequence, either leading to a single amino acid substitution (Gln-19→Pro, strain 60-mut1) or even deletions of 44 and 6 nucleotides (strains 59-mut1 and 59-mut3, respectively). Surprisingly, the two strains (59-mut2 and 60-mut2) with the highest *ramA* overexpression values harboured a similar genotype: a 6- and 16-nucleotide deletions, respectively, in the *ramA* promoter. Lastly, and as previously

246 reported[21] strain 59-64 showed a single-nucleotide change also located in the *ramA*
247 promoter.

248 Previous reports have revealed that mutations or gene interruptions can be either
249 acquired within *ramR* or in the *ramA* promoter.[11,28,29] However, no association has ever
250 been made between the type of mutation and transcription levels of *ramA*. Our study
251 points out that severe nucleotide deletions located in the *ramA* promoter have a higher
252 impact on increasing the expression of this regulator, whereas mutations within *ramR*
253 have a lesser effect. This situation can be explained by the published information
254 regarding this promoter. RamR has been reported to bind as homodimer to two RamR
255 binding sites located in the *ramA* promoter (Figure 2).[35] Thus, important deletions
256 occurring in these locations seriously impair the RamR repressive activity by preventing
257 RamR binding. On the contrary, mutations or deletions occurring in RamR do not seem
258 to abolish repression to the same extent. Our results also suggest that single nucleotide
259 changes in these binding sites have an effect similar to that reported for *ramR*
260 mutations.

261 In no strain did we find any mutation in any of the other regulatory sequences
262 analysed in the present study. Consequently, we are unable to explain the increased *soxS*
263 transcription reported in 59-64 and 60-mut2. Concerning *acrF* overexpression, previous
264 results have associated it with mutations within the *acrS* gene or in the *acrEF*
265 promoter.[9] However, in the present study no mutation in the *acrSE* regulatory region
266 could explain our findings. Instead, and as previously mentioned and reinforced by our
267 results, overexpression of this efflux component is related to the levels of *ramA*
268 transcription.[30] High levels of *ramA* expression trigger *acrF* overexpression whereas
269 intermediate levels do not. In line with these results, a previous study has also
270 associated nucleotide deletions in the *ramA* promoter with *acrEF* overexpression.[36] In

view of these findings, the regulatory network that controls the expression of genes involved in MDR still needs further research to completely understand the bacterial response for survival under antimicrobial exposure.

Conclusions

In this study we provide further evidence of the prevalence of *ramRA* mutations versus other *acrB* regulators in the acquisition of MDR. Nonetheless, heterogeneity was observed in the types of mutations acquired, which may be associated with different levels of *ramA* transcription. Large deletions affecting the RamR binding sites in the *ramA* promoter lead to higher *ramA* expression, which is ultimately associated with the highest levels of *acrB*, *tolC*, *marA* and *acrF* expression, hence making a major contribution to the MDR phenotype.

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297

298 **TRANSPARENCY DECLARATION**

299 The authors declare no conþicts of interest.

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301

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FIGURES:

Figure 1. Expression levels obtained by RT-PCR analysis. Single and double asterisks refer to high levels of *ramA* overexpression, 66- and 74.2-fold, respectively, which are out of scale to facilitate the visualisation of the results.

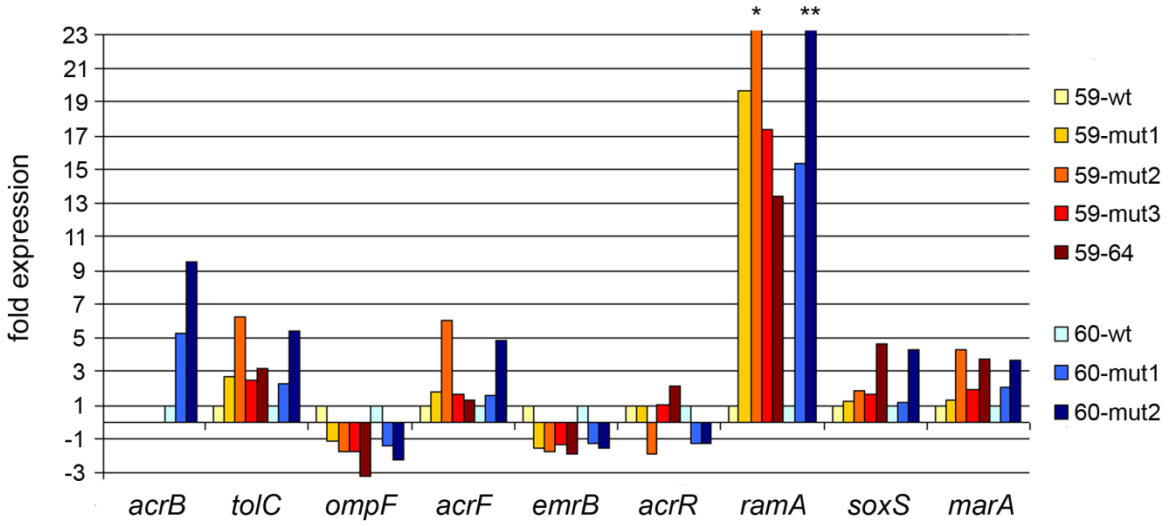
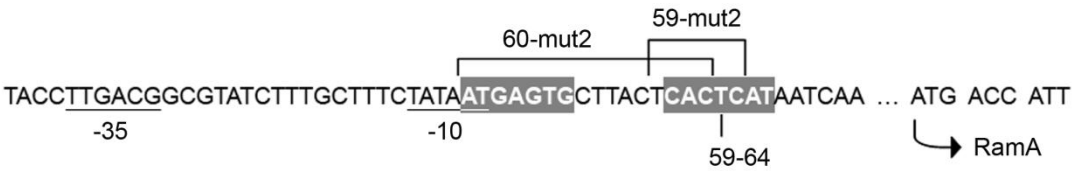


Figure 2. Representative location of the RamR binding sites and the MDR-related mutations detected in the *ramA* promoter. White letters and grey boxes indicate the two RamR binding sites. The -35 and -10 boxes of the *ramA* promoter are underlined. Ellipses indicate DNA sequences not shown. The black arrow is used for the initiation of translation.



436 **TABLES**:

437 **Table 1.** Primers used in the RT-PCR analysis.

Genes	Primers	Sequence 5'-3'	Reference
Internal control			
16S rRNA	16S_RT_F	GCGGCAGGCCTAACACAT	[37]
	16s_RT_R	GCAAGAGGCCCGAACGTC	
Structural genes			
acrB	AcrB_RT_F	TTTTGCAGGGCGCGGTCAGAATAC	[11]
	AcrB_RT_R	TGCGGTGCCCAGCTCAACGAT	
tolC	TolC_RT_F	GTGACCGCCCGCAACAAC	[24]
	TolC_RT_R	ATTCAGCGTCGGCAGGTGAC	
acrF	SacrF.RT.1	TACCCAGGACGACATCTCTGA	[24]
	SacrF.RT.2	CACACCATTCAGACGGCTGAT	
emrB	EmrB_RT_F	CCGTCGTCCTGATGACGTTA	[24]
	EmrB_RT_R	CCGTTCGGTATGCGTTTCAC	
ompF	SompF.RT1	GGGCGCGACTTACTACTTCAAC	This study
	SompF.RT2	TCGTTTTCGTCCAGCAGGTT	
Regulatory genes			
acrR	SacrR.RT1	AGAACGACGCCGCTTATTGA	[12]
	SacrR.RT2	GCGCCTGTTGAACCACAAC	
ramA	SramA.RT1	CTCGACACCGACCAGAAGGT	[12]
	SramA.RT2	GTAAAAATGCGCGTAAAGGTTTG	
soxS	SsoxS.RT1	CATATCGACCAACCGCTAAACA	[12]
	SsoxS.RT2	CGAAACATCCGCTGCAAATA	
marA	SmarA.RT1	ATTCCAAATGGCACCTGCAA	This study
	SmarA.RT2	CATTTTACGGCTGCGGATGT	

438

Table 2. Susceptibility testing of all the strains and ciprofloxacin concentrations used for the selection of mutants.

Strain	[CIP] (mg/L) at selection	MICs (mg/L)[b]									
		CIP	NOR	NAL	AMX	CRO	FOX	TET	CHL	ERY	KAN
59-wt	---[a]	0.012	0.094	4	>256	0.094	2	64	>256	32	1.5
59-mut1	0.06	0.125	2	32	>256	0.190	6	128	>256	128	1.5
59-mut2	0.25	0.38	6	96	>256	0.5	12	128	>256	256	1.5
59-mut3	2	8	16	8128	>256	0.25	4	96	>256	128	1.5
59-64	64	256	512	8128	>256	1	96	256	>256	>256	1.5
60-wt	---	0.016	0.094	3	1	0.032	3	3	3	32	1
60-mut1	0.015	0.047	0.19	6	1.5	0.064	8	8	8	192	1
60-mut2	0.03	0.094	0.38	24	3	0.125	12	12	24	>256	1

[a] ---, clinical isolate not exposed to ciprofloxacin *in vitro*.

[b] CIP, ciprofloxacin; NOR, norfloxacin; NAL, nalidixic acid; AMX, amoxicillin; CRO, ceftriaxone; FOX, cefoxitin; CHL, chloramphenicol; TET, tetracycline; ERY, erythromycin; KAN, kanamycin.

Table 3. Mutations acquired in the quinolone target genes and the *ramRA* regulatory region.

Strains	QRDR mutations					*ramRA* mutations	
	GyrA		GyrB	ParC		*ramR*/RamR[a]	*ramA* promoter[b,c]
59-wt	---	---	---	---	---	---	---
59-mut1	---	---	E466D	---	---	Del C_{514}-G_{557}	---
59-mut2	---	---	E466D	---	---	---	**Del T_{-162}-C_{-157}**
59-mut3	S83Y	---	E466D	S80R	---	Del A_{346}-G_{352}	---
59-64	S83Y	D87G	E466D	S80R	F115S	---	$T_{-158}A$
60-wt	---	---	---	---	---	---	---
60-mut1	---	---	---	---	---	Q19P	---
60-mut2	---	---	---	---	---	---	**Del A_{-174}-C_{-159}**

[a] Del, deletion. Mutations are indicated by either the nucleotide positions deleted relative to the translation start site or the amino acid substitution.

[b] Del, deletion. Numbers indicate the upstream positions relative to the translation start site.

[c] Mutations leading to the maximum *ramA* expression values are represented in bold.

2. Relationship between quinolone resistance acquisition and a decrease in the virulence traits.

Paper III

Impact of quinolone-resistance acquisition on biofilm production and fitness in *Salmonella enterica*

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Previous studies have reported an association between the acquisition of quinolone resistance and a decreased expression of virulence factors in *S. enterica in vitro*-selected mutants. In these studies, virulence was evaluated by testing the expression of genes involved in the invasion process of host cells, assessing growth rate, motility and eukaryotic cell invasion assays [210–212]. Moreover, in clinical isolates of other species, such as *E. coli* and *A. baumannii* the frequency of biofilm producers resistant to nalidixic acid or ciprofloxacin has been shown to be lower than in non-biofilm producers [213,214].

The aim of this work was to evaluate the potential relationship between quinolone resistance acquisition and biofilm production in *S. enterica* clinical samples as well as in *in vitro*-selected quinolone-resistant *S*. Typhimurium mutants. In the first part of the study, nalidixic acid susceptibility and biofilm production ability was tested in a collection of 122 *S. enterica* clinical isolates from the Public Health Laboratory (Oviedo, Spain). In the second part of the study, a *S.* Typhimurium clinical strain (59-wt) isolated at the Hospital Clínic (Barcelona, Spain) was exposed to increasing concentrations of ciprofloxacin, and serial *in vitro* mutants were obtained (59-0.015, 59-0.03, 59-0.06, 59-0.25, 59-2, 59-16 and 59-64). Despite having made 50 consecutive passages of the most resistant strain, 59-64, in the absence of ciprofloxacin, no revertants were recovered.

These strains were analyzed in terms of quinolone resistance mechanisms, MDR phenotype and the ability to form biofilm using different approaches.

The results obtained in relation to biofilm production (assessed by Crystal Violet staining and further quantification) among serovars in the collection of clinical isolates, indicated that nearly half of the *S.* Enteritidis strains were negative for this feature (49% *vs*. 51%). However, the majority of *S.* Typhimurium isolates were biofilm producers (74% vs. 26%). In *S*. Enteritidis no association was seen between nalidixic acid susceptibility and the ability to form biofilm; in contrast, all *S.* Typhimurium were susceptible to nalidixic acid, and most (74%) were biofilm producers. In addition to these two serovars, only one isolate belonged to the Muenchen serovar. This strain was susceptible to nalidixic acid and was able to form biofilm, while another isolate consisted of a nalidixic acid-resistant strain of *S*. Hadar and did not form biofilm.

In the second part of the study, characterization of the (fluoro)quinolone and multidrug resistance profile of the *S.* Typhimurium *in vitro*-selected mutants revealed the following results. A progressive increase was reported in the MIC of nalidixic acid and the fluoroquinolones ciprofloxacin and norfloxacin, consisting of 4, 0.094 and 0.012 μg/mL, respectively, in 59-wt and 8192, 256 and 512 μg/mL in the last mutant 59-64. Screening of quinolone resistance mechanisms revealed a progressive acquisition of mutations in the QRDR of the target genes: the first amino acid substitution was found in the GyrB subunit (E466D) of 59-0.03, two additional changes were acquired in 59-2 in GyrA (S83Y) and ParC (S80R), and finally, a second change in GyrA (D87G) and in ParC (F115S) was seen in the most resistant mutant, 59-64. Regarding susceptibility to other non-related antibiotics, an increase in the MIC was seen at different steps; a first increase ranging from 1.5-to 3-fold in the MIC of tetracycline, erythromycin, ceftriaxone and cefoxitin was reported for 59-0.06, followed by 59-0.25, with an additional 2-8-fold increase in 59-16. This pattern was also seen upon the addition of PAβN. Consistent with these results, ciprofloxacin accumulation assays were assessed for 59-wt, 59-2, 59-16 and 59-64. The results revealed that the 59-2 mutant accumulated less ciprofloxacin than the original strain, and a further decrease was reported in 59-16. These results implied the contribution of efflux, and thus, the major efflux system

AcrAB/TolC and its regulators were investigated. Sequencing of the regulatory genes *soxRS*, *ramR*, *acrR* and *marRAB* revealed changes in *acrA* and *ramR*. A mutation in the RamR binding site located in the *ramA* promoter was detected in 59-64 and was likely the cause of the *ramA* gene overexpression seen by RT-PCR. Additionally, 59-wt and 59-64 strains were reported to have a 1-bp nucleotide deletion at position 139 within the *acrA* gene that led to a frameshift mutation resulting in a truncated protein. Moreover, SDS-PAGE revealed a decrease in the protein expression of both AcrA and AcrB in the two strains, whereas overexpression of TolC in 59-64 compared to 59-wt was seen at transcriptional and post-transcriptional levels. Nevertheless, no changes were reported in the gene expression of *acrEF* in both strains, thereby ruling out its contribution to efflux.

Regarding biofilm formation, a progressive decrease in this virulence feature was observed inversely to antibiotic resistance acquisition. A significant 3.6-fold reduction in biofilm production occurred between 59-2 and 59-16. The rdar morphotype, visualized after culturing the mutants in LB agar containing Congo red and Coomassie brilliant blue, was in accordance with the results obtained from the Crystal Violet approach. However, no differences were seen between the strains when strains were grown in calcofluor-containing agar plates and visualized under UV light. Accordingly, a 23-fold reduction in the gene expression of *csgB*, the minor subunit of curli fibers, a component of the biofilm structure, was reported in 59-16 compared to 59-2. Therefore, these results indicate a decrease in the curli fiber production (seen in the rdar morphotype and by gene expression of *csgB*) but no changes in cellulose (provided by the calcofluor approach).

Growth impairment was also seen in the two most resistant mutants and biofilm-defective strains, 59-16 and 59-64. The resistant mutants showed a diminished growth rate since a longer lag phase was observed before the exponential phase.

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Impact of quinolone-resistance acquisition on biofilm production and fitness in *Salmonella enterica*

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Objectives: To investigate the potential relationship between quinolone resistance and biofilm production in a collection of *Salmonella enterica* clinical isolates and in *S. enterica* serovar Typhimurium serial mutants with increasing resistance to ciprofloxacin.

Methods: Nalidixic acid susceptibility and biofilm formation were assessed in a collection of 122 *S. enterica* clinical isolates. An *in vitro* quinolone-resistant mutant, 59-64, was obtained from a biofilm-producing and quinolone-susceptible clinical isolate, 59-wt, in a multistep selection process after increasing ciprofloxacin concentrations. The quinolone resistance mechanisms [target gene and multidrug resistance (MDR) regulatory mutations, MICs of several antibiotics, cell envelope protein analysis, real-time PCR and ciprofloxacin accumulation] were characterized for mutant strains. In addition, analysis of fitness, biofilm formation, rdar morphotype and expression of biofilm-related genes by real-time PCR were also determined.

Results: Nalidixic acid-susceptible *S. enterica* strains were more prevalent in producing biofilm than the resistant counterparts. Strain 59-64 acquired five target gene mutations and showed an MDR phenotype. AcrAB and *acrF* overexpression were ruled out, whereas TolC did show increased expression in 59-64, which, in addition, accumulated less ciprofloxacin. Consistently, increased *ramA* expression was seen in 59-64 and attributed to a mutation within its promoter. Reduced biofilm production related to diminished *csgB* expression as well as reduced fitness was seen for 59-64, which was unable to form the rdar morphotype.

Conclusions: Quinolone resistance acquisition may be associated with decreased production of biofilm due to lower *csgB* expression. Efflux, biofilm production and fitness seem to be interrelated.

Keywords: *Salmonella* Typhimurium, rdar morphotype, *csgB*, efflux, *ramA*

Introduction

The levels of morbidity, mortality and burden of disease caused by *Salmonella enterica* are of substantial importance worldwide. Host susceptibility and infecting *S. enterica* serovar determine disease manifestation.[1,2] Serovars Typhimurium and Enteritidis are those most frequently reported as causing enterocolitis in humans. Fluoroquinolones and extended-spectrum cephalosporins are usually the treatment of choice for these infections.[2,3] However, decreased susceptibility to both ciprofloxacin, which is associated with nalidixic acid resistance, and extended-spectrum cephalosporins is steadily increasing among *Salmonella* species.[4–7] Consequently, the success of antimicrobial therapy may be compromised. Therefore, the mechanisms leading to resistance to such compounds need better understanding at a molecular level.

Quinolones inhibit the activity of the DNA gyrase and topoisomerase IV, two essential enzymes involved in the replication and transcription of DNA. Each enzyme is a heterotetramer composed of two identical A subunits (GyrA and ParC, respectively) and two identical B subunits (GyrB and ParE, respectively).[8,9] Resistance to such compounds is attributable to mutations within the so-called quinolone resistance-determining regions (QRDRs) characterized in each subunit. Other chromosomally encoded mutations lead to a decreased intracellular accumulation of quinolone, either by means of the overexpression of efflux pump(s) (e.g. AcrAB/TolC) or by a decrease in the expression of porins.[10] Additionally, plasmid-encoded determinants decreasing

fluoroquinolone susceptibility have also been characterized.[11] However, QRDR mutations and increased AcrAB/TolC are the most important and prevalent mechanisms. This pump has been reported to play a key role not only in conferring resistance to quinolones, but also in multidrug resistance (MDR),[12–14] a term used for resistance to four or more antimicrobials in the case of non-typhoidal *Samonella*.[15] In *Salmonella*, AcrAB expression has been reported to be under the control of several regulators: the local repressor, AcrR,[16] and three transcriptional activators, MarA, belonging to the *marRAB* operon,[17] SoxS, belonging to the *soxRS* region[18] and RamA, repressed by the *ramR* gene.[19,20]

On the other hand, the ability to form a biofilm in response to unfavourable growth conditions is among the virulence properties described for *S. enterica*, since this phenotype contributes to conferring MDR and can help bacteria to survive in hostile or suboptimal environments.[21,22] Curli fimbriae, also designated as thin aggregative fimbriae (Csg or Agf), and cellulose production have been well characterized as contributing to biofilm formation and the rdar morphotype (for red, dry and rough colony morphology).[23–27] Recently, evidence showing an association between the acquisition of quinolone resistance and a decreased expression of virulence factors has been published. Several studies have revealed that ciprofloxacin-resistant *S. enterica* mutants show a decreased expression of invasion genes, particularly those encoded within the *Salmonella* pathogenicity island 1 (SPI-1), in association with decreased invasion ability in *in vitro* models.[14,28,29] In addition, a relationship has been found between *in vitro* biofilm formation and resistance to quinolones not only among uropathogenic *Escherichia coli* clinical strains, but also among *Acinetobacter baumannii* clinical isolates. In these studies, biofilm producers are significantly less frequently resistant to nalidixic acid or ciprofloxacin.[30,31]

In this study, a collection of 122 *S. enterica* clinical isolates, including nalidixic acid-susceptible and -resistant strains, was analysed to detect whether or not there is also a relationship between biofilm formation and quinolone resistance in *S. enterica*. Moreover, a fluoroquinolone-resistant *Salmonella* Typhimurium mutant was obtained from a susceptible and biofilm producer clinical isolate in an *in vitro* multistep selection procedure. Thus, the possible relationship between quinolone resistance acquisition and decreased biofilm production was also investigated in these mutants.

Materials and methods

Bacterial strains and selection of resistant mutants

A collection of 122 *S. enterica* clinical isolates was studied, 101 belonging to serotype Enteritidis, 19 to serovar Typhimurium, 1 to serovar Hadar and 1 to serovar Muenchen (Public Health Laboratory, Oviedo, Spain). Furthermore, one *Salmonella* Typhimurium clinical isolate, strain 59-wt, was independently recovered from a stool sample in the Department of Clinical Microbiology at the Hospital Clinic of Barcelona, Spain. Strain 59-wt was grown at 37°C on MacConkey plates in the presence of ciprofloxacin in a multistep selection process to obtain strain 59-64, a ciprofloxacin-resistant mutant. Ciprofloxacin (Fluka, Steinheim, Germany) was only present in the agar plates during the selection procedures, starting at 0.007 mg/L (half of the MIC for 59-wt) and increasing 2-fold each step, until a maximum concentration of 64 mg/L was reached. Single colonies were selected at each step and named according to the ciprofloxacin concentration of selection (e.g. strain 59-0.015 was selected at a ciprofloxacin concentration of 0.015 mg/L). Certain intermediate mutants (59-0.015, 59-0.03, 59-0.06, 59-0.25, 59-2 and 59-16) were also included in the study. Furthermore, strain 59-64 was grown in the absence of ciprofloxacin for 50 consecutive steps to obtain strain 59-rev.

Biofilm production

Biofilm production was assessed according to a previously described protocol with some modifications.[32] Briefly, overnight bacterial cultures grown at 37°C with shaking, adjusted to the same optical density (OD) at 600 nm, were used to inoculate 1 mL of Luria-Bertani (LB) broth (without NaCl) in small polystyrene tubes (1/100 dilution). Incubation followed at 28°C for 48 h without shaking. The medium was then discarded and the tubes stained with 1.5 mL of 1% Crystal Violet for 5 min. Finally, they were washed three times with water and left to dry. An isolate was categorized as a biofilm producer when a violet ring was visible in the tube. When biofilm production was determined for strain 59-wt and the derivative mutants, the Crystal Violet ring was dissolved with 96% ethanol in order to measure the OD at 620 nm in an automatic spectrophotometer (Anthos Reader 2001, Innogenetics, Spain). This assay was carried out in triplicate.

Congo Red and calcofluor phenotype assays

Strains were grown overnight in LB medium at 37°C with shaking. Cultures were adjusted to the same OD at 600 nm and then 10 μL was spotted onto LB plates (without NaCl) containing 40 μg/mL Congo Red and 20 μg/mL Coomassie Brilliant Blue or 400 μg/mL calcofluor. The drops were allowed to dry prior to incubation of the plates at 28°C for 48 h. The rdar morphotype was assessed on Congo Red plates, since absorption of this dye is indicative of curli and cellulose production.[33] A fluorescence of colonies under UV light indicated calcofluor binding, which is mainly indicative of cellulose production.[33]

Susceptibility testing

The MICs of ciprofloxacin, norfloxacin, nalidixic acid, chloramphenicol, tetracycline, erythromycin, amikacin, amoxicillin, ceftriaxone and cefoxitin were determined by Etest (AB Biodisk) according to the manufacturer's recommendations. The broth microdilution method was used to evaluate the MICs of ciprofloxacin, norfloxacin and nalidixic acid when maximum Etest values were reached. The MICs were determined according to CLSI guidelines.[34] The MICs of quinolones, tetracycline and erythromycin were also determined in the presence of 20 μg/mL of the efflux pump inhibitor phenyl-arginine-β-naphthylamide (PAβN) (Sigma-Aldrich, St Louis, MO, USA) in Mueller Hinton (MH) plates.

Detection of mutations within the QRDRs and regulatory loci

Amplification of the QRDRs of *gyrA*, *gyrB*, *parC* and *parE* as well as the *soxRS*, *marRAB*, *acrR* and *ramR* MDR regulatory loci was performed by PCR amplification as previously described.[14] The PCR products were recovered and sent to Macrogen Inc. (Seoul, South Korea) for sequencing to allow comparison with the genome of *Salmonella* Typhimurium LT2 as the reference strain (RefSeq NC_003197.1).

Cell envelope protein gel electrophoresis

Protein samples were obtained as previously reported by our group.[14] Bacterial pellets from overnight cultures were rinsed and resuspended in chilled Tris-Mg buffer. Samples were centrifuged after sonication, and the supernatant was centrifuged again. Pellets were finally frozen at

−20°C. A 12% SDS-PAGE was run and the protein bands of interest were recovered and sent to the Parc Cientific of Barcelona (Barcelona, Spain), where the proteins were characterized through matrix-assisted laser desorption ionization–time of flight analysis.

Western blotting

Western blot detection was performed as previously reported.[14] Cell envelope proteins were extracted and resuspended in 1× PBS. Amounts of 10 μg of each protein sample were loaded onto an 8% SDS-PAGE and transferred onto a nitrocellulose membrane on ice. The membranes were appropriately treated and incubated overnight at 4°C with the primary antibody against TolC diluted 1/500. Secondary antibody, anti-rabbit IgG, diluted 1/2000, was added for 1 h of incubation. Finally, the membranes were washed again and processed for chemiluminescence detection.

RNA extraction and real-time PCR

Four independent RNA extractions were carried out following the previously described protocol.[14] Exponential phase cultures were grown in LB at 37°C with shaking until the OD values at 600 nm were similar and between 0.5 and 0.6. For the stationary phase analysis, subcultures were grown overnight in LB in the absence of NaCl at 28°C and without shaking.

cDNA synthesis and RT–PCR were performed as described elsewhere.[35] The genes selected for analysis in exponential phase cultures were *acrR*, *soxS*, *marA*, *ramA* and *acrF*, whereas *csgB*, *csgD*, *adrA* and *bapA* were tested in stationary phase cultures. Relative gene expression was evaluated using the $2^{-\Delta\Delta CT}$ method, and the 16S rRNA gene was used as an internal control to normalize the transcriptional levels. Each RNA sample was tested in triplicate. A total of four independent assays were performed. The primers used are reported in Table 1.

Ciprofloxacin accumulation

The intracellular accumulation of ciprofloxacin was determined according to the protocol previously reported by our group and adapted from that described by Mortimer and Piddock.[35,36] Briefly, bacteria were grown in LB to late-log phase at 37°C with shaking (OD at 600 nm = 0.8–0.9) and a pellet was obtained and washed in PBS. A final concentration of 10 mg/L of ciprofloxacin was added and aliquots of 500 μL were taken at different time intervals for 10 min. After timepoint 5, 100 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), an energy uncoupler, was added to the reaction. Aliquots were washed and lysed overnight in 0.1 M glycine hydrochloride (pH 3.0) before fluorescence was measured. A standard curve in 0.1 M glycine hydrochloride (pH 3.0) was tested to infer the concentration of the drug.

Fitness

Strains were incubated overnight in LB at 37°C with shaking and then diluted to a similar OD at 600 nm. A 1/100 dilution in fresh LB broth followed and bacterial growth was allowed with shaking (540 rpm) in sterile 96-well microplates and assessed in an iEMS Multiskan MF Reader (Thermo Fisher Scientific). Bacterial curves were calculated for both conditions: 37°C in LB and 28°C in LB without NaCl. OD at 620 nm was determined every 15 min for 24 h. Four independent assays were performed for each strain and standard deviation agreed to within 10%.

Statistical analysis

Normal distribution was checked in order to apply parametric or non-parametric tests. When a normal distribution was found, differences between groups were detected by applying parametric tests: ANOVA (with contrasts) when more than two strains were compared or a paired *t*-test when comparing only two strains. Otherwise, Kruskal–Wallis and Mann–Whitney tests were applied to detect significant differences. Categorical data were compared using the χ^2 test.

All statistical analyses were performed using IBM SPSS Statistics software, version 20 (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.) and the level of significance was established at 0.05 (two-sided).

Results

Biofilm production and nalidixic acid susceptibility among clinical strains

The *in vitro* capacity to form biofilm was studied among 122 *S. enterica* clinical isolates by testing the ability to form a visible ring. Sixty-seven (55%) isolates were biofilm positive and 55 (45%) were biofilm negative. Among the 101 *Salmonella* Enteritidis isolates, 52 (51%) were positive for biofilm and 49 (49%) were negative. Most of the 19 *Salmonella* Typhimurium isolates ($n=14$) were positive for biofilm formation (74% versus 26%). Finally, the only isolate belonging to serovar Muenchen

Table 1. Primers used for RT–PCR analysis

Gene	Primer	Sequence 5′–3′	Temperature (°C)	Reference
16S	16S.RT.F	GCGGCAGGCCTAACACAT	60	62
	16S.RT.R	GCAAGAGGCCCGAACGTC		
acrF	SacrF.RT.1	TACCCAGGACGACATCTCTGA	60	35
	SacrF.RT.2	CACACCATTCAGACGGCTGAT		
csgB	ScsgB.RT.1	TCGACCAGGCAGGGAATTAT	60	this study
	ScsgB.RT.2	TTTGCGATATACTGGCATCGTT		
csgD	ScsgD.RT1	AACACGTGGTCAGCGGATTACAG	60	this study
	ScsgD.RT2	CGACCTCGCGATTTCATTATTAGA		
adrA	SadrA.RT1	GGAACGCCGGCAGACAGC	60	this study
	SadrA.RT2	CGCCATATCCGCAGACTTTAGC		
bapA	SbapA.RT1	TTCCCGCCGACAATAGCAGTAG	60	this study
	SbapA.RT2	CGTCAGCGGCGGCGTAGTA		

had the capacity to form biofilm, whereas the isolate belonging to serovar Hadar did not form.

Concerning the quinolone resistance phenotype of these 122 strains, 82 (67%) isolates were susceptible to nalidixic acid (MIC $<$16 mg/L), whereas the other 40 (33%) were resistant (MIC $\geq$64 mg/L). When the capacity to form biofilm was compared with nalidixic acid susceptibility, a relationship, albeit not significant, was observed between the two characteristics: susceptible strains were more frequently found to form biofilm than the resistant isolates (74.5% versus 25.4%, $P=0.054$).

Acquisition of fluoroquinolone resistance, strain 59-64

In order to study the possible relationship between the acquisition of quinolone resistance and decreased biofilm production, a quinolone-susceptible and biofilm-producing *Salmonella* Typhimurium clinical isolate, strain 59-wt, was chosen and its high-level fluoroquinolone resistant mutant, strain 59-64, was obtained *in vitro* by exposure to increasing concentrations of ciprofloxacin. Intermediate mutants were also selected and studied (strains 59-0.015, 59-0.03, 59-0.06, 59-0.25, 59-2 and 59-16). Regarding all these strains, sequencing of the QRDRs of the four target genes (*gyrA*, *gyrB*, *parC* and *parE*) was performed, as was the MIC determination for several quinolones (ciprofloxacin, norfloxacin and nalidixic acid) in the absence and presence of PAβN (Table 2).

Strain 59-wt showed an MIC of ciprofloxacin of 0.012 mg/L that rose to 256 mg/L in the high-level resistant mutant 59-64. A total of five QRDR mutations were found in 59-64, which were gradually acquired: strain 59-0.03 showed a substitution in GyrB (E466D), strain 59-2 acquired two amino acid changes, one in GyrA (S83Y) and another in ParC (S80R), and finally, strain 59-64 revealed two further modifications, one in GyrA (D87G) and one in ParC (F115S). Increments in the MICs of the three quinolones tested were observed in these three strains (4- to 16-fold for ciprofloxacin, 4- to 8-fold for norfloxacin and 6- to 64- for nalidixic acid), and attributed to the mutations described, since a parallel increase in these MICs was also observed in the presence of PAβN. There was only one exception, strain 59-64, which showed no increase concerning the MIC of nalidixic acid (8192 mg/L). Otherwise, strains 59-0.06, 59-0.25 and 59-16 did not acquire any QRDR mutation but showed increasing MICs of quinolones. Strain 59-16 displayed the largest increments, 5.3-fold for ciprofloxacin and 6-fold for norfloxacin. Since this strain showed the same MIC values in the presence of PAβN as 59-2, the previous resistant mutant, these increments are likely to be the result of the overexpression of a PAβN-susceptible efflux pump(s). Intriguingly, in comparison with 59-2, the two most resistant strains, 59-16 and 59-64, showed no change in the already high MIC of nalidixic acid (8192 mg/L) (Table 2).

Finally, strain 59-64 was grown on agar plates in the absence of ciprofloxacin in order to obtain a strain with a reverted phenotype. However, after 50 consecutive steps, strain 59-rev was selected and analysed, with no difference in the resistance phenotype being detected in comparison to 59-64.

MDR

The occurrence of an MDR phenotype is usually concomitant with the acquisition of fluoroquinolone resistance.[37,38] Thus, we determined the MICs of several unrelated antibiotics (chloramphenicol, tetracycline, erythromycin, amikacin, amoxicillin, ceftriaxone, cefoxitin) for strains 59-wt and 59-64. The results showed an increase ranging from 4- to 32-fold in the MICs of all these antibiotics in strain 59-64 when compared with the susceptible isolate. There were only three exceptions: chloramphenicol, amoxicillin and amikacin. Strain 59-wt was already resistant to chloramphenicol and amoxicillin (>256 mg/L), while no increase in the MIC of amikacin was found (1.5 mg/L) (Table 3). Furthermore, the MICs of tetracycline, erythromycin, ceftriaxone and cefoxitin were also determined for all intermediate mutants. The results showed two consecutive 1.5- to 3-fold increments for strains 59-0.06 and 59-0.25, and a more noteworthy 2- to 8-fold increase in the case of 59-16. Moreover, the MICs of tetracycline and erythromycin were also determined in the presence of PAβN and revealed that increased efflux inhibition was detected in strain 59-0.06, higher inhibition values were seen for 59-0.25, and maximal inhibition was achieved in 59-16 and 59-64.

Table 2. MIC determinations in the presence and absence of PAβN and QRDR mutations

	MIC (mg/L)[a]						Amino acid substitution[b]					
Strain	CIP		NOR		NAL		GyrA		GyrB	ParC		ParE
59-wt	0.012	(0.012)	0.094	(0.094)	4	(0.25)	—	—	—	—	—	—
59-0.015	0.012	(0.012)	0.094	(0.094)	4	(0.25)	—	—	—	—	—	—
59-0.03	0.047	(0.047)	0.38	(0.25)	24	(1)	—	—	E466D	—	—	—
59-0.06	0.19	(0.064)	1	(0.25)	48	(1)	—	—	E466D	—	—	—
59-0.25	0.38	(0.125)	2	(0.5)	128	(1)	—	—	E466D	—	—	—
59-2	6	(1)	16	(8)	8192	(128)	S83Y	—	E466D	S80R	—	—
59-16	32	(1)	96	(8)	8192	(128)	S83Y	—	E466D	S80R	—	—
59-64	256	(8)	512	(32)	8192	(128)	S83Y	D87G	E466D	S80R	F115S	—

CIP, ciprofloxacin; NOR, norfloxacin; NAL, nalidixic acid.
[a]Numbers in parentheses represent the MICs determined in the presence of PAβN (20 μg/mL).
[b]—, no mutation found.

Table 3. Characterization of the MDR phenotype

	MIC (mg/L)[a,b]								
Strain	CHL	TET		ERY		AMK	AMX	CRO	FOX
59-wt	>256	64	(12)	32	(6)	2	>256	0.094	2
59-0.015	—	64	(12)	32	(6)	—	—	0.094	2
59-0.03	—	64	(12)	32	(6)	—	—	0.094	2
59-0.06	—	96	(16)	96	(12)	—	—	0.190	4
59-0.25	—	128	(16)	>256	(12)	—	—	0.5	12
59-2	—	128	(16)	>256	(12)	—	—	0.5	12
59-16	—	>256	(16)	>256	(16)	—	—	1	96
59-64	>256	>256	(16)	>256	(16)	1.5	>256	1	96

CHL, chloramphenicol; TET, tetracycline; ERY, erythromycin; AMK, amikacin; AMX, amoxicillin; CRO, ceftriaxone; FOX, cefoxitin.
[a]Numbers in parentheses represent the MICs determined in the presence of PAβN (20 μg/mL).
[b]—, not determined.

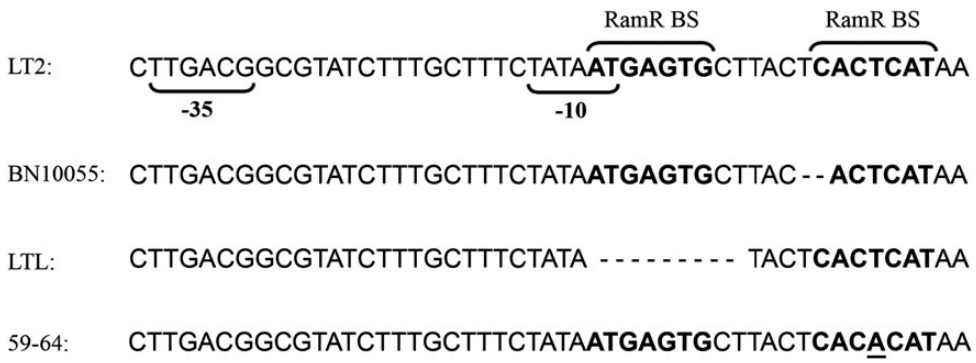

Figure 1. Schematic representation of the *ramA* promoter. The predicted −35 and −10 promoter regions as well as the putative RamR-binding sites (RamR BS) are represented in the sequence of the LT2 strain. Deletions previously reported for BN10055 and LTL strains are shown below. The underlined nucleotide corresponds to the mutation reported in the present study for the mutant 59–64.

Altogether, these findings suggest that a broad-spectrum mechanism such as increased efflux is involved in strains 59-0.06, 59-0.25 and particularly 59-16, as mentioned above.

Analysis of the MDR regulatory genes

Mutations within the regulatory genes that lead to increased AcrAB transcription have been reported to trigger both quinolone resistance and MDR phenotypes.[16,20,39,40] Thus, sequencing of the *acrR*, *soxRS*, *marRAB* and *ramR* regulatory loci and their corresponding promoters was performed for 59-wt and 59-64. The sequencing results revealed the acquisition of two mutations. The first was a 1 bp nucleotide deletion (adenine at position 139) within *acrA* leading to a frameshift mutation in the amino acid at position 47 in both the 59-wt and 59-64 strains. This mutation would cause a premature stop codon to appear at position 72, triggering a truncated protein. The second mutation detected was a point mutation within the *ramA* promoter in the mutant 59-64 when compared with 59-wt. Previous studies have characterized different strains (e.g. BN10055[20] and LTL[41]) carrying deletions affecting the putative RamR-binding sites and have associated them with increased expression of the *ramA* gene. Similarly, the mutation detected here is located within one of these binding sites and is assumed to impair basal *ramA* repression (Figure 1). A parametric test consistently detected significantly increased expression (>12-fold) of the *ramA* gene by RT–PCR analysis in the resistant mutant 59-64 in comparison with 59-wt when assessing exponential phase cultures ($P=0.014596$) (data not shown).

Expression analysis of AcrAB, TolC and AcrEF

Several methodologies were carried out to study the increased expression and hence putative involvement of efflux-related proteins. SDS-PAGE analysis was performed using a cell envelope protein extract from strains 59-wt and 59-64. In agreement with the *acrA* frameshift mutation, the bands corresponding to AcrB and AcrA virtually showed the same reduced expression levels in the two strains in comparison with the positive control strain 50-64, a *Salmonella* Typhimurium mutant previously reported to overexpress AcrAB[14] (Figure 2a). Western blotting using antibodies against TolC was then performed. The results revealed an increased TolC expression in 59-64 when compared with the basal levels of 59-wt (Figure 2b).

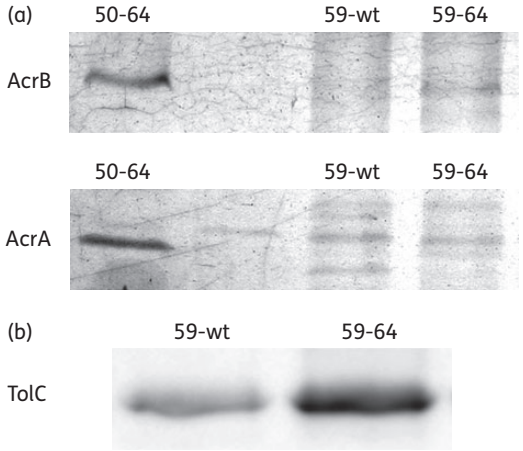

Figure 2. Cell envelope protein extracts analysed by (a) SDS-PAGE and (b) western blotting with antibodies against TolC.

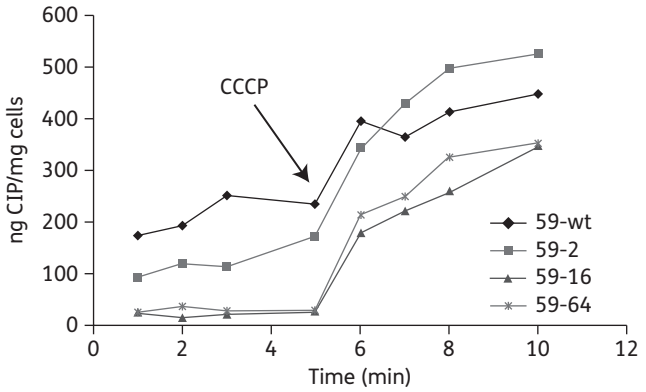

Figure 3. Ciprofloxacin accumulation. Aliquots were taken at 1, 2, 3, 5, 6, 7, 8 and 10 min after incubation. CCCP (100 μM) was added at the time indicated by the arrow. CIP, ciprofloxacin.

Several studies performed with *E. coli* or *Salmonella* Typhimurium mutants that do not express AcrAB have shown an up-regulation of the alternative efflux pump AcrEF that may compensate the absence of AcrAB.[42–44] Therefore, we studied the possibility that AcrEF may act in conjunction with TolC and lead to the MDR phenotype of 59-64. *acrF* expression was evaluated by RT–PCR analysis of the exponential growth phase of strains 59-wt and 59-64. However, the results showed no change among these strains, suggesting no role in the resistance phenotype for AcrEF (data not shown).

Ciprofloxacin accumulation

In order to accurately evaluate whether or not increased efflux was selected during the resistance procedure, particularly in the case of the mutant 59-16, the intracellular accumulation of ciprofloxacin was measured. The strains tested included 59-wt, 59-2, 59-16 and 59-64. On comparing the results with 59-wt, a decrease in the accumulation levels was observed for 59-2, followed by a consecutive reduction for 59-16. These low levels of intracellular ciprofloxacin were maintained in 59-64. In order to confirm the contribution of efflux to the resistance phenotype, the energy uncoupler CCCP was added to the samples, leading to a substantial increase in ciprofloxacin accumulation in all strains, despite the values of 59-16 and 59-64 not reaching those observed for 59-wt and 59-2 (Figure 3). Thus, increased efflux seems to have been acquired at several steps in the resistance procedure. The first step may be the result of the cumulative effects in the mutants previously selected to 59-2 (likely to be 59-0.06 and 59-0.25 as mentioned above), and the second step is related to 59-16. Nonetheless, the contribution of efflux seems to be equal in both steps.

Biofilm formation and related phenotypes

Biofilm production was determined for 59-wt and all the derivative mutants. The results showed a progressive decrease in this virulence trait that occurred inversely to the resistance values observed. Significant differences were determined applying non-parametric tests. The most significant and representative decrease was observed between 59-2 and 59-16 ($P=0.000032$), leading to a 3.6-fold reduction (Figure 4a). To further study which of the elements involved in biofilm formation had impaired expression, production of curli fibres and cellulose was tested through several approaches.

First, colony morphology was tested in all strains on Congo Red plates at 28°C. This dye has been reported to be indicative of curli and cellulose production.[33] The results showed a pattern similar to that observed for biofilm production: a progressive reduction in the rdar morphotype with a more important decrease for strains 59-16 and 59-64, which were unable to produce this trait (Figure 4b). Second, the phenotype of all strains after growth on plates with calcofluor was analysed. This fluorochrome has been reported to be primarily indicative of cellulose production.[33] However, in this situation no difference was observed under UV light between all the strains. These results suggested an impaired expression of curli fibres but no change in cellulose production.

Expression of biofilm-related genes

Finally, expression of the genes involved in biofilm production was assessed by the RT–PCR analysis of overnight cultures of 59-wt, 59-2, 59-16 and 59-64, since the most important decrease in biofilm formation was seen for 59-16. The genes tested were: *csgB*, nucleator and minor subunit of curli fibres,[27] *adrA*, post-transcriptional activator of cellulose biosynthesis,[45] *bapA*, large surface adhesin,[45] and *csgD*, master regulator of biofilm.[46] The most consistent and reproducible results were obtained for the *csgB* gene, which were analysed using parametric tests. Initially, a 2.2-fold reduction was observed for 59-2 in comparison with 59-wt, albeit without significance. Nonetheless, a major and statistically significant decrease of 23-fold was seen for 59-16 when compared with 59-2 ($P=0.000081$) (Figure 5). These results suggest that the decrease in biofilm production and the rdar morphotype observed in 59-16 and 59-64 are attributable to reduced *csgB* expression.

Fitness

Bacterial growth was evaluated for 59-wt and all mutant strains by measuring the OD at 620 nm every 15 min for 24 h at 28°C and

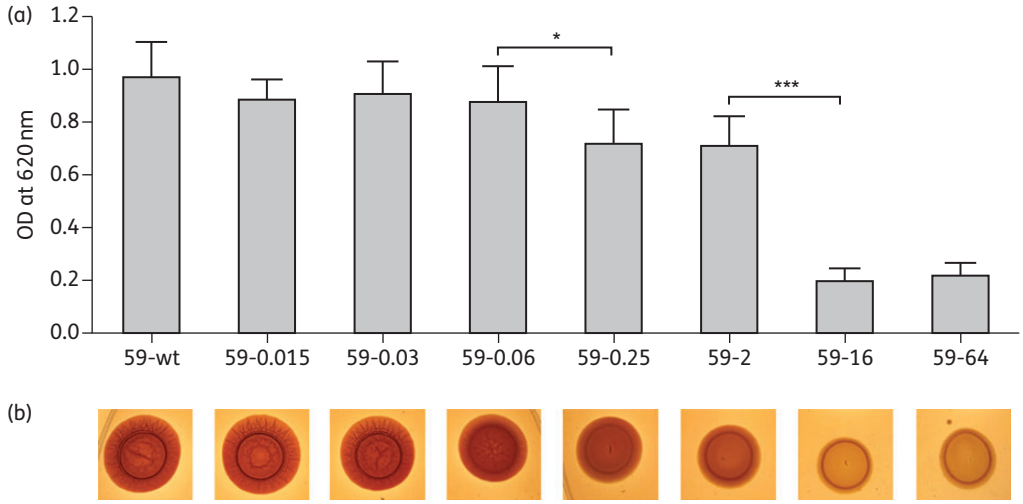

Figure 4. Production of biofilm by measuring the OD at 620 nm (a) in correlation with the rdar morphotype (b). Data are presented as mean ± SD. Asterisks indicate statistically significant differences between strains (*$P<0.05$; **$P<0.01$; ***$P<0.001$). This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

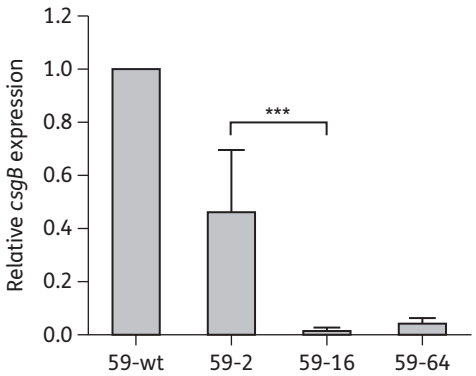

Figure 5. RT–PCR analysis showing the relative expression values of the *csgB* gene. The *16S* gene was used as the internal control. Data are presented as mean ± SD. Asterisks indicate statistically significant differences between strains (*$P<0.05$; **$P<0.01$; ***$P<0.001$).

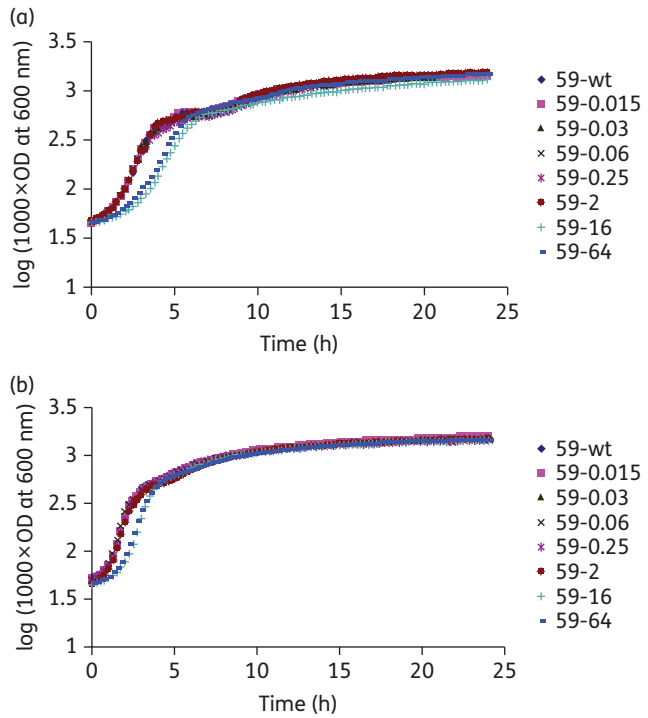

Figure 6. Bacterial fitness evaluated at two different temperatures: 28°C (a); and 37°C (b). This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

37°C. On comparing the growth curves, a significant difference was detected between the two most resistant mutants, 59-16 and 59-64, and the rest of the strains. The resistant mutants showed a diminished ability to grow since a longer lag phase was observed before the exponential growth. These differences were observed at the two temperatures tested. Once bacteria reached the stationary phase, the differences between all the strains disappeared (Figure 6). Moreover, the specific growth rate (μ) was calculated according to $\mu=(\ln N-\ln N_0)/(t-t_0)$. The resulting data obtained for both temperatures, 28°C and 37°C, were analysed applying non-parametric or parametric tests, respectively. Significant differences were detected between 59-2 and 59-16 in both conditions [$P=0.021$ (28°C) and $P=0.000004$ (37°C)].

Discussion

In this study, we evaluated the biofilm-forming ability of a collection of 122 *S. enterica* clinical isolates. Among these, 82 strains were reported to be nalidixic-acid susceptible, whereas the remaining 40 strains were resistant. The results are in agreement with those of previous studies performed by our group and showed a relationship between nalidixic acid susceptibility and biofilm production, with susceptible strains more frequently producing biofilm than resistant strains.[30,31]

In order to better understand this relationship, a quinolone-susceptible *Salmonella* Typhimurium clinical isolate, 59-wt, was selected to obtain *in vitro* mutants with increasing MICs to quinolones. Strain 59-64 was the most resistant mutant, displaying a ciprofloxacin MIC of 256 mg/L. The mechanisms of quinolone resistance acquisition were studied first. Strain 59-64 acquired up to five QRDR mutations, three leading to well-described amino acid substitutions at the most frequently affected positions (S83Y and D87G in GyrA and S80R in ParC, in strains 59-2 and 59-64).[47–49] Substitutions within GyrB and ParE have been reported, although their prevalence is much less frequent and they have sometimes been related to a minor contribution to resistance.[50,51] In this study, the first amino acid modification, which has only recently been reported,[29] was detected in GyrB (E466D) in strain 59-0.03. Although the replacement residue did not change the functional group, the MICs of the three quinolones tested concomitantly increased. Otherwise, the fifth substitution (59-64) occurred in ParC (F115S) and has not been previously described. However, its contribution to the resistance phenotype cannot be completely elucidated since it was acquired simultaneously with the second amino acid substitution in GyrA.

Increased efflux in *S. enterica* (i.e. AcrAB overexpression) has been reported as usually representing the first step in the process of quinolone resistance acquisition, whereas QRDR mutations as well as enhanced efflux activity are acquired in following steps.[13,14,47,52] In the present study, however, the first step (59-0.03) was explained by the acquisition of a mutation in the *gyrB* gene in strain 59-0.03. In contrast, the ciprofloxacin accumulation results indicated that increased efflux was acquired later and in at least two separate steps, 59-2 and 59-16. Since the increments in resistance observed in strain 59-2 are likely to be the result of two target gene mutations, this increased efflux may have been acquired before, in strains 59-0.06 and 59-0.25, according to the MIC values of both quinolones and other unrelated antibiotics observed in the absence and presence of PAβN. Of note, no further increase in the MIC of nalidixic acid was observed in the two most resistant strains (59-16 and 59-64), even though the GyrA amino acid substitution D87G (59-64) is associated with a >4-fold increase in the MICs of these quinolones.[13,14,47] These results suggest the existence of a plateau in the levels of resistance to nalidixic acid (MIC of 8192 and 128 mg/L in the absence and presence of PAβN, respectively). Similar results related to an upper limit to drug resistance have been reported in *E. coli*.[53]

This study has also shown that MDR concurred with the fluoroquinolone resistance phenotype. The wide range of exportable substrates shown by 59-64 is similar to that shown by AcrAB-TolC. Nevertheless, our results revealed no overexpression of this major efflux pump attributable to a frameshift deletion within *acrA* in both the 59-wt and 59-64 strains. Despite the reported difficulty in selecting ciprofloxacin-resistant mutants in the absence of *acrB*,[54] strain 59-64 displayed an MDR phenotype comparable to that reported for mutants overexpressing AcrAB.[14,29] Evidence that other efflux systems are likely to be involved in conferring quinolone resistance and MDR phenotypes have previously been reported.[14,29,52] Among the alternatives, several studies have revealed that after *acrB* inactivation, in either *E. coli* or *Salmonella* Typhimurium strains, overexpression of *acrEF* can be detected and may relay the usual AcrAB overexpression.[42–44] However, the results obtained in this study ruled out a possible *acrF* overexpression in 59-64. Even though AcrAB does not play a role in the present study, on analysing the MDR regulatory genes, which usually activate this major efflux pump, the findings revealed increased expression of the *ramA* gene attributable to a mutation within one of the two RamR-binding sites located in its own promoter. Thus, at least one efflux system involved in conferring these resistance phenotypes, likely to be acting in conjunction with TolC, is under the positive control of RamA, although it has yet to be characterized.

Finally, biofilm production was evaluated for strain 59-wt and its derivative mutants in order to further study whether the acquisition of quinolone resistance was related to a decrease in biofilm formation. Production of biofilm was shown to progressively diminish as far as quinolone resistance increased, although a more significant reduction was observed for the two most resistant strains, 59-16 and 59-64. Similarly, a parallel decrease was observed in terms of the rdar morphotype. Both features could be explained by the decreased expression of the *csgB* gene, more importantly detected in these two strains.

Several studies have focused on the putative relationship between antimicrobial resistance and virulence. Several approaches need to be considered. First, the selection of fluoroquinolone resistance usually correlates with impaired fitness,[14,29,55] this phenotype being attributed to the acquisition of mutations leading to increased efflux.[56] Second, this resistance phenotype, supported by target gene mutations, AcrAB overexpression and reduced fitness, has been associated with a decreased expression of virulence factors and a consequent reduction in *in vitro* invasiveness.[14,29,57] Third, the inactivation of efflux pump components, such as *acrB* and particularly *tolC*, leads to impaired virulence, including *in vitro* invasion and *in vivo* colonization.[58–60] Moreover, both inactivation and high overexpression of the *ramA* gene, and consequently of *acrAB*, leads to impaired expression of virulence determinants, reduced macrophage survival and diminished virulence in *in vivo* models.[61] Concerning biofilm production, individual inactivation of both *tolC* and *acrB* triggers a reduction in biofilm formation.[32]

In this study, we report that an increasing quinolone resistance phenotype, combining target gene mutations and increased efflux, was inversely related to a reduction in formation of biofilm and the rdar morphotype as well as diminished expression of the *csgB* gene. The results suggest two stages, the initial stage of which would be progressively acquired and represent the cumulative effects of mutations acquired in several strains (from 59-wt to 59-2). In contrast, the second stage would be more drastic (strains 59-16 and 59-64). These findings could be explained by the type of mutations acquired in 59-16. The reduced biofilm production detected in this strain may appear as a consequence of the noteworthy increased efflux, a property that may also explain the impaired fitness observed. As previously reported, an appropriate production of efflux systems seems to play a key role in determining the expression of virulence properties, since both absence and overexpression appear to be detrimental for virulence.[58–60] The diminished virulence observed, in terms of biofilm production, may also reflect bacterial adaptation to the increased energy consumption, supported by increased efflux, by turning off such virulence properties that are dispensable for bacterial survival. Further work is needed in order to determine the efflux pump(s) involved in the resistance phenotype, thereby allowing a more precise evaluation of the relationship between quinolone resistance and biofilm formation.

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Transparency declarations

None to declare.

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Paper IV

Attenuation of *in vitro* host-pathogen interactions in quinolone-resistant *Salmonella* Typhi mutants

Clara Ballesté-Delpierre, Anna Fàbrega, Mario Ferrer-Navarro, Ramkumar Mathur, Sankar Ghosh and Jordi Vila

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The relationship between the acquisition of quinolone resistance and a decrease in virulence traits has been previously studied in non-Typhi serovars [210–212]. However, little information is available regarding *S*. Typhi. The aim of this study was to investigate if virulence was also affected in terms of host cell invasion and the induction of immune responses in *S*. Typhi mutants upon acquisition of quinolone resistance. Moreover, the molecular mechanisms involved in antibiotic resistance were also analyzed in depth.

A *S*. Typhi clinical isolate from the Hospital Clínic (Barcelona, Spain), named Ty_wt, was exposed to increasing concentrations of ciprofloxacin in order to obtain mutants able to survive upon antimicrobial pressure; as a result, two mutants were obtained (Ty_c1 and Ty_c2) showing intact growth rates. Unfortunately, no revertants were obtained after 35 consecutive passages.

Quinolone susceptibility revealed that the parental strain was already resistant to nalidixic acid (MIC 250 µg/mL) even though a sequential 4-fold increase was noted in Ty_c1 with a further 2-fold in Ty_c2. Moreover, the MIC of norfloxacin also rose from 1 µg/mL in Ty_wt to 8 µg/mL in Ty_c1, finally reaching 24 µg/mL in Ty_c2. However, the MIC of ciprofloxacin was almost unaltered in Ty_wt and Ty_c1, and increased 8-fold in Ty_c2. Complete sequencing of the quinolone target genes (*gyrA*, *gyrB*, *parC* and *parE*) revealed a single amino acid change in GyrA (S83F) already present in the clinical isolate, meaning that acquisition of the fluoroquinolone resistant phenotype was not attributed to target gene modification. In order to check if resistance to other drugs belonging to different antibiotic classes was also acquired, susceptibility testing was assessed in a broader collection of

antibiotics including erythromycin, ampicillin, ceftriaxone, cefoxitin, chloramphenicol, tetracycline, trimethoprim and kanamycin. Upon comparing Ty_wt with the first mutant Ty_c1, the latter strain showed the highest increase in the MIC of ampicillin (8-fold), modest increments (2-4-fold) in the MICs of tetracycline, trimethoprim, cefoxitin and ceftriaxone, whereas no major changes (<1.5-fold) were seen for chloramphenicol and erythromycin. However, Ty_c2 showed an MDR phenotype as it became resistant to cefoxitin and chloramphenicol with a 32-fold increase in the MIC compared with Ty_c1. In the case of the remaining antibiotics, the MICs increased from 3- to nearly 5-fold, although they did not reach the resistant breakpoints. Kanamycin was the only antibiotic for which no variation was recorded in any of the three strains studied.

These results pointed out the contribution of increased efflux as an antibiotic resistance mechanism. For this reason, gene expression of the *acrB*, *tolC*, *acrF*, *emrB* and *ompF* genes was tested by RT-PCR in the three strains. The results revealed no major changes in Ty_c1 in any of these genes despite statistically significant differences being seen for *acrF* and *emrB* (almost 2-fold decrease). In contrast, overexpression of *acrB* and *tolC* was detected in Ty_c2 compared to Ty_wt (RQ=3.91 for *acrB* and RQ=3.42 for *tolC*) as was a repression greater than 6-fold of the *ompF* porin. Surprisingly, the alternative efflux-related genes, *acrF* and *emrB*, were repressed in this strain compared to Ty_wt (3- and 3.9-fold, respectively). Outer-membrane protein analysis confirmed the findings related to TolC and AcrA as these proteins were overproduced in Ty_c2. In addition, a decreased production of the OmpC porin was detected.

In a further step, the regulatory regions of AcrAB were sequenced (*acrR*, *marRAB*, *soxRS* and *ramR*) and gene expression was evaluated. Interestingly, an important alteration of the *marR* locus was detected in Ty_c2 and corresponded to a deletion of 445 nucleotides. This shift included the deletion of almost the entire *marR* gene and 34 nucleotides upstream of the gene; however, Site I, one of the two MarR binding sites as well as the -35 and -10 signals of the *marRAB* promoter were completely preserved. Consistent with these findings, *marA* was highly overexpressed in this mutant (RQ 108.19 ± 0.16). Hence, the absence of repression triggered by the lack of a functional MarR caused the overexpression of *marA*

eventually affecting the overproduction of the efflux system AcrAB/TolC and also explaining the downregulation of the OmpF porin. Nevertheless, despite no further changes being seen in the sequences of the other regulators, a repression of *acrR, soxS* and *ramA* ranging from 2- to almost 6-fold was observed in Ty_c2. No changes were seen in any regulatory gene of the Ty_c1 isolate.

Virulence aspects were investigated and revealed the following results. First, motility was assessed and showed a sequential decrease from Ty_wt to Ty_c2. This was confirmed by the gene expression levels of the flagellin-encoding gene *fliC,* for whichTy_c1 showed a 10-fold reduction in comparison with Ty_wt, and an additional 10-fold decreased expression for Ty_c2 (RQ=0.01).

The ability to invade eukaryotic cells was assessed by infecting HeLa cells with the different strains. The invasion rates corresponded to: 7.31% for Ty_wt, Ty_c1 invaded 1.69%, around 4 times less, and the lowest levels of invasion, 0.27%, were reported for Ty_c2, being 6 times less than the previous mutant and 27 times less than Ty_wt. Internalization in macrophages was also tested and a similar pattern was seen in the mutants, albeit to a lesser extent: almost a 3-fold reduction was seen in the number of internalized bacteria in Ty_c1 compared to Ty_wt, and an additional 1.6-fold was seen for Ty_c2, which corresponded to 4.37-fold less than Ty_wt. In terms of bacterial survival inside macrophages (at 24h post-infection), similar results were observed for the two mutants, with a 4-fold reduction compared to the parental strain.

The gene expression of representative virulence factors (*invA*, *hilD*, *hilA*, *fimA* and *tviA)* was studied and showed a progressive decrease in the expression levels of *invA*, *hilD*, *hilA* and *fimA* in the two mutants. As a first step, decreased expression of 2.5- to 3.75-fold was seen in Ty_c1 followed by an additional 1.89- to 3.67-fold in Ty_c2. This profile was in accordance with the above-mentioned results reflecting the reduced ability to invade epithelial cells and be internalized and survive inside macrophages. A different result was seen for the Typhi-specific virulence determinant *tviA*, for which an important reduction of almost 30-fold was only seen in the expression of Ty_c2.

The potential to induce immune response in infected cells was assessed by evaluating cytokine levels (TNF-α and IL-1β), and the activation of the nuclear transcription factor NF-κB.TNF-α was measured in the supernatants and revealed a gradual a significant decrease in the two mutants compared to Ty_wt, with the levels ranging from 5,181 µg/mL in Ty_wt to 3,256 µg/mL in Ty_c2. A greater reduction was seen for IL-1β (checked at 24h post-infection) showing a reduction of more than 50% in both mutants compared to Ty_wt. Activation of NF-κB was significantly diminished in Ty_c1 (a decrease of 16.67 % was seen compared to Ty_wt) and was much more important for Ty_c2 (71% reduction in comparison with Ty_wt). These findings demonstrated that the two mutants elicited a more discrete immunogenic reaction than the parental strain.

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Attenuation of *in vitro* host–pathogen interactions in quinolone-resistant *Salmonella* Typhi mutants

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Objectives: The relationship between quinolone resistance acquisition and invasion impairment has been studied in some *Salmonella enterica* serovars. However, little information has been reported regarding the invasive human-restricted pathogen *Salmonella* Typhi. The aim of this study was to investigate the molecular mechanisms of quinolone resistance acquisition and its impact on virulence in this serovar.

Methods: Two antibiotic-resistant mutants (Ty_c1 and Ty_c2) were generated from a *Salmonella* Typhi clinical isolate (Ty_wt). The three strains were compared in terms of antimicrobial susceptibility, molecular mechanisms of resistance, gene expression of virulence-related factors, ability to invade eukaryotic cells (human epithelial cells and macrophages) and cytokine production.

Results: Multidrug resistance in Ty_c2 was attributed to AcrAB/TolC overproduction, decreased OmpF (both mediated by the *mar* regulon) and decreased OmpC. The two mutants showed a gradually reduced expression of virulence-related genes (*invA*, *hilA*, *hilD*, *fliC* and *fimA*), correlating with decreased motility, reduced infection of HeLa cells and impaired uptake by and intracellular survival in human macrophages. Moreover, Ty_c2 also showed reduced *tviA* expression. Additionally, we revealed a significant reduction in TNF-α and IL-1β production and decreased NF-κB activation.

Conclusions: In this study, we provide an in-depth characterization of the molecular mechanisms of antibiotic resistance in the *Salmonella* Typhi serovar and evidence that acquisition of antimicrobial resistance is concomitantly detected with a loss of virulence (epithelial cell invasion, macrophage phagocytosis and cytokine production). We suggest that the low prevalence of clinical isolates of *Salmonella* Typhi highly resistant to ciprofloxacin is due to poor immunogenicity and impaired dissemination ability of these isolates.

Introduction

Typhoid fever is a human-restricted systemic infection caused by *Salmonella enterica* serovar Typhi. This pathogen causes ~21 million infections causing typhoid fever each year, leading to ~200 000 deaths.[1] The highest incidence rates are seen in Asia, where this pathogen remains a major public health problem. The transmission route is through exposure to food and water contaminated with human faeces. Although it belongs to the same species as *S. enterica* serovar Typhimurium, their pathogenesis substantially differs. While salmonellosis caused by *Salmonella* Typhimurium infection is usually self-limiting, typhoid fever is a systemic infectious disease with much more severe consequences. Once bacteria reach the small intestine, they adhere to the mucosa and mostly invade M cells, which mediate internalization of the pathogen and allow transportation to Peyer's patches. This process is followed by phagocytosis by dendritic cells and macrophages, thus favouring dissemination through the mesenteric lymph nodes and eventual spread to the liver, spleen and bone marrow where *Salmonella* Typhi is able to survive and replicate.[2–4]

The treatment of typhoid fever has been changing and adapting together with the spread of drug-resistant strains. Chloramphenicol, trimethoprim/sulfamethoxazole and ampicillin were initially used until the emergence and spread of plasmid-mediated resistance to these antimicrobial agents. As a consequence, these drugs were replaced by ceftriaxone and ciprofloxacin as well as other fluoroquinolones such as ofloxacin.[5] However, dissemination in the past decades, mostly in Asia, of strains showing both resistance to nalidixic acid (MIC >256 mg/L) and decreased ciprofloxacin susceptibility (MIC range, 0.125–1 mg/L) has occurred.[5–7] In addition, some reports have already shown the

emergence of highly fluoroquinolone-resistant isolates.[8–10] This scenario is forcing the introduction of new-generation fluoroquinolones (e.g. gatifloxacin) and alternative drugs such as azithromycin.[5,11]

Quinolones act through inhibition of DNA gyrase and topoisomerase IV, hampering DNA replication and transcription as well as interfering with cell division. In *S. enterica*, resistance to quinolones is mainly due to point mutations in the quinolone resistance-determining regions (QRDRs) of the target genes, with amino acid substitutions at positions 83 and 87 of GyrA being the most frequently reported.[4,12,13] Mutations affecting the internal accumulation of the drug, by decreasing the expression of porins (e.g. OmpF and OmpC) and/or increasing the expression of efflux pumps such as AcrAB/TolC,[13–15] are also of great concern. Expression of the efflux pump AcrAB is controlled by its local repressor AcrR and by three homologous transcriptional activators, MarA, SoxS and RamA. The contribution of these regulators to the MDR phenotype has been reported in laboratory mutants and clinical isolates of *S. enterica*.[16–20] There is only a single exception, since results concerning the role of MarA have been demonstrated in *Escherichia coli*,[21–23] but not yet in clinical isolates of *Salmonella*. Similarly, activation of the transcription of *micF*, an antisense RNA that inhibits synthesis of the outer membrane porin OmpF,[24] has yet to be supported with clinical data for *Salmonella*. In addition, plasmid-encoded genes [e.g. *aac(6′)-Ib-cr* and the *qnr* genes] are also responsible for quinolone resistance in *Salmonella* spp.[13]

Pathogenicity is primarily mediated by a number of virulence factors, the genes of which are organized within particular regions of the genome, named *Salmonella* pathogenicity islands (SPIs). To date, 15 SPIs have been identified in *Salmonella* Typhi, among which SPI-1, homologous to that described in *Salmonella* Typhimurium, contains genes encoding the type III secretion system-1 (T3SS-1) and secreted effector proteins needed for the invasion process.[25–27] Furthermore, *Salmonella* Typhi-specific SPI-7 contains genes involved in the biosynthesis, regulation and export of the Vi capsular antigen. This virulence factor plays a key role in the attempt of *Salmonella* Typhi to avoid host defences and is therefore important in enhancing infectivity and virulence.[28] Moreover, innate immune responses such as inflammation are also activated through the interaction of pathogen-associated molecular patterns, e.g. LPS and flagellin, with the Toll-like receptors (TLRs) TLR-4 and TLR-5, respectively.[4,27,29] Stimulation of TLRs induces activation of the transcriptional regulator NF-κB, thus triggering the production of proinflammatory cytokines such as TNF-α, IL-6 and IL-1β.[30]

Previous reports conducted in non-Typhi *S. enterica* have shown that acquisition of quinolone resistance is related to decreased expression of virulence factors and impaired adherence to and invasion into the host cell.[31,32] Even though there is an epidemiological relatedness between Typhi and non-Typhi serovars, many differences have been reported in terms of virulence and colonization behaviour, suggesting that adequate experiments should be conducted to address this relationship in *Salmonella* Typhi. Thus, the main objective of this study was to investigate if this loss of virulence, in terms of invasion and induction of host immune responses, also occurred in *Salmonella* Typhi upon acquisition of quinolone resistance. Moreover, despite the above-mentioned mechanisms of quinolone resistance being well studied in non-typhoidal *Salmonella*, an in-depth characterization of the molecular mechanisms involved in antibiotic resistance of two *Salmonella* Typhi mutants was also carried out.

Materials and methods

Strains

A clinical isolate of *Salmonella* Typhi was recovered from a patient diagnosed with spondylodiscitis at the Hospital Clínic of Barcelona (Spain). This isolate (Ty_wt) was used to generate *in vitro* antibiotic-resistant mutants in a multistep selection process, as previously described,[31] by exposure of the bacteria to doubling concentrations of ciprofloxacin, starting at 0.25 mg/L. The mutants studied in this work were selected at 1 and 2 mg/L ciprofloxacin, named Ty_c1 and Ty_c2, respectively. When overnight cultures were required, strains were grown for 16–18 h.

Antimicrobial susceptibility

Antimicrobial susceptibility to nalidixic acid, ciprofloxacin, norfloxacin, ampicillin, ceftriaxone, cefoxitin, erythromycin, chloramphenicol, tetracycline, trimethoprim and kanamycin was assessed using Etests (bioMérieux) on Mueller–Hinton II plates (Becton Dickinson) following the manufacturer's recommendations. At least three replicates of each susceptibility test were performed.

Sequencing of quinolone resistance genes

DNA amplification of the target genes *gyrA*, *gyrB*, *parC* and *parE* as well as the global regulators *acrR*, *marRAB*, *soxRS* and *ramR* was performed using the primers listed in Table S1 (available as Supplementary data at *JAC* Online). Sequencing was performed by Beckman Coulter Genomics (Essex, UK) and the sequences analysed by alignment with the template sequence of *Salmonella* Typhi (RefSeq NC 016832.1).

Bacterial growth

Fresh cultures were grown at 37°C with shaking, as previously described,[31] and OD readings at 600 nm (OD_{600}) were done every 15 min for 24 h by means of an iEMS Multiskan Reader MF (Thermo Fisher Scientific). Each plate included four replicates of each sample and the assay was repeated three times.

Motility

Bacterial cultures were grown overnight in LB medium at 37°C with shaking and 10 μL was 'stab inoculated' into soft agar plates containing 0.5% agar. Plates were incubated for 7 days at 37°C in a humid chamber. Motility was assessed by measuring and comparing the growth diameter of the three tested strains.

Relative expression of resistance and virulence genes

RNA extraction from exponential cultures of Ty_wt, Ty_c1 and Ty_c2 was performed as reported by Fàbrega *et al*.[31] A two-step real-time PCR was performed as previously described by our group[33] following the $2^{-\Delta\Delta CT}$ method. Briefly, the obtained RNA was reverse transcribed to cDNA using the PrimeScript RT Reagent Kit (Takara) followed by PCR (SYBR® Premix Ex Taq Tli RNase H Plus Kit, Takara) under universal thermal cycling conditions. Expression of the efflux-related genes *acrB*, *tolC*, *emrB* and *acrF*, the *marA* regulator, the *ompF* porin and the virulence-related genes *hilA*, *hilD*, *invA*, *fliC*, *fimA* and *tviA* was studied. The primers used (Table S2) were designed using Primer Express® software (Applied Biosystems). The $2^{-\Delta\Delta CT}$ method was applied to measure the gene expression, defined as relative

quantification (RQ) of the target genes in the two mutant strains. The 16S rRNA was used as the reference gene for normalization and Ty_wt was the calibrator strain. Five independent extractions were performed and differences of >2-fold were considered relevant. Standard deviation was calculated and only reported when this value was >0.1.

Epithelial cell invasion assay

The invasion assay was performed according to Fàbrega *et al.*[31] with some modifications. Briefly, HeLa cells (ECACC 84211901) were seeded into 6-well tissue culture-treated plates (Corning) in order to obtain a monolayer corresponding to $\sim 5 \times 10^5$ cells/well at 37°C in a 5% CO_2 atmosphere. Infection was then performed with bacteria grown overnight at 37°C without shaking at an moi of 100. Plates were incubated at 37°C/5% CO_2 for 2 h followed by an additional 2 h of incubation in the presence of 100 mg/L gentamicin (Life Technologies) to kill extracellular bacteria. Wells were then washed and 1 mL of chilled sterile water was added and kept at 4°C for 30 min to lyse the cells. This volume was recovered for intracellular bacterial counting by plating several dilutions in LB agar. At least three independent experiments were performed with intraexperiment duplicates. The invasion ability of each strain was determined by calculating the ratio between the number of intracellular bacteria and the inoculum.

Human macrophage infection assay

Human-derived monocytes (U-937 cell line) were used upon differentiation into adherent macrophages with 50 μg/L phorbol 12-myristate 13-acetate. A total of 5×10^5 cells/well were seeded into 24-well plates (Becton Dickinson) and incubated at 37°C/5% CO_2 with RPMI-1640 (Life Technologies) supplemented with 10% FBS (Life Technologies). After 24 h, wells were washed three times with PBS (Life Technologies) and new medium was added in order to eliminate the non-adhered cells. Infection was carried out with *Salmonella* Typhi strains grown overnight at 37°C without shaking at an moi of 50. Plates were then centrifuged for 5 min at 2500 **g** at room temperature and incubated for 20 min at 37°C/5% CO_2. Afterwards, cells were washed again and new medium containing 100 mg/L gentamicin was added before incubating for 2 h under the same conditions as described above. After this time, bacterial entry into host cells was determined by eliminating the supernatant, incubating the cells for 15 min at 4°C with 1 mL of chilled sterile water and then plating the appropriate dilutions on LB agar plates in order to allow bacterial counting. Alternatively, to determine the uptake rate, after 2 h of incubation with 100 mg/L gentamicin, the medium was replaced with new RPMI containing a lower dose of gentamicin (12 mg/L) and maintained for 24 h. Then, wells were washed and treated with 1 mL of chilled sterile water for cell lysis and bacterial counting. The intracellular survival ability was determined by calculating the ratio between the number of bacteria recovered at 24 and 2 h post-infection.

Cytokine assay

Production levels of TNF-α and IL-1β were measured in the supernatants during the human macrophage infection assay. After the two incubation periods (2 and 24 h), supernatants were collected and levels of TNF-α (from the 2 h post-infection plates) and IL-1β (from the 24 h post-infection plates) were measured by ELISA according to the manufacturer's recommendations (BD OptEIA).

NF-κB luciferase assay

HEK293 cells stably transfected with an NF-κB luciferase reporter construct, previously obtained by Koblansky *et al.*,[34] were plated onto 48-well plates. After 24 h of incubation at 37°C/5% CO_2, cells were stimulated with heat-killed *Salmonella* Typhi as well as with 0.2 μg/L TNF-α as a positive control for 6 h. Luciferase activity was measured with the Luciferase Reporter Assay System (Promega). Heat-killed bacteria were prepared from an overnight culture adjusted to $OD_{600}=0.7$. An 800 μL aliquot of the bacterial culture was centrifuged at 13 500 **g** for 10 min, washed twice with PBS and resuspended in 1 mL of the same solution. Then, following 1 h of incubation at 65°C, the bacterial solution was vortexed for 3 min and centrifuged for 5 min at 2500 **g** to discard protein debris. For stimulation, 25 μL of the supernatant was added to each well. Each sample was tested in triplicate and three independent assays were performed.

Outer membrane protein analysis

Extraction of outer membrane proteins was performed with *N*-lauroyl sarcosinate. Briefly, bacteria were harvested from 200 mL of exponential culture ($OD_{600}=0.6$) by centrifugation at 4°C and 3500 **g**. Cells were washed twice in PBS in order to remove any residual medium. Then, cells were resuspended in 6 mL of 10 mM Tris, pH 8.0/1% NaCl. At this point, cells were sonicated for 15 min (cycles of 59 s 'on' and 59 s 'off'). After cell disruption, samples were centrifuged at 4°C and 3500 **g** in order to remove any cell debris. Supernatants were collected and transferred into ultracentrifugation tubes and samples were centrifuged at 100 000 **g** for 1 h at 4°C in a Sorvall MS-150 microultracentrifuge (Thermo Scientific) using an S50-ST rotor. After this centrifugation step, the supernatant was discarded and the pellet resuspended in 1% sarcosyl solution and incubated for 60 min at room temperature with gentle agitation. After incubation, samples were again centrifuged at 100 000 **g** for 1 h at 4°C and the resulting pellet was cleaned twice with 10 mM Tris, pH 8.0/1% NaCl. Finally, the pellet was carefully resuspended in 500 mL of milliQ water and the protein concentration estimated with the 2D-Quant Kit (GE Healthcare). Protein extracts were separated by SDS–PAGE on 12.5% isocratic Laemmli gels using a mid-size gel casting system (Hoefer SE600 Chroma). Gels were run at 25 mA following the manufacturer's recommendations. Bands showing differential expression between strains were recovered and further identified through MALDI-TOF MS performed using an Ultraflex instrument (Bruker Daltonics).

Statistics

Data were analysed using IBM SPSS Statistics 20 software. As data were normally distributed, multiple comparisons were performed using the one-way ANOVA test. *P* values <0.05 were considered to be significant.

Results

Selection of the strains

A *Salmonella* Typhi clinical isolate (Ty_wt) was selected to generate mutants able to grow at inhibitory concentrations of ciprofloxacin. First, Ty_wt was plated onto MacConkey agar plates containing 0.25 mg/L ciprofloxacin, corresponding to 0.5× its MIC (0.5 mg/L). From this point onwards, the concentration of ciprofloxacin was doubled at each step, in which a single colony was selected and plated again in the following selection stage, reaching a ciprofloxacin concentration of 16 mg/L. The mutants selected for the study were Ty_c1 and Ty_c2, which corresponded to colonies grown on plates containing 1 and 2 mg/L ciprofloxacin with MICs of ciprofloxacin of 1 and 8 mg/L, respectively. The mutant recovered at 4 mg/L ciprofloxacin, with an MIC of 8 mg/L, was not considered for the study as it showed the same antimicrobial susceptibility profile as Ty_c2, suggesting that no additional resistance mechanisms were selected. Mutants able to grow at greater concentrations (8 and 16 mg/L) were also recovered

(MICs 12–24 mg/L), although they could not be further studied as they showed important growth impairment. Resistant colonies appeared at frequencies of 2×10^{-6} and 7×10^{-6} mutants per cfu for Ty_c1 and Ty_c2, respectively. When bacteria were exposed to a concentration of 8 mg/L ciprofloxacin, the frequency of selection decreased to 5.5×10^{-7} and in the presence of 16 mg/L it was further reduced to 6×10^{-8} mutants per cfu.

To assess reversion of the resistance phenotype, the selected resistant mutants Ty_c1 and Ty_c2 were plated onto MacConkey agar plates without antibiotic. However, no revertant colonies were obtained after 35 consecutive passages.

Bacterial growth

A link between fluoroquinolone resistance and fitness has been stated previously. Several studies carried out in *Salmonella* Typhimurium and *S. enterica* serovar Enteritidis have shown that acquisition of high levels of fluoroquinolone resistance have a fitness cost.[31,35,36] In order to evaluate this feature in our strains, bacterial growth was examined. No differences between the three strains were observed, indicating that acquisition of antimicrobial resistance in these mutants (ciprofloxacin MICs of 1 and 8 mg/L) did not impose an energy cost on bacterial growth. However, the mutants with ciprofloxacin MICs of 12 and 32 mg/L (named Ty_c8 and Ty_c32) showed a much longer lag phase than the previous strains and Ty_c32 did not reach the same OD in the stationary phase as the previous mutants (Figure 1). Moreover, these mutants reached stationary phase ($OD_{600}=0.6$) after 8 h of incubation at 37°C with shaking, compared with 3 h for Ty_wt, Ty_c1 and Ty_c2. In addition to the altered bacterial growth, the colonies were phenotypically different from the others: they were much smaller and did not grow well on MacConkey agar plates. Taking into account all these observations, Ty_c8 and Ty_c32 were excluded from the present study.

Characterization of the antimicrobial resistance mechanisms

Quinolone resistance profile

The MICs of nalidixic acid and the fluoroquinolones ciprofloxacin and norfloxacin were determined (Table 1). As expected, the MICs of the quinolones tested sequentially increased for the two mutants, except for the MIC of ciprofloxacin for which a slight difference was recorded between Ty_wt and Ty_c1, whereas the MIC for Ty_c2 increased 8-fold. In contrast, a 4- and 8-fold increase was seen for Ty_c1 in the MICs of nalidixic acid (MIC 1000 mg/L) and norfloxacin (MIC 8 mg/L), respectively, and a further 2- and 3-fold increase for Ty_c2.

In order to study the molecular mechanisms of quinolone resistance, sequencing of the QRDRs of the target genes *gyrA*, *gyrB*, *parC* and *parE* was carried out. The results revealed a single amino acid substitution in GyrA (S83F) in all three strains and no additional mutations were found in the QRDRs of the mutants. As mutations outside the QRDR region of *gyrA* were found in a

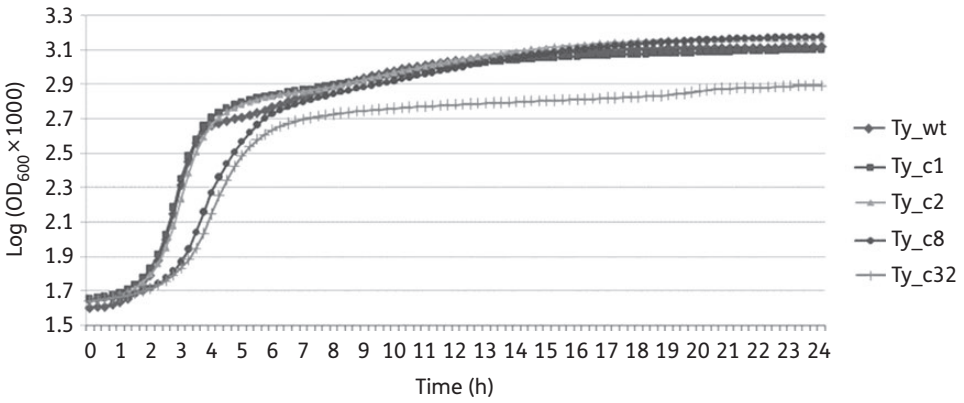

Figure 1. Mutants Ty_c8 and Ty_c32 show impaired growth. Bacterial growth of all the mutants obtained was assessed. The most resistant mutants (Ty_c8 and Ty_c32) were rejected as they presented growth deficiencies. Results correspond to the mean of three independent experiments.

Table 1. Antimicrobial susceptibility testing of the three strains studied

	MIC (mg/L)										
Strain	NAL	CIP	NOR	ERY	CHL	TET	TMP	AMP	FOX	CRO	KAN
Ty_wt	250	0.5	1	96	4	0.5	0.094	0.094	1	0.064	1
Ty_c1	1000	1	8	128	4	1.5	0.25	0.75	4	0.125	1
Ty_c2	2000	8	24	>256	128	4	0.75	4	128	0.5	1

NAL, nalidixic acid; CIP, ciprofloxacin; NOR, norfloxacin; ERY, erythromycin; CHL, chloramphenicol; TET, tetracycline; TMP, trimethoprim; AMP, ampicillin; FOX, cefoxitin; CRO, ceftriaxone; KAN, kanamycin.

previous study and were suggested to contribute to the quinolone resistance phenotype,[37] sequencing of the entire target genes (*gyrA*, *gyrB*, *parC* and *parE*) was also performed, but no additional change was found.

Multidrug resistance profile

The absence of new mutations acquired in the target genes of the two mutants suggested the acquisition of broad-spectrum resistance mechanisms such as increased efflux. In this context, the antimicrobial susceptibility profile of Ty_wt, Ty_c1 and Ty_c2 was assessed against a broader collection of antibiotics including erythromycin, ampicillin, ceftriaxone, cefoxitin, chloramphenicol, tetracycline, trimethoprim and kanamycin. The results obtained, reported in Table 1, showed a progressive, but different, increase in the MIC of most of the antibiotics tested. In comparison with Ty_wt, Ty_c1 showed the highest increase in the MIC of ampicillin (8-fold), modest increments (2- to 4-fold) in the MICs of tetracycline, trimethoprim, cefoxitin and ceftriaxone, whereas no major change (<1.5-fold) was seen for chloramphenicol and erythromycin. Contrarily, a different response was observed for strain Ty_c2. This mutant could be classified as MDR according to the ECDC.[38] It acquired resistance to cefoxitin and chloramphenicol with a 32-fold increase in both MICs compared with Ty_c1. Moreover, and even though the MICs of the remaining drugs tested did not reach the resistance breakpoints, increases of 3- to 5-fold were recorded in all cases. The only antibiotic tested for which no changes in susceptibility were seen in any of the mutants was kanamycin.

Efflux and permeability are altered in Ty_c2

In *Salmonella* Typhimurium, the predominant transport system involved in multidrug resistance is AcrAB/TolC.[15] Other less frequently detected drug transporters such as AcrEF, highly homologous to AcrAB, and EmrAB have also been reported to extrude quinolones[13,39] and have been shown to be overexpressed in quinolone-resistant *Salmonella* Typhimurium mutants.[40] Expression of the *acrB*, *tolC*, *acrF*, *emrB* and *ompF* genes was tested in the three strains by real-time PCR. Results revealed overexpression of *acrB* and *tolC* only in Ty_c2 (RQ=3.91 for *acrB* and RQ=3.42 for *tolC*). Surprisingly, the other efflux-related genes, *acrF* and *emrB*, were repressed in this strain compared with Ty_wt (3.06- and 3.96-fold, respectively). Additionally, the porin *ompF* was >6-fold repressed in Ty_c2. In the case of Ty_c1, despite the fact that statistically significant differences were seen for *acrF*, *emrB* and *ompF*, no relevant results were detected for any of these genes since all expression values were <2-fold. Thus, these results suggest the involvement of AcrAB/TolC and OmpF in the multidrug resistance profile of Ty_c2 (Table 2).

Analysis of the bacterial outer membrane proteins further confirmed part of these changes in gene expression: two bands identified as AcrA and TolC were overproduced only in Ty_c2, together with a decreased production of the outer membrane protein OmpC (Figure 2).

Role of MarA in the antimicrobial resistance profile of Ty_c2

Mutations in AcrR have been shown to impair its repressive effect, hence leading to derepression of the *acrAB* genes.[16–18] The

Table 2. Transcriptional levels of genes involved in antimicrobial resistance and virulence

	Efflux-related genes					Regulatory genes				Virulence genes				
Strain	*acrB*	*tolC*	*acrF*	*emrB*	*ompF*	*marA*	*soxS*	*ramA*	*acrR*	*hilD*	*hilA*	*invA*	*tviA*	*fimA*	*fliC*
Ty_wt	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ty_c1	−1.08	−1.64	−1.60*	−1.90**	−1.20*	−1.28	1.25	−1.32*	−1.89**	−2.50**	−2.59**	−3.75**	−1.26*	−3.22**	−9.89**
Ty_c2	3.91**	3.42**	−3.06*	−3.96**	−6.37**	108.19**	−2.38**	−2.70**	−5.88**	−9.19**	−5.04**	−8.55**	−27.85**	−6.09**	−121.09**

*$P<0.5$ and **$P<0.01$.

repressors MarR and RamR and the activator SoxR are regulators of transcriptional factors MarA, RamA and SoxS, respectively. Mutations in RamR and SoxR have been reported in different *Salmonella* serovars to increase expression of the homologue activators and, therefore, increase protein levels of AcrAB and downregulate OmpF.[16–20] However, studies describing mutations in MarR in strains selected in the clinical setting have been reported for *E. coli*, but not for *Salmonella* spp.[21,22]

In this study, sequencing of the known regulatory regions of AcrAB expression (*acrR*, *marRAB*, *soxRS* and *ramR*) was carried out. Indeed, an important alteration in the *marR* locus was detected in the Ty_c2 mutant corresponding to a deletion of 445 nucleotides. The fragment deleted included the first 411 nucleotides of the *marR* gene and 34 nucleotides upstream of the gene (Figure 3). In *E. coli*, the existence of two binding sites (Site I and Site II) for MarR in the *marO* region has been reported.[41] In Ty_c2, the entire Site II was deleted although Site I was completely preserved as well as the −35 and −10 elements of the promoter. Interpretation of this finding suggests that MarA expression is not only possible since the promoter is still intact, but also overexpression is likely to be detected due to a lack of the MarR protein. Consistently, real-time PCR transcription analysis of *marA* revealed high overexpression (RQ 108.19 ± 0.16) in the MDR strain Ty_c2 compared with Ty_wt. Nonetheless, despite no further changes being seen in the sequences of the other regulators, repression of *acrR*, *soxS* and *ramA* ranging from 2- to almost 6-fold was observed in Ty_c2 (Table 2). The results obtained in this section suggest that overexpression of MarA is responsible, at least in part, for the resistance phenotype observed in Ty_c2.

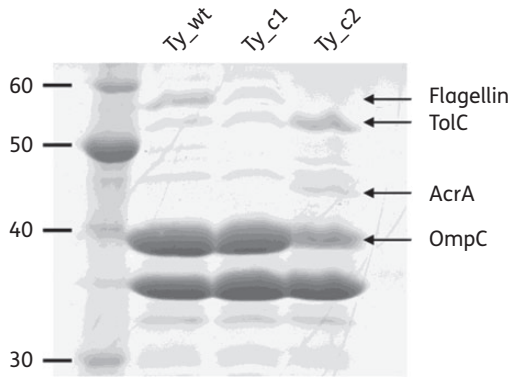

Figure 2. Ty_wt, Ty_c1 and Ty_c2 have different outer membrane protein profiles. Proteins showing differential expression are indicated by arrows.

Evaluation of virulence in the quinolone-resistant Salmonella Typhi mutants

Gradual reduction of motility is seen in Ty_c1 and Ty_c2

Intact motility of *Salmonella* Typhi has been determined to participate in the invasion process and is therefore related to the virulence potential of this pathogen.[42,43] For this reason, the motility of the three strains was measured by inoculation into soft agar plates. Although the clinical strain (Ty_wt) was already poorly motile, with a colony diameter of 13 mm, a decrease in motility was seen for the mutants, showing diameters of 9 mm for Ty_c1 and 7 mm for Ty_c2 (data not shown).

As flagellin is the major component of the bacterial flagellar filament, the expression profile of the flagellin-encoding gene *fliC* was analysed by real-time PCR and revealed statistically significant differences between the parental strain and the mutants (Table 2). Ty_c1 showed a 10-fold reduction in *fliC* expression levels in comparison with Ty_wt and an additional >10-fold decreased expression was seen for Ty_c2 (RQ = 0.01). These results are in accordance with the phenotype observed in the soft agar assay.

In order to determine whether the differences in *fliC* expression could be attributed to the acquisition of mutations within the gene, *fliC* as well as the regulators *fliA* and *flhDC*, involved in flagella biosynthesis,[35,44] were sequenced. Amino acid substitutions at particular positions of the conserved site of flagellin have been reported to be essential for protofilament assembly, bacterial motility and TLR-5 recognition.[45] A single mutation in *fliC* was found in both Ty_c1 and Ty_c2 leading to the amino acid substitution T187I, whereas no mutations in any of the regulators were detected. This change detected in the mutants is located outside of the conserved region, likely suggesting it to be trivial for the function and structure of flagellin.[45]

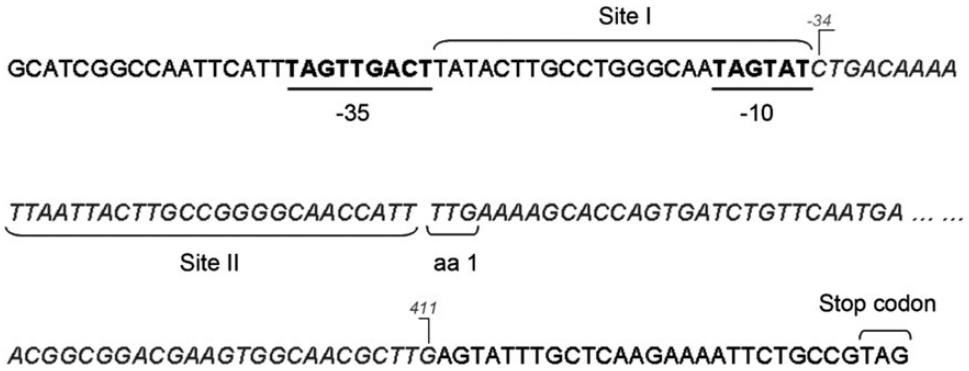

Figure 3. Schematic representation of the deleted region located in the *marRAB* regulatory region of Ty_c2. The predicted −35 and −10 promoter regions are shown in underlined bold letters. The two MarR-binding sites (Site I and Site II) are also indicated and extrapolated from *E. coli*.[41] Letters in italics correspond to the deletion detected and numbers in grey indicate the first and last position of the deleted nucleotides.

Salmonella Typhi mutants are less able to invade epithelial cells

As described previously in several serovars of *S. enterica*,[31,32,46] acquisition of quinolone resistance has been related to decreased invasion ability in an *in vitro* eukaryotic cell model. For this reason, *in vitro* invasion of HeLa cells was examined with the three studied strains (Ty_wt, Ty_c1 and Ty_c2) and revealed a gradual reduction in the invasion ability of the mutants compared with the parental isolate. The invasion rate of Ty_wt was 7.31%, that of Ty_c1 was 1.69% (~4 times lower) and the lowest rate of invasion (0.27%) was reported for Ty_c2, which was 6 times lower than the Ty_c1 mutant and 27 times lower than Ty_wt (Figure 4a).

Salmonella Typhi mutants show impaired internalization and survival in macrophages

Internalization of *Salmonella* Typhi by phagocytes once *Salmonella* Typhi has reached the submucosa as well as survival of the pathogen inside these host cells are crucial processes for systemic infection.[2] Thus, differentiated U-937 human macrophages were infected with the three strains. In order to study their ability to be phagocytosed by macrophages, the number of intracellular bacteria was determined at 2 h post-infection. Results were expressed as percentage values of recovered bacteria with respect to the total number of infecting bacteria. A reduction of 2.75-fold in Ty_c1 compared with Ty_wt and an additional 1.6-fold for Ty_c2, which corresponded to 4.37 times less than Ty_wt, was seen (Figure 4b). Moreover, the intracellular survival rate was also determined and expressed as the percentage of bacterial count at 2 h versus bacterial load at 24 h post-infection. Similar results were observed for the two mutants and corresponded to a 4-fold reduction compared with the parental strain (Figure 4c). These results showed a gradual inability of the mutants to be recognized by macrophages and therefore be internalized. In addition, the internalized bacteria were less able to survive inside macrophages, showing no significant differences between the two mutants.

Transcription levels of virulence factors are low in Salmonella Typhi mutants

Among the virulence factors reported to play a key role in *Salmonella* Typhi–host interactions, several loci have been described. The invasion process of *Salmonella* is mainly driven by virulence determinants contained in SPI-1,[25,26,36] such as the effector *invA* and the key regulators *hilD* and *hilA*.[47] Type 1 fimbriae are important for adhesion to eukaryotic cells and are encoded by *fim* genes, with *fimA* being responsible for the production of the major fimbrial subunit.[36] The Vi capsular antigen, a *Salmonella* Typhi-specific determinant encoded by the *tviA* gene located in SPI-7, is involved in systemic dissemination.[25] In this study, the gene expression profiles of *invA*, *hilD*, *hilA*, *fimA* and *tviA* were evaluated through real-time PCR analysis (Table 2). The results reflect sequential gene repression in the two mutants compared with the original strain for almost all the genes tested. In Ty_c1, *invA*, *hilD*, *hilA* and *fimA* were 2.5- to 3.75-fold less expressed than in Ty_wt. On comparing Ty_c2 versus Ty_c1, expression of these genes was reduced by 1.89- to 3.67-fold. Contrary to the results observed for these genes, *tviA* expression in Ty_c1 was comparable to that seen for the parental strain (RQ=0.79). However, almost 30-fold less expression was observed in Ty_c2 (Table 2). These results suggest involvement of *invA*, *hilD*, *hilA* and *fimA* in the progressive loss of virulence in the two mutants; however, as *tviA* only showed a decreased expression in Ty_c2, this gene would only be related to the phenotype of this latter mutant.

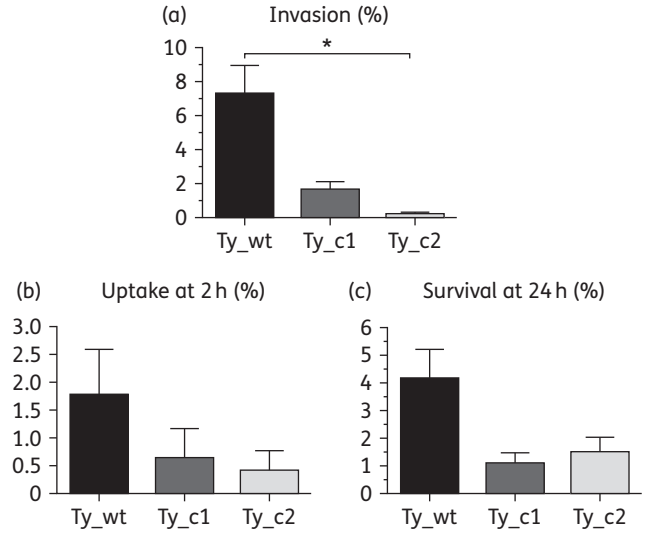

Figure 4. Ability to invade HeLa cells and uptake by and survival in macrophages is compromised upon infection with Ty_c1 and Ty_c2. Invasion was performed by infecting HeLa cells with the different bacteria (a). Uptake at 2 h (b) and survival at 24 h (c) was assessed by infecting human differentiated macrophages with the three strains studied. Results correspond to the mean of three independent experiments ± standard error. Differences between groups were assessed using the one-way ANOVA test. *$P < 0.05$.

Induction of the immune response is compromised in Salmonella Typhi mutants

The levels of the proinflammatory cytokines TNF-α and IL-1β produced by infected U-937 macrophages were measured. Levels of TNF-α were checked in the supernatants collected after 2 h of infection and revealed a gradual and significant decrease in the two mutants compared with Ty_wt. The levels of this cytokine consisted of 5181 μg/mL in Ty_wt, almost 4000 μg/mL in Ty_c1 and 3256 μg/mL in Ty_c2 (Figure 5a). A greater reduction was seen for IL-1β, checked in the supernatants collected at 24 h post-infection: Ty_wt reached 3082 pg/mL, whereas the levels in Ty_c1 and Ty_c2 were reduced by >50%, showing values of 1167 and 1067 pg/mL, respectively (Figure 5b).

Since transcription of these two cytokines is controlled by NF-κB,[2,27] activation of this nuclear transcription factor was checked upon stimulation of HEK293 cells with heat-killed bacteria by means of a luciferase reporter assay. Activation of NF-κB significantly diminished in Ty_c1 compared with the parental strain and corresponded to a decrease of 16.67%. This ratio was much more significant for Ty_c2, with a 71.72% reduction of the NF-κB activity in comparison with Ty_c1 (Figure 5c). The results obtained in this section indicate that *Salmonella* Typhi

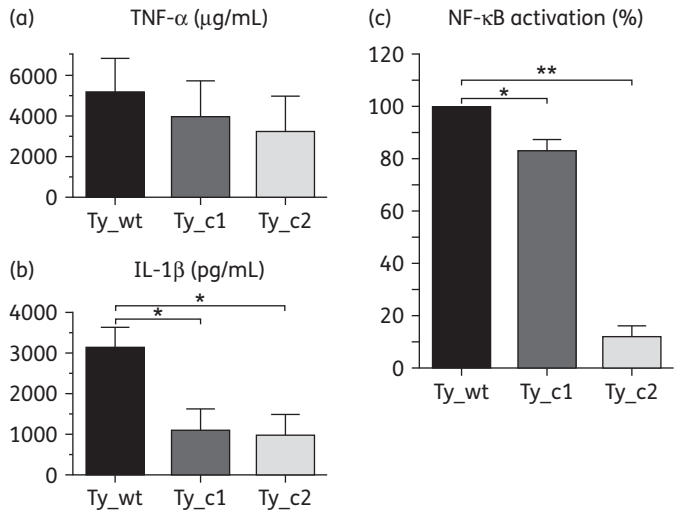

Figure 5. *Salmonella* Typhi mutants are less immunogenic than Ty_wt. Levels of TNF-α (a) and IL-1β (b) were measured in infected macrophages and activation of NF-κB (c) was assessed in stimulated HEK293 cells. Results correspond to the mean of three independent experiments ± standard error. Differences between groups were assessed using the one-way ANOVA test. *$P < 0.05$ and **$P < 0.01$.

mutants elicit a more discrete immunogenic reaction *in vitro* than the original clinical isolate, with Ty_c2 being significantly less reactive.

Discussion

In this study, we evaluated the process of quinolone resistance acquisition and the impact on virulence properties in the pathogen *Salmonella* Typhi. In the first part of the study, we characterized the molecular mechanisms of resistance since they have not been extensively studied in this *Salmonella* serovar. We obtained two mutants, Ty_c1 and Ty_c2, with increased MICs of ciprofloxacin (1 and 8 mg/L, respectively) selected from a clinical isolate (ciprofloxacin MIC of 0.5 mg/L). All studies reporting resistance or decreased susceptibility to ciprofloxacin (MIC >0.25 mg/L) in *Salmonella* Typhi associate this phenotype with mutations in the QRDRs of the target genes, particularly in *gyrA*.[37,48] In this study, we found that Ty_wt already harboured a GyrA substitution (S83F), but no additional change was detected in the two mutants on sequencing the full locus of each quinolone target gene. The profile of increased MICs was different for the two mutants. A moderate increase in MICs of several unrelated compounds was seen in Ty_c1, whereas more important increases were reported for Ty_c2 affecting almost all compounds tested and leading to an MDR phenotype.

According to the results obtained from gene expression and protein analyses, we found that alteration of membrane permeability was seen in Ty_c2 due to a reduction in the production of OmpF and OmpC. Although this alteration in porin expression has been previously shown in *E. coli* and non-Typhi *Salmonella*,[31,49,50] it has not been reported in *Salmonella* Typhi. More importantly, this MDR phenotype was primarily associated with hyperproduction of the major efflux pump AcrAB/TolC. Only a few studies, such as that of Chiou *et al*.,[10] have previously reported increased efflux (based on the use of an efflux pump inhibitor) among *Salmonella* Typhi clinical isolates with resistance or decreased susceptibility to ciprofloxacin (MICs of 0.12–16 mg/L). Nonetheless, this is the first time that the efflux pump AcrAB/TolC has been characterized in this serovar. Our findings together with the data of Chiou *et al*.[10] contrast with the results of Baucheron *et al*.,[51] in which no involvement of efflux mechanisms was seen in a collection of *Salmonella* Typhi clinical isolates. It is worth mentioning that the majority of these latter strains showed MICs of ciprofloxacin ranging from 0.125 to 1 mg/L, although a single strain had a higher MIC of 8 mg/L, attributed to the acquisition of two further target gene mutations. In the study by Chiou *et al*.,[10] clinical isolates for which increased efflux activity was reported had ciprofloxacin MICs of >4 mg/L. In our study, Ty_c2 had a ciprofloxacin MIC of 8 mg/L. Therefore, we suggest that selection of increased efflux is acquired at ciprofloxacin MICs of 4–8 mg/L as a general trend. To better confirm this hypothesis, characterization of larger collections of ciprofloxacin-resistant clinical isolates is needed to accurately evaluate the relevance and prevalence of this mechanism. In an attempt to understand the increased levels of AcrAB/TolC detected in Ty_c2, we found that this was due to alterations in the *marRAB* regulatory region. A deletion of almost the entire *marR* gene detected in this mutant likely resulted in the absence of transcription of the repressor, hence leading to hyperproduction of MarA. This phenotype, in addition to causing increased amounts of AcrAB/TolC, would also explain the down-regulation of the *ompF* gene detected in Ty_c2. This is the first demonstration in *Salmonella* Typhi of the molecular regulation of this efflux system, as it has been previously described only in non-typhoidal *Salmonella*.[13,23,52] In addition, and to the best of our knowledge, it is also the first description of a *marR* mutation reported in *S. enterica* leading to increased AcrAB/TolC, since most of the reported mutations are located in *ramR*[17,53] and, less frequently, in *acrR*[54] and *soxRS*.[19]

Surprisingly, the transcription levels of the regulators *acrR*, *soxS* and *ramA* were repressed in Ty_c2. Additionally, the alternative efflux pump candidates *emrB* and *acrF*, reported to be overexpressed in quinolone-resistant *Salmonella* Typhimurium isolates,[40] were also studied and revealed down-regulation only in this mutant. A similar observation has also been reported by Kang and Woo,[55] where expression levels of efflux pump-related genes were tested in ciprofloxacin-resistant *S. enterica* serovar Istanbul mutants. In all of the mutants, not only *soxS*, but also *acrF*, were down-regulated compared with the parental strain, whereas *acrB*, *ramA* and/or *marA* were overexpressed. Despite another study conducted *in E. coli*[56] reporting an increase in the expression of *soxS* in first-step mutants selected with tetracycline, a decrease in the expression levels of this gene was observed in the most resistant strain. Therefore, the authors suggested that the SoxS response could be activated at the early stages of antibiotic resistance adaptation. Considering this proposal, the decreased expression levels of *soxS* observed in Ty_c2 could be partly explained by this phenomenon. According to all these results, including the findings reported in the present study, it seems that overexpression of AcrAB may lead to reduced expression of some of their regulators and/or alternative transport systems to avoid redundancy in efflux transportation, resulting in a reduction of the energy cost of the bacteria.

Contrary to the scenario reported for Ty_c2, the increased MICs detected for the intermediate resistant mutant Ty_c1 could not be

attributed to the enhanced expression of AcrAB/TolC. Moreover, despite statistically significant results being detected for *acrF*, *emrB* and *ompF*, these results were considered irrelevant as repression was <2-fold. These results suggest that unknown TolC-independent mechanisms of resistance with reduced impact are likely to be involved in antibiotic resistance.

In the second part of this work, the association between virulence and quinolone resistance was investigated. This relation has been studied based on data reporting a rise in the number of *Salmonella* Typhi clinical isolates with decreased ciprofloxacin susceptibility, considered endemic in countries such as India,[57] but a lower incidence of strains showing high levels of ciprofloxacin resistance. A study conducted in the UK from 2001 to 2004 reported low incidence (1%–2%) of ciprofloxacin-resistant *Salmonella* Typhi isolates (MIC ≥1 mg/L) in contrast to an increase in the number of strains with decreased ciprofloxacin susceptibility (from 35% in 2001 to 47% in 2004).[58] Nonetheless, some cases reporting high levels of ciprofloxacin resistance in *Salmonella* Typhi have already emerged.[9,59]

Moreover, previous studies conducted in different serovars of *S. enterica* have already associated quinolone resistance with a decreased invasion profile. Wang *et al.*[32] reported a reduction in the expression of SPI-1 genes, decreased ability to invade epithelial cells and low replication inside macrophages for *Salmonella* Typhimurium and *Salmonella* Choleraesuis resistant mutants. Similarly, studies conducted with *Salmonella* Typhimurium and *Salmonella* Enteritidis also showed an association between resistance, reduced virulence and impaired expression of SPI-1 genes in quinolone-resistant mutants.[16,31] Consistent with these findings, our results showed that upon acquisition of resistance to quinolones, the two mutants showed a progressively reduced ability to invade epithelial cells and macrophages. Moreover, the survival rate inside macrophages was also diminished. Regulation of these virulence determinants has been well established in *Salmonella* Typhimurium[36] and demonstrated to be mostly homologous in *Salmonella* Typhi by Faucher *et al.*[26] They identified that SPI-1 genes were involved in the invasion process when infecting macrophages. Moreover, Bishop *et al.*[60] showed that *Salmonella* Typhi strains lacking *invA* were 1000-fold less able to invade epithelial cells. Thus, the results of our gene expression analysis agreed with the phenotype observed, since several SPI-1-related genes (*invA*, *hilA* and *hilD*) showed a gradual reduction in their expression levels.

In an attempt to find an explanation for this effect on virulence, several hypotheses should be considered. First, DNA supercoiling has been proposed to influence the transcription process.[61] Thus, acquisition of mutations in DNA gyrase has been considered to impair regular supercoiling activity and hence modify gene expression patterns.[32] Nonetheless, our results do not support this as our parental strain already had a mutation in GyrA, but no other QRDR mutation was acquired in the mutants. Second, impaired bacterial growth has been described for quinolone-resistant mutants of *Salmonella* Typhimurium and *Salmonella* Enteritidis.[31,46,62] This reduced ability to grow may shut off dispensable bacterial functions such as virulence properties. Nonetheless, the three strains reported in this study did not show any difference in terms of bacterial growth. On the contrary, a recent study performed in *Salmonella* Typhi associated mutations in the QRDRs of the target genes with fitness benefits, with the S83F mutation in GyrA, which leads to decreased ciprofloxacin susceptibility, being the most advantageous.[63] The presence of this mutation in our strains may explain the unchanged growth observed. Third, high production levels of AcrAB/TolC have been suggested to be the reason for impaired virulence.[46] As quorum-sensing signal molecules have been shown to be extruded by efflux pumps[64] and such molecules can activate virulence genes,[65] an impaired quorum-sensing homeostasis triggered by increased efflux activity may lead to impaired gene transcription. To strengthen this hypothesis, a study conducted by Bailey *et al.*[66] showed that high overexpression of *ramA* was accompanied by overexpression of efflux pumps as well as a decrease in the expression of virulence genes and impaired host–pathogen interactions. Taking into account these findings, we suggest that a similar situation could be happening in the present work triggered by the high overexpression of *marA* (>100-fold) in the Ty_c2 mutant.

Additional virulence genes were also studied in the present work. On the one hand, *Salmonella* Typhimurium and *Salmonella* Typhi have been demonstrated to share a number of pathogenesis determinants, such as the bacterial flagellum. Mutations in the flagellar regulatory genes *flhDC* and *fliA* have been associated with severely decreased entry into eukaryotic cells and reduced cytotoxicity in macrophages.[67] Moreover, uptake and survival defects in macrophages have also been seen in *fliC* and *flhCD* mutants as reported by Sabbagh *et al.*[68] A correlation between reduced motility, diminished *fliC* levels and impaired invasion of epithelial cells was seen for our mutants. On the other hand, *Salmonella* Typhi-specific virulence genes have also been identified. Among these factors, the most relevant are the genes located in the *Salmonella* Typhi-specific pathogenicity island SPI-7.[25,69] The Vi capsular polysaccharide is encoded by the *viaB* locus, from SPI-7, and has been reported to be necessary for *Salmonella* Typhi survival in macrophages, serum resistance and systemic dissemination.[25,70] An important repression of the *tviA* gene was reported in the MDR mutant Ty_c2. Thus, it seems reasonable that repression of these virulence factors (FliC and TviA) has also contributed to the impaired virulence phenotype observed in the two mutants, concerning invasion of epithelial cells as well as uptake by and survival inside human macrophages.

It has been previously reported that TviA represses important virulence factors including genes encoding flagella and T3SS-1 through the *flhDC–fliZ–hilD–hilA* axis.[60,71] In our study, however, repressed transcription of *tviA* was only considered in Ty_c2, as values seen in Ty_c1 were <1.3, whereas the expression levels of *fliC* and the SPI-1 encoded genes, were already decreased in the Ty_c1 mutant. Thus, these results support the existence of a *tviA*-independent regulation of flagellin in Ty_c1, in accordance with the different regulators governing flagella expression reported in other *Salmonella* serovars. However, in Ty_c2, TviA certainly seems to play a role in *fliC* regulation.

In the last part of this work, we evaluated the ability of the mutants to trigger an immunogenic response when infecting human macrophages. The flagellin protein FliC has been reported to activate TLR-5 inside macrophages, thus decreasing the signalling pathway of NF-κB, which elicits cytokine production (TNF-α and IL-1β). Our results showed that Ty_c1 and Ty_c2 triggered a poor innate immune response as reflected by a decrease in the production of these two proinflammatory cytokines as well as in the nuclear transcriptional regulator NF-κB. This attenuated

immunogenic profile is most likely linked to reduced levels of FliC, as described previously.[72,73] Moreover, we attribute the more noticeable reduction observed for IL-1β (>2-fold), compared with TNF-α, to the additive effects derived from *fliC* repression, mediated by both the TLR-dependent pathway directly stimulated by *fliC* and the alternative TLR-independent secretion pathway through caspase-1 activation, which is also stimulated by flagellin.[30]

Conclusions

The results obtained in the present study provide evidence, for the first time in *Salmonella* Typhi, of a link between the MDR phenotype and increased levels of AcrAB/TolC caused by the hyperproduction of its regulator MarA. Moreover, we determined that when *Salmonella* Typhi acquires resistance to quinolones and other unrelated antibiotics it becomes less virulent. Concerning the hypothesis to explain this impaired virulence and host–pathogen interactions, our results rule out previously reported explanations such as acquisition of QRDR mutations or reduced growth. Our findings, particularly those obtained from Ty_c2, reinforce the idea that high efflux pump activity may affect quorum-sensing homeostasis, eventually leading to changes in virulence gene expression. Although more research is needed to clarify this phenomenon and consider also the situation reported for Ty_c1, our results contribute to the complex understanding of quinolone resistance and virulence. On the other hand, with the aim to explain the lower prevalence of ciprofloxacin-resistant *Salmonella* Typhi causing illness in the clinical setting, we hypothesize that, in comparison with susceptible bacteria, when quinolone-resistant *Salmonella* Typhi reaches the gut only a small proportion of the bacterial load is able to cross the mucosa and survive inside the host due to: (i) poor invasion of enterocytes due to a mild activation of the invasion machinery; (ii) reduced phagocytosis by dendritic cells and macrophages; and (iii) reduced survival rate inside macrophages. This situation, together with decreased activation of the innate immune response, may result in impaired dissemination of the resistant pathogen. However, the emergence of isolates able to overcome antibiotic pressure needs to be taken into account and efforts should be made to fight against them.

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Transparency declarations

None to declare.

Supplementary data

Tables S1 and S2 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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3. Study of genes potentially related to quinolone resistance and/or virulence. Identification of *yedF*, a novel presumably virulence-related gene

Manuscript

Evaluation of the pleiotropic role of *yedF*, a novel gene of *S.* Typhimurium

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Salmonella possesses a number of virulence determinants that allow the bacteria to replicate under extreme conditions and invade the host cell in order to cause disease.

The aim of this work was to identify novel putative genes involved in virulence and/or antibiotic resistance. In order to do so, genes were selected from a previous study performed in our group [210], in which a susceptible clinical isolate of *S*. Typhimurium (50-wt) and a multidrug-resistance mutant (50-64), also showing a severely reduced *in vitro* invasion ability, were transcriptionally compared by means of microarrays. Among the differentially expressed genes (either up-regulated or down-regulated in the resistant strain) four were predicted to be outer (*ydiY)* or inner (*ybdJ*, *STM1441* and *ynaJ)* membrane proteins. Additionally, a putative transcriptional factor, *yedF*, was also selected. Mutants overexpressing and lacking these genes were obtained from the reference strain SL1344 and their involvement in the antibiotic susceptibility profile was evaluated. The results revealed that none of these mutants was involved in the antibiotic resistance phenotype as no differences were reported in the MICs of a collection of 6 different antibiotics (nalidixic acid, ciprofloxacin, tetracycline, cefoxitin, erythromycin and trimethoprim). Then, in an attempt to see if these genes contributed to the virulence phenotype, the *in vitro* invasion ability was evaluated. Among the four putative outer or inner proteins, *ydiY* was the only gene showing consistent results: invasion by the *ydiY*-deficient mutant was almost 2-times lower than that of the parental strain even in the absence of statistically significant differences. These results

correlated well with the repression of *ydiY* seen in the less virulent strain 50-64, for which a 5-fold decrease in the expression levels was previously reported compared to the wild-type strain (50-wt). However, when *ydiY* was overexpressed, this phenotype was not reverted. Thus, the study of the putative efflux-related genes was discontinued.

In the case of *yedF*, the absence of this gene led to a significant reduction in the invasion ability compared to the wild-type strain SL1344 (10.16% *vs* 27.51%). When the gene was reintroduced, the capability of this strain to invade epithelial cells was even greater than the reference strain achieving 40.32% of invasion.

In order to further understand the role of *yedF* in S. Typhimurium, we compared the transcriptomic profile of the reference strain SL1344 and the mutant lacking *yedF* (Δ*yedF)*. Our results revealed that the genes involved in virulence showed a decrease in the expression levels in the *yedF*-deficient mutant compared to the wild-type strain. More than 2-fold repression of the flagellum-related genes (FliC, FliR, and FlgA) was observed in the Δ*yedF* mutant compared to SL1344. Surprisingly, the *flhB* gene, the protein of which is needed for the formation of the flagellum, was 2.6-fold activated in the mutant. However, an unexpected down-regulation of the operon composed of the flagellin repressor *fljA* and the phase 2 flagellin *fljB* encoding genes was reported (-11.48- and -5.97-fold, respectively) in the Δ*yedF* mutant compared to the wild-type strain. These results seem to be contradictory as it has been reported that either phase 1 (FliC) or phase 2 (FljB) flagellin are transcribed [215–217].Nevertheless, the fimbrial-related genes *fimA* and *fimZ* were 5- and 3-fold repressed in the strain lacking *yedF*, respectively. In accordance with the general inactivation of the genes involved in the approach and attachment to the host cell, a general repression was also seen of the genes involved in the invasion process and encoded in SPI-1, SPI-2, SPI-4 and SPI-5 in the Δ*yedF* mutant. Accordingly, the key regulators *hilA* and *hilC* showed a significant repression of ~3-fold in the *yedF*-deficient strain compared to the wild-type SL1344 and an almost 2-fold change was seen for *hilD*.

The virulence determinant LPS was also seen to be altered, as revealed in the transcriptional levels of the genes involved in LPS biosynthesis. A repression ranging from a 2- to 6.8-fold change of the genes involved in the O-antigen polysaccharide assembly (*rfb* genes) as well as in the core-oligosaccharide composition (*rfa* genes) was reported in the

mutant lacking the *yedF* gene compared to the wild-type strain SL1344. In agreement with this finding, a decrease was also detected in the global content of LPS in the *yedF*-deficient.

Transcriptomic analysis also revealed a general overexpression of the genes involved in anaerobic respiration in the Δ*yedF* mutant compared to SL1344. The genes affected by the absence of *yedF* included the nitrite and nitrate reductases (8- to 40-fold increase), the anaerobic dimethyl sulfoxide reductase SL1344 (~15-fold overexpression) and the anaerobic ribonucleoside-triphosphate reductase (12.5-fold up-regulated). Additionally, the genes involved in the production of hydrogen from formate (formate dehydrogenase H and the proteins HypABCDE) as well as the transcriptional activator *fhlA*, were also importantly activated in the mutant lacking *yedF* compared to the wild-type strain (2.7- to 10.4-fold).

Consistent results were seen at a protein level. A decrease, ranging from 2- to 5-fold, in the production of proteins involved in the approach and attachment to the host cells, including chemotaxis (CheV, CheW, CheA, CheZ, SL1344_3189 and SL1344_4464), flagellar-related (FlgE, FliG and FliC) and fimbrial (FimA and FimC) proteins was observed in the *yedF*-deficient mutant compared to the wild-type strain. Additionally, the SPI-1 and SPI-5 encoded proteins SipC and SigD, respectively, were also less produced in the *yedF*-deficient mutant than the wild-type strain (5.7- and 2.1-fold change, respectively). However, contrary to the transcriptional profile, 3 proteins involved in anaerobic metabolism (the glycyl radical cofactor, the ornithine decarboxylase and the formate dehydrogenase α-subunit) showed decreased protein levels ranging from 2.5- to almost 6-fold in the Δ*yedF* mutant compared to SL1344.

All of these results taken together suggest that *yedF* plays a role in activating the transcription of the genes involved in the invasion process, including those needed in the approach, attachment, invasion and survival in the host cell. Moreover, the alteration of the LPS architecture, an important virulence determinant, reinforces the fact that *yedF* contributes to the virulent state of *S*. Typhimurium. Additionally, our data also reveal that *yedF* is involved in the repression of anaerobic metabolism in the presence of oxygen.

Evaluation of the pleiotropic role of *yedF*, a novel gene of *S.* Typhimurium

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ABSTRACT

Salmonella possesses a number of virulence determinants that allow replication under extreme conditions and invasion of the host cell in order to cause disease. The objective of this study was to identify novel putative genes involved in virulence and/or antibiotic resistance. To do this, genes were selected from a previous study performed by our group in which a susceptible clinical isolate of *S*. Typhimurium (50-wt) and a multidrug-resistant mutant (50-64), also showing severely reduced *in vitro* invasion ability, were transcriptionally compared using microarrays. Among the differentially expressed genes (either up- or down-regulated in the resistant strain) four were predicted to be outer (*ydiY)* or inner (*ybdJ*, *STM1441* and *ynaJ)* membrane proteins. Additionally, a putative transcriptional factor, *yedF*, was selected. Mutants overexpressing and lacking these genes were obtained from the reference strain SL1344 and their involvement in the antibiotic susceptibility profile was evaluated. The results revealed that none of the genes were involved in the antibiotic resistance

phenotype as no differences were found in the MICs of unrelated antibiotics. We then assessed the contribution to the virulence phenotype in terms of *in vitro* invasion ability and revealed that *ydiY* and *yedF* showed consistent results; a reduction in the invasion ability was observed in the mutants lacking the genes, as seen in 50-64, and the invasive phenotype was reverted only in the case of *yedF* when the gene was restored. In the absence of *yedF*, transcriptomic and proteomic approaches revealed a decrease in virulence-related determinants represented by the genes involved in motility, chemotaxis, attachment and survival inside the host cell. A decrease in the levels of expression of the global regulators involved in the invasion process (*hilA*, *hilC* and *hilD*) was also observed. Moreover, LPS biosynthesis was compromised in the *yedF*-deficient strain as demonstrated at both transcriptional and phenotypical levels. An increase in the expression of the genes involved in the anaerobic metabolism pathway was also detected in the absence of *yedF*. Taking all of this into account, we propose that *yedF* is involved in the complex regulation related to *Salmonella* pathogenesis and contributes to the activation of the virulence machinery. Moreover, we suggest that when oxygen is available, *yedF* contributes to sustain repression of the anaerobic pathway.

INTRODUCTION

Salmonella Typhimurium is an enteric foodborne pathogen responsible for causing salmonellosis associated with acute diarrhea upon ingestion of contaminated food or water. *Salmonella* possesses a number of virulence determinants that allow the bacteria to replicate under extreme conditions and invade the host cell in order to cause disease. Indeed, bacteria are able to survive stomach passage and reach the intestine where they cause inflammation and penetrate the mucosa. In the first steps of the infectious process, bacteria require motility through flagella in order to reach the intestinal lumen and cross the mucus layer of the intestinal epithelia. The flagellum is composed of three structural parts and includes a basal body anchored to the cell envelope, a hook complex that constitutes the molecular motor, and a filament composed of flagellin monomers encoded by the *fliC* gene. Adhesion to the host cell is mainly mediated by fimbriae such as the Type 1 pilus system encoded by the *fimAICDH* operon [1]. During invasion, proteins encoded by the *prgHIJK*, *spaMNOPQRS*, and *invABCEFGH* operons located in the *Salmonella* Pathogenicity Island 1 (SPI-1), which are highly conserved regions in the genome of the bacteria, constitute a Type-3 Secretion System across which effector proteins, such as the SPI-1 encoded proteins SipA, SipC, and the inositol phosphatase SigD/SopB, encoded in SPI-5, are translocated to the host cytosol. This causes intracellular changes that allow engulfment of the bacteria and induction of immune response eliciting mucosal inflammation [2–4]. The survival and replication of intracellular *Salmonella* inside *Salmonella*-containing vacuoles (SCVs) is mainly mediated by SPI-2 located genes [2].

Immune response is activated through recognition of specific pathogen-associated molecular patterns (PAMPs) by specific receptors expressed on the surface and/or

inside different cell types such as macrophages, intestinal epithelial cells, dendritic cells and neutrophils [1,5,6]. The most important PAMPs are flagellin, in particular the FliC subunit highly expressed on the bacterial surface and the lipopolysaccharide (LPS) [7].

Oxygen availability is limited in the inflamed gut whereas other compounds such as hydrogen sulfide (H_2S) [8] and H_2 [9] are produced in large quantities by colonic bacteria. Moreover, when bacteria penetrate the intestinal mucosa and invade and survive inside the SCVs of the host cell, nutrients are limited in this environment and reactive oxygen and nitrogen species may be found [10].

In anaerobic conditions, *Salmonella* is able to survive by using alternative energy sources such as nitrate or fumarate. This is possible thanks to the expression of genes encoding for specific enzymes of the anaerobic respiratory pathways including nitrate (*narGHJI*) and nitrite (*nirBDC*) reductases [11], fumarate reductase (*frdABCD*), DMSO reductase (*dmsABC*) [12,13] and respiratory hydrogenases (*HybABCDEF*) [9,14]. In order to overcome these extreme conditions and replicate inside the host, a sophisticated virulence armamentarium is activated. The key genes involved in this pathogenic process are also encoded in SPIs; however, other important virulence-related determinants are located elsewhere in the genome but are transcriptionally regulated by SPI-encoded genes. One of the master regulators of the virulence machinery is HilA, which is encoded in SPI-1. It is positively regulated by HilC and HilD, also located within the same SPI, and RtsA the gene of which is located outside this island [3].

In a previous study published by our group, a susceptible clinical isolate of *S*. Typhimurium and a multidrug-resistant mutant were transcriptionally compared using microarrays [15]. In addition to having increased MICs of several unrelated compounds, the resistant mutant showed a severely reduced invasion ability. In order to analyze

the results, in the present study we focused our attention on those genes showing impaired expression (either up- or down-regulated in the resistant strain) which, in addition, had an unknown function; however, due to its intracellular location or predicted function these genes could play a role in the acquired resistance phenotype or in the repressed virulence observed. Thus, the *yedF* gene of unknown function was chosen and investigated in the present study to evaluate its putative role in virulence and/or antimicrobial resistance. Moreover, 4 putative outer and inner membrane proteins were also selected and further studied to evaluate their involvement in these phenotypes.

MATERIALS AND METHODS

Clinical isolates and genes selected. In a previous work performed in our group and published by Fàbrega *et al.* [15], a *Salmonella enterica* serovar Typhimurium clinical isolate, 50-wt, was recovered from a stool sample in the Department of Clinical Microbiology at Hospital Clínic (Barcelona, Spain). This clinical strain was used to generate an antibiotic-resistant mutant, 50-64, by exposure to increasing concentrations of ciprofloxacin. The selection of 5 unknown genes potentially involved in antibiotic resistance/virulence was made based on previously performed microarray analyses and considering a significant >2-fold difference in the expression between the two strains [15]. These genes included three putative inner membrane proteins: *STM1441* (Gene ID: 1252959), *ynaJ* (Gene ID: 1253180) and *ybdJ* (Gene ID: 1252102); one putative outer membrane protein named *ydiY (*Gene ID: 1252845) and one hypothetical transcription factor, *yedF* (Gene ID: 1253487). All sequences were obtained from the S. Typhimurium LT2 reference strain (RefSeq NC_003197.1).

Construction of *STM1441*, *ynaJ*, *ybdJ, ydiY and yedF* mutants. Individual inactivation of the genes of interest was done in the reference strain SL1344 following the Datsenko & Wanner method [16]. Briefly, the chloramphenicol resistant cassette (*cat*) flanked by homologous sequences of the gene of interest was electroporated in the reference strain SL1344 carrying the pKD46 plasmid. This plasmid encodes an arabinose-inducible phage lambda Red recombinase which allows the recombination of the electroporated fragment with the homologous region of the wild-type gene. The specific fragments were generated by PCR amplification using the primers listed in **Table 1** and the pKD3 plasmid as the template. Selection of the knock-out colonies was made by antibiotic selection using Luria-Bertani (LB) plates supplemented with 8 mg/L

chloramphenicol (Sigma-Aldrich) and 10mM arabinose (Panreac). Confirmation of the deleted region was done by PCR and the absence of gene expression of the disrupted genes was checked by RT-PCR (**Table 2**).

Obtainment of strains carrying recombinant plasmids. Constructions of the cloning vector pBAD33 carrying the full genes *STM1441*, *ynaJ*, *ybdJ, ydiY and yedF* were made as follows: the genes were amplified in such a way that they acquired the restriction enzyme sites for XbaI (at 5' end) and SacI (at 3' end) which was included in their respective primers (listed in **Table 1**). These fragments, as well as the vector pBAD33, were purified and digested with the restriction enzymes selected. The digested fragments were then ligated to pBAD33, using the T4 DNA ligase and the correct constructs were confirmed by PCR and DNA sequencing. The primers used for amplification of the inserted fragment in the pBAD33 vector were pBAD_F (5'-CTGTTTCTCCATACCCGTT-3') and pBAD_R (5'-CTCATCCGCCAAAACAG-3') published by Guzman *et al.* [17]. Five mutants of SL1344 carrying the different recombinant plasmids (named *STM1441*_pBAD33, *ynaJ*_pBAD33, *ybdJ*_pBAD33*, ydiY*_pBAD33 and *yedF*_pBAD33), as well as the SL1344 with the empty plasmid pBAD33 (named SL1344_pBAD33) were obtained by electroporation and selected in LB plates containing 30 mg/L of chloramphenicol (Sigma-Aldrich). Gene expression was induced with 10 mM arabinose (Panreac) and overexpression was confirmed by RT-PCR using the primers shown in **Table 2**.

Complementation of the expression of *yedF* in the SL1344 *yedF* mutant. The Δ*yedF* strain*,* with a disrupted *yedF,* was complemented in *trans* with p9817, a low copy number plasmid, carrying the gene of interest (named Δ*yedF*_p9817*yedF).* Both plasmid p9817 and the amplified fragment of the gene of interest containing the

restriction enzyme sites for NdeI and BamHI, as previously described, were digested with these enzymes. Then, prior to ligation of the digested plasmid to the fragment, p9817 was incubated 1 hour at 37ºC with alkaline phosphatase. Confirmation of the recombinant plasmid containing the gene of interest was made by PCR and DNA sequencing using the primers 1681.for (5'-CCCCAGGCTTTACACTTTATGCTTCC-3') and 1030.rev (5'-GCGGATGCCGGGAGCAGACAAGCCC-3'), at an annealing temperature of 57ºC. Once confirmed, transformation of the resulting plasmid carrying the *yedF* gene was made by electroporation into the Δ*yedF* strain and positive colonies were selected in LB plates containing 50 mg/L of ampicillin (Sigma-Aldrich). Additionally, the Δ*yedF* strain was electroporated with the empty vector p9817 and named Δ*yedF*_p9817, and the same was done with SL1344 (SL1344_p9817). The mutants carrying p9817 obtained were also selected in ampicillin-containing LB plates.

Antimicrobial susceptibility testing. The MICs of the antibiotics nalidixic acid, ciprofloxacin, tetracycline, cefoxitin, erythromycin and trimethoprim were determined by Etests (Biomérieux) on Mueller Hinton-II plates (Becton Dickinson) following the manufacturer's recommendations. Three replicates of each susceptibility test were performed.

Gene expression by real-time PCR. RNA of the strains studied was obtained from exponential cultures using the Maxwell ® 16 Research Instrument (Promega). Bacterial cultures were grown in LB broth supplemented with the appropriate antibiotic and arabinose when needed until they reached an OD_{600}=0.6. Then, 12 mL of RNA Protect (Qiagen) were added to 9 mL of the exponential culture and a pellet was obtained and further dissolved in 200 µL of TE supplemented with 3 mg/mL lysozyme. This was followed by ten minutes of incubation at 32ºC with intense vortex every 2 minutes. At

this point, RNA extraction was performed using the Maxwell® 16 LEV simplyRNA Blood Kit according to the manufacturer's recommendations.

A two-step real-time PCR was performed in a StepOne™ Real-Time PCR System (Applied Biosystems) as previously described by our lab [18]. Briefly, retro-transcription of the RNA obtained was performed and followed by the PCR reaction (SYBR®Premix Ex Taq TliRNase H Plus Kit, Takara) under Universal Thermal Cycling conditions. The primers used to confirm the expression of the selected genes for the obtention of mutants, as well as the virulence-related genes (*hilA*, *hilD*, *invA*, *fliC* and *fimA*) are shown in **Table 2**. The relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method and *16S rRNA* was considered as the endogenous gene for normalization. Three independent extractions and an alyses were performed.

Bacterial growth. Overnight cultures were diluted 1/100 as described previously [15] and OD at 620 nm was determined by means of an iEMS Multiskan Reader MF (Thermo Fisher Scientific) every 15 minutes of incubation at 37ºC for 24 hours. Each plate included four replicates of each sample, and the assay was repeated three times.

Gentamicin protection assay. The invasion assay by bacterial infection of HeLa cells was performed according to Fàbrega *et al.* [15] with some modifications. Briefly, HeLa cells were grown to obtain a monolayer of 55% confluence after 24h of incubation at 37ºC in a 5% CO_2 atmosphere. Then, bacteria from an o/n culture without shaking were used to infect the eukaryotic cells at a multiplicity of infection of approximately 100. After 2 hours of incubation at 37ºC- 5% CO_2, an additional step of 2 hours of incubation in the presence of 100 mg/L of gentamicin (Life Technologies) was followed in order to kill extracellular bacteria. At this point, cells were lysed to count the intracellular bacteria by plating several dilutions in LB agar. At least three independent

experiments were performed with intra-experiment duplicates. The ratio between the number of intracellular bacteria and the infection load was calculated to evaluate the percentage of invasion ability of the strains tested. Induction of gene expression in the strains carrying the gene of interest in the pBAD33 construct was done with the addition of 10 mM arabinose to the medium.

Endotoxin detection assay. Measurement of the endotoxin content of SL1344_p9817, Δ*yedF*_p9817 and Δ*yedF*_p9817*yedF* was performed by means of the kinetic-chromogenic LAL Kinetic-QCL kit (Lonza). Briefly, o/n cultures were adjusted to OD=1 in LB followed by centrifugation of 1 mL; then the pellet was diluted 1/100.000 and used for the assay. Lectures were done spectrophotometrically at 404 nm and the concentration was extrapolated from a Standard curve. Three independent assays were performed.

RNA-seq. RNA extraction of 3 biological replicates of each condition studied (wild-type SL1344 strain and Δ*yedF* mutant) was obtained as described in the **Gene expression by real-time PCR** section of **Material and Methods.** After extraction, the RNA was quantified using a Quantus Fluorometer (Promega) and integrity was assessed with a 2100 Bioanalyzer (Agilent Technologies). Ribosomal RNA was depleted with the Ribo-Zero ^{TM} Magnetic Kit for Gram-negative bacteria (Epicentre) and eluted in 18 µL of "Fragment, Prime, Finish" mix followed by fragmenting and priming for cDNA synthesis. Starting at the "First strand cDNA synthesis" step of the protocol for TruSeq Stranded mRNA Sample Prep Kit (Illumina), samples were converted into a library suitable for cluster generation and DNA sequencing. Library quality was again assessed as previously mentioned and quantified by qPCR (KAPA Library Quantification Kits, Kapa Biosystem's) prior to normalization of the libraries. cDNA transcripts (2 × 75 bp) were

sequenced with the Illumina MiSeq platform. An average of 4 million reads per sample was obtained considering an average Phred quality score of 37. All of them passed the filter with an expected false discovery rate (base-calling error) of 0.05% based on the quality control plot. The paired-end sequence reads were mapped onto the reference genome (*Salmonella enterica* subsp. enterica serovar Typhimurium str. SL1344 version NC_016810.1) using the EDGEpro software [19]. The resulting count datasets for each gene expressed of the individual samples were exported to the DESeq2 module in the R platform [20] where they were normalized, and pair-wise differential expression was carried out for each gene. Only those genes for which the fold values changed by more than ± 2 between the two conditions were taken into account, considering a p value < 0.05. Gene Ontology and Pathway analysis according to the KEGG database were conducted with DAVID [21,22] for all significant genes.

Comparative proteomic analysis i-TRAQ. Identification of proteins with differences in their abundance between SL1344 and *ΔyedF* strains was performed using the isobaric tag for relative and absolute quantitation (iTRAQ) technology. Four independent replicas were prepared for each strain in order to obtain statistically significant results. Both strains were grown independently in LB medium at 37ºC with shaking until they reached the exponential phase (OD_{600}=0.6). Then, the cultures were centrifuged, the supernatants were removed and the cell pellets were washed three times with PBS. Finally, they were resuspended in 8 M urea, 2 M thiourea 2.5% 3-[(3-cholamidopropyl) dimethylammonio]-1 propanesulfonate (CHAPS), 2% ASB-14, 40 mM Tris-HCl, pH 8.8. The cells were then disrupted by sonication during 15 minutes (in on and off cycles of 59 seconds each) followed by centrifugation at 4 °C and 3500 xg in order to remove any cell debris. The proteins extracted were trypsin digested and peptides were

labeled with 8-plex iTRAQ. Pooled peptides were fractionated off-line in a strong cation exchange spin column and the fractions were analyzed by liquid chromatography coupled to mass spectrometry (LC-MS/MS) using a LTQ-Orbitrap Velos mass spectrometer. The Proteome Discoverer was employed to perform the database search and reporter ion intensities extraction using Sequest search engine. Ion intensity normalization, protein ration calculation and statistical analysis were carried out using R (R Development Core Team/ Inferno RND software).

Statistical analysis. The one-way ANOVA test was used for multiple comparisons as the data was normally distributed, and the IBM SPSS Statistics 20 software was used. P-values < 0.05 were considered as significant.

RESULTS

Inactivation and overexpression of four potentially efflux-related genes is neither linked to antimicrobial susceptibility nor involved in virulence.

Four genes were selected. These were derived from a microarray analyses performed in a previous work [15] in which an antibiotic resistant *S*. Typhimurium strain (50-64) was compared to a susceptible clinical isolate (50-wt). All the genes selected showed significant differences in the gene expression levels between the two strains and had not been studied previously. Little information was available in the NCBI database based on sequence homology. Among the three genes annotated as being putative inner membrane proteins, *ybdJ* was highly overexpressed in 50-64 compared to the original strain (33-fold increase), followed by a 5.5- and 3-fold increase in *STM1441* and *ynaJ*, respectively. On the contrary, the gene *ydiY,* encoding for a putative outer membrane protein showed a 5-fold decrease in the resistant strain. After confirming the expression levels of these genes by RT-PCR (data not shown), mutants of SL1344 carrying the disrupted genes (Δ*ybdJ*, Δ*STM1441*, Δ*ydiY* and Δ*ynaJ)* as well as mutants overexpressing these genes were obtained by cloning into the arabinose-inducible vector pBAD33 (*ybdJ*_pBAD33*, STM1441*_pBAD33*, ydiY*_pBAD33 and *ynaJ*_pBAD33). In order to study their involvement in antimicrobial resistance, antimicrobial susceptibility tests of the different mutants to a collection of 6 different antibiotics (nalidixic acid, ciprofloxacin, tetracycline, cefoxitin, erythromycin and trimethoprim) was assessed. Unfortunately, no differences were observed in any mutant compared to the reference strain SL1344 (data not shown) ruling out the idea of a possible role in efflux and thus, in antimicrobial resistance in any of the genes selected.

Another relevant feature seen in the 50-64 resistant strain compared to 50-wt was the decrease in the virulence phenotype [15]. *In vitro* invasion ability was evaluated in an attempt to evaluate if the genes selected, which showed altered expression levels in both strains, could somehow contribute to virulence, As seen in **Figure 1**, statistically significant differences in the ability to invade HeLa cells were only seen for Δ*STM1441* and corresponded to a 2-fold reduction compared to the reference strain SL1344. Overexpression of *STM1441* resulted in a 1.66-fold increase in this invasion ability compared to the wild-type strain, with this difference not being statistically significant. As an increase in the transcriptional levels of *STM1441* was reported in the poorly virulent mutant (50-64) compared to the wild-type strain, the results obtained were contrary to what was expected. According to our data, overexpression of this gene would contribute to a reduction in the invasion ability whereas its deletion would restore it.

On the other hand, the Δ*ydiY* mutant showed almost 2-fold lower invasion ability than the parental strain even in the absence of statistically significant differences. These results correlated well with the decrease in the transcriptional expression of *ydiY* seen in 50-64. Nevertheless, when *ydiY* was overexpressed, this phenotype was not reverted as no differences in the invasion ability were seen compared to SL1344_pBAD33.

The inconclusive results presented above led to discontinuation of the study of these genes.

Antimicrobial susceptibility is not altered in the mutant lacking *yedF*.

Another novel gene also found to be differentially expressed in the resistant strain (50-64) compared to its susceptible clinical isolate (50-wt) in the DNA microarray analysis was *yedF*. This gene was almost 4-fold decreased in 50-64 [15], and this was further confirmed by RT-PCR (data not shown). Based on sequence alignment, this gene has been predicted to code for a TusA-like protein of unknown function with a 31% homology in *E. coli* [23]. TusA is a sulfur transfer protein involved in tRNA modification and molybdenum cofactor biosynthesis in *E. coli* [23,24]; however, according to Dahl *et al.*, *yedF* was not able to replace the TusA functions in anaerobic conditions, as demonstrated in an *E. coli* mutant lacking *yedF* [23]. From a structural point of view, the presence of the CPxP conserved motif in the N-terminal region, stabilizing the first helix, together with a similar folding structure in the C-terminal of the translation initiation factor IF3C of *E. coli* supports the idea of possible involvement in mRNA binding [25,26]. We studied the *yedF* gene at different levels in *S.* Typhimurium due to the lack of consistent information available in the literature regarding the function of this gene and the absence of data in *Salmonella.*

The Δ*yedF* strain with a disrupted *yedF,* as well as a strain carrying a pBAD33 with a cloned *yedF* (*yedF*_pBAD33) were obtained. In order to see if this gene was related to antimicrobial resistance, the susceptibility to different antibiotics (nalidixic acid, ciprofloxacin, tetracycline, cefoxitin, erythromycin and trimethoprim) was determined. Neither inactivation nor overexpression of *yedF* triggered any change in the antimicrobial susceptibility profile (data not shown).

***In vitro* invasion ability is compromised in the mutant lacking *yedF*.**

To evaluate whether *yedF* is involved in virulence, the invasion ability of SL1344 and *ΔyedF* was assessed by infection of HeLa cells. The *ΔyedF* strain showed an average invasion ability of only 8.21 % whereas SL1344 achieved 26.64 %, being this reduction statistically significant ($p = 0.019$). Furthermore, when the *yedF*_pBAD33 mutant was assessed, an invasion ability above the levels of the reference strain (SL1344_pBAD33) was seen (45.17% *vs.* 33.4%, respectively), although the differences were not statistically significant. These results were coherent with the expression levels seen in the resistant and poorly virulent isolate 50-64, suggesting the involvement of *yedF* in this virulence-associated phenotype.

Complementation of the knock-out strain was subsequently obtained by transforming Δ*yedF* with the vector p9817 carrying the wild-type gene (named Δ*yedF* _p9817*yedF)* in the presence of ampicillin. Complementation with the pBAD33 was not possible as the antibiotic used for the selection of both systems was chloramphenicol. For this reason, the *in vitro* invasion assay was repeated with the new collection of mutants: SL1344_p9817 (empty vector), Δ*yedF*_p9817 (empty vector) and the complemented strain Δ*yedF* _p9817*yedF*. The results obtained were consistent with the previous findings. The absence of *yedF* led to a statistically significant reduction in the invasion ability compared to the wild-type strain (10.16% *vs* 27.51%). When the gene was reintroduced, the capability of this strain to invade epithelial cells was even greater than the reference strain achieving 40.32% of invasion (see **Figure 2**).

Transcriptomic analysis reveals that virulence-related genes are affected by *yedF*.

The results obtained from the transcriptomic comparison using the RNA-Seq approach between the SL1344 strain and the Δ*yedF* mutant revealed that 630 genes were repressed, and 321 were overexpressed ($p < 0.05$) in the Δ*yedF* mutant compared to the wild-type strain according to the threshold defined in **Materials and Methods**. The complete list of genes with the corresponding fold-change values is reported as Supplementary data **S1** and a hierarchical cluster with the significant genes allowed the samples to be separated (wild-type *versus* Δ*yedF* mutant), although some variability was observed in the SL1344_1 replicate (**Figure 3**).

A decrease in the expression levels of genes involved in the invasion process is observed in the absence of yedF. In accordance with the results obtained in the HeLa infection assay, an important number of the repressed genes were related to virulence, and a list of the most representative genes involved in this feature is shown in **Table 3**. Repression of flagellum-related genes was observed in the Δ*yedF* mutant: differential expression of the genes encoding for the late flagellin proteins FliC, the type III secretion apparatus FliR, and FlgA, involved in flagellar assembly [27,28] (fold change of -2.17, -2.77 and -2.4, respectively) was observed.

Unexpectedly, transcription of the operon composed by the flagellin repressor FljA and the phase 2 flagellin *fljB* encoding genes was markedly repressed (-11.48- and -5.97-fold, respectively) in the Δ*yedF* mutant compared to the wild-type strain. The concomitant repression of *fliC* together with *fljA and fljB* observed is contradictory: when flagellar phase variation is induced, FljA is expressed and inhibits the translation of the FliC mRNA through binding to its operator region, thus resulting in the transcription of phase 2 flagellin FljB and the absence of FliC [31–33]. In addition to

motility, mediated by flagella, several chemotaxis genes, required in the approach step [3] were also downregulated in the Δ*yedF* mutant (**Table 3**). Moreover, genes related to attachment to the host cell were also differentially expressed: the type-1 fimbrial gene *fimA* and the regulatory gene *fimZ* were 5- and 3-fold repressed in the strain lacking *yedF*, respectively.

Consistently with the transcriptional profile observed in genes related to approach and attachment to the host cell, a general repression of the genes encoded in the different SPIs involved in the invasion process (SPI-1, SPI2, SPI-4 and SPI-5) was also seen in the Δ*yedF* mutant, mostly explained by the levels of the global regulators. Accordingly, a significant repression of the key regulators *hilA* and *hilC* was observed consisting in a 3.10- and 3.57-fold decreased expression, respectively, in the *yedF*-deficient strain compared to the wild-type SL1344 whereas an almost 2-fold change was seen for *hilD*, albeit with a p value of 0.07.

A selection of the above-mentioned genes was confirmed by RT-PCR in order to validate the results obtained by RNA-Seq. As shown in **Table 3**, all the genes tested (*fliC*, *fimA*, *hilA*, *hilD* and *invA)* were repressed in the *yedF*-deficient strain in accordance with the previous results, although lower expression levels were seen when RT-PCR was performed.

Transcription levels of virulence-related genes are restored upon complementation of the ΔyedF mutant with the wild-type yedF. In order to evaluate the effect on invasion gene transcription on reintroduction of *yedF*, RT-PCR of the genes previously mentioned (*fliC*, *fimA*, *hilA*, *hilD* and *invA)* was also carried out in the complemented strain and compared to the parental strain and the *yedF*-deficient mutant. As expected, the expression levels of the invasion genes were restored, reaching even higher values

than the reference strain SL1344_p9817 (see Supplementary Data **Figure S1**). The highest expression levels in the complemented strain were seen for *invA* and *fliC*, with a 4- and 3.9-fold increase compared to the reference SL1344_p9817; being this overexpression 7 and 7.9-fold higher than the knock-out mutant The regulatory genes *hilA* and *hilD* also showed high levels of expression (2.67- and 3.57-fold, respectively) compared to the parental strain (SL1344_p9817) and corresponded to a 4.4-fold (*hilA*) and 5.5-fold (*hilD)* increase compared to the levels seen in Δ*yedF.* Surprisingly, a repression of 10-fold was observed for the fimbria-encoding gene *fimA* in the complemented strain, being these levels below those seen in the knock-out strain (-1.46-fold).

Genes involved in LPS biosynthesis are repressed in the ΔyedF mutant. In addition to the above-mentioned genes, another relevant virulence determinant also affected was the lipopolysaccharide (LPS). This structure can be recognized by specific receptors of the host cells and promotes innate pathways of inflammation [5,6]. LPS is composed of repeated units of the O-antigen, constituting the most exposed part of this structure and connected to lipid A, anchored to the outer membrane of the bacteria by a core oligosaccharide [34]. As shown in **Table 3**, transcription of the genes involved in O-antigen polysaccharide assembly (*rfb* genes), as well as in core-oligosaccharide composition (*rfa* genes) [35] was repressed in the mutant lacking the *yedF* gene. The transcription values of this latter strain ranged from a -2- to -6.8-fold change compared to the wild-type strain SL1344.

A decrease in the LPS content is observed in the absence of *yedF*.

In order to validate the transcriptomic data regarding the genes involved in LPS biosynthesis, the amounts of this virulence determinant were calculated in the wild-

type strain, the mutant with the disrupted *yedF* and the complemented strain. As expected, a decrease in the concentration of endotoxins was observed in the knock-out strain compared to the SL1344 ($4.99x10^4$ EU/mL vs $7.51x10^4$ EU/mL), and these levels increased to $6.46x10^5$ EU/mL when *yedF* expression was restored.

The gene *yedF* is involved in the transcriptional regulation of the anaerobic metabolism pathway.

Transcriptomic analysis of the genes overexpressed in Δ*yedF* compared to SL1344 revealed that all of these genes encoded for enzymes and related proteins involved in anaerobic respiration. In the absence of oxygen, *Salmonella* is able to survive by performing fermentations and/or by using alternative electron acceptors such as nitrate or fumarate [13]. In the present study, the genes affected by the absence of *yedF,* among others, listed in **Table 4** included the nitrite and nitrate reductases, the anaerobic dimethyl sulfoxide reductase, and the anaerobic ribonucleoside-triphosphate reductase. The genes most affected were, above all, the determinants involved in anaerobic respiration and genes related to nitrate/nitrite metabolism. Transcription of the operon *narGHIJ,* which encodes for the membrane-bound nitrate reductase and is responsible for NO production in NO_3^- conditions [36], was highly activated in the *yedF*-defective strain. The expression levels of the different genes of this operon in the mutant lacking *yedF* showed an increase ranging from 8- to 30-fold compared to the wild-type strain. Moreover, an overexpression of almost 40-fold was observed in *ΔyedF* within the *nir* operon, which encodes for the cytoplasmic nitrite reductase NirB. However, although the implication of this enzyme in NO production has been reported in *E. coli* [37], Gilberthorpe *et al.* did not find the same to be true for

NirB in *S.* Typhimurium [36]. Transcriptional levels of the genes encoding for the subunits of the anaerobic dimethyl sulfoxide reductase were also highly activated in the *yedF*-deficient strain compared to SL1344 (~15-fold overexpression) as were the levels of the *nrdDG* operon encoding for the class III reductase NrdD (*nrdD* was 12.5-fold up-regulated in the *ΔyedF* mutant). The genes encoding for the formate dehydrogenase H (FdhF), a component of the formate hydrogenlyase (FHL) complex, together with the genes encoding for the maturation proteins HypABCDE that catalyze the production of hydrogen from formate [38] were also activated in the strain lacking *yedF,* with an increase in the levels ranging from 2.7- to 10.4-fold compared to the wild-type strain. Accordingly, the transcriptional activator of the *fdhF* gene, named *fhlA*, was also up-regulated by 5.33-fold in Δ*yedF*. Similar transcription activation was observed in this latter strain for the *glpABC* operon, encoding for the anaerobic glycerol-3-phosphate dehydrogenase subunits (from 2.5- to 5.3-fold change). This enzyme has been reported in *E. coli* to be essential for anaerobic growth on glycerol-3-phosphate [39,40]. The up-regulation of the transcription of all the genes involved in anaerobic metabolism is in accordance with a previous study conducted in *E. coli* [23]. In this work, deletion of the sulfur transferase *tusA* triggered a similar situation to that observed in the present study regarding hydrogenases and the *nar* and *nap* operons in aerobic conditions. In the absence of *tusA,* the expression of these enzymes was increased suggesting that *yedF* in *S.* Typhimurium has a similar role in the regulation of these proteins.

Differential proteomic profile between SL1344 and *ΔyedF*.

The proteomic approach allowed the identification of 16 proteins with a reduced production in the Δ*yedF* mutant compared to the wild-type strain SL1344 considering a fold change < -2 and $p < 0.05$, whereas only 5 were overproduced in the same strain

(fold change > 1.5; $p < 0.05$). Most of the proteins showing decreased production were involved in the invasion process except 3 that were related to anaerobic processes, as shown in **Table 5**.

Proteins involved in invasion are produced less in the yedF-deficient strain. A decrease in the production of 6 chemotaxis proteins (CheV, CheW, CheA, CheZ, SL1344_3189 and SL1344_4464) ranging from a -2- to almost -5-fold change was observed in the strain lacking *yedF* compared to SL1344. Similarly, the flagellar hook protein FlgE and the flagellar motor switch protein FliG were both produced in lesser amounts in the mutant lacking *yedF* than the wild-type strain (2.4- and 2.1-fold change, respectively), with flagellin FliC showing an even lower production (5.4-fold change). According to the fimbrial proteins FimA and FimC, both showed production levels under -4-fold in the Δ*yedF* mutant compared to the wild-type strain SL1344. Additionally, the production of the two invasion-related proteins SipC, encoded in the SPI-1, and SigD, encoded in the SPI-5, was lower in the *yedF*-deficient mutant than the wild-type strain (5.7- and 2.1-fold change, respectively).

The production of proteins related to anaerobic metabolism is decreased in the absence of yedF. The remaining 3 proteins identified as being less produced in the mutant strain compared to the wild-type strain were the glycyl radical cofactor (5.9-fold change), ornithine decarboxylase, also known as SpeF, showing 2.6-fold change and the formate dehydrogenase α-subunit (2.5-fold change). Interestingly, the two latter enzymes are involved in anaerobic respiration, and the glycyl radical is formed by the class III ribonucleotide reductases (RNRs), which have a central role in DNA synthesis and repair, only in the absence of oxygen [41]. The ornithine decarboxylase is an amino acid decarboxylase that buffers the extracellular environment under acid

conditions and has recently been demonstrated to act only in anoxic environments [42]. Moreover, the formate dehydrogenase α-subunit is part of the formate hydrogenlyase (FHL) complex the function of which is to produce hydrogen from formate in anaerobic conditions [38].

Upon comparing the proteomic analyses with the results obtained in the transcriptomic assay, a partial correlation was found between the two approaches. Among the 16 proteins identified as being less produced in the the Δ*yedF* mutant compared to the wild-type strain, 7 were also seen to be repressed at a transcriptional level considering the same statistical threshold. These consisted of proteins the functions of which were involved in the invasion process, including chemotaxis proteins, flagella and fimbria-related determinants. However, it is worth mentioning that among the remaining 9 genes, none showed statistically significant differences considering the transcriptomic approach (-1.8 to -7.35-fold change) except for 2. The 7 non-significantly repressed genes included four chemotaxis genes, two flagellin-related genes and the ornithine decarboxylase. The only 2 proteins that showed ly discordant results between the two techniques were the formate dehydrogenase α-subunit and the glycyl radical cofactor showing gene expression levels with a 2.09- and 7.4-fold change, respectively, in the Δ*yedF* mutant compared to the wild-type strain. Discordance between transcriptomic and proteomic data is often observed as there is no direct correlation between the amounts of mRNA and proteins.

Overproduced proteins in the ΔyedF mutant are involved in different processes.

In regard to the proteins with increased production levels in the *ΔyedF* mutant compared to the wild-type strain, 5 proteins involved in different processes were identified. Two of these proteins were part of the cold-shock protein family and

consisted of CspJ (1.6-fold change) and CspA (1.5-fold change). This latter protein was reported to be important for optimal growth at a low temperature in *E. coli* [43,44]. Additionally, the ribosomal large subunit pseudouridine synthase RluB, involved in the post-transcriptional modifications in rRNA, was 1.58-fold less produced in the strain lacking *yedF*. The S-formylglutathione hydrolase, for which its role in yeast methanol metabolism is documented [45] but no information is available about its function in *Salmonella*, and a putative lyase, were the two proteins most highly overproduced, showing 1.7 and 1.8-fold changes, respectively.

However, a lack of correlation was seen on comparing these data with the transcriptional analysis. Among the overexpressed genes involved in anaerobic metabolism in *ΔyedF* mutant, only HypB and FrdA were detected in the proteomic approach, albeit with no differences between the two strains; interestingly, none of the remaining proteins was identified in any of the samples.

DISCUSSION

The results obtained in the first part of the present work show that none of the four putative outer and inner membrane proteins studied led to conclusive results, and their role could not be elucidated. Possible reasons explaining the lack of differences concerning antimicrobial susceptibility could be due to the fact that they play a minor role in the efflux, thus an alteration of these proteins is not sufficient to see a relevant phenotype. On the other hand, they may be unrelated to virulence and/or antimicrobial resistance, and rather are a collateral effect derived from adaptation of the bacteria to antibiotic pressure.

However, the data obtained in this study reveal different pathways in which the *yedF* gene is potentially involved in *S.* Typhimurium. Structure prediction from amino acid sequence homology with the *E. coli tusA* suggest that YedF may be a TusA-like protein inferring that both genes could share the same functions, at least partly [26]. In *E. coli*, TusA functions as a sulfur transferase providing sulfur, delivered from the IscS protein, to diverse molecules involved in different pathways [46]. The sulfur transported through TusA is needed for the formation of 2-thiouridine in tRNA and for the molybdenum cofactor biosynthesis [23,47]. Moreover, a previous study has demonstrated that an *E. coli* mutant lacking TusA presented severe growth defects [48,49] suggesting the involvement of TusA in the general physiology of the bacteria.

On comparing our data with the role of TusA in *E. coli*, some of the functions seem to be redundant. Our results indicate that deletion of *yedF* triggers an increase in the gene expression of enzymes involved in anaerobic respiration, similar to what was demonstrated by Dahl *et al.* in a *tusA* deleted mutant of *E. coli* [23]. In this previous study, *yedF* was also investigated; nitrate reductase activity was tested in both aerobic and

anaerobic conditions using a strain lacking *tusA*, *yedF* and a triple mutant lacking these genes in addition to another TusA-like gene (*yeeD*). The objective was to test a possible redundant role of *tusA* and the novel genes, but the results revealed that neither *yedF* nor *yeeD* were able to replace even partially the functions of TusA [23]. However, according to our results, *yedF* seems to play a relevant role in the regulation of proteins involved in anaerobic metabolism in *S*. Typhimurium, although another TusA-like gene with a protein sequence identity of 90% is present in this bacteria (GI:486186069). In order to evaluate the contribution of both the *TusA* homolog and *yedF* in *Salmonella*, further studies using single and double mutants of both genes are needed.

Interestingly, we observed that all the genes showing transcriptional activation in the *ΔyedF* mutant compared to the wild-type strain (**Table 4)** are regulated by FNR, the main transcriptional regulator of the adaptive response of *S. enterica* to the lack of oxygen [9,13,23,36,38–40,50]. This protein contains a 4Fe4S cluster in its sensory domain that is converted to 2Fe2S under aerobic conditions, resulting in an inactive form of FNR [51]; our results show that the expression of this transcriptional regulator was not affected by the presence/absence of YedF. Accordingly, we found that even when oxygen is available, when most of the FNR is inactive, an important activation of the genes encoding for the anaerobic enzymes occurs in the absence of *yedF,* suggesting its contribution to the regulation of the expression of these enzymes needed in the anaerobic process. The differences in gene expression were not reflected at a protein level, as only the hydrogenase HypB and the fumarate reductase FrdA were detected but did not show differential amounts between the wild-type and the strain lacking *yedF.* These results are expected as in aerobic conditions protein synthesis of enzymes

involved in the anaerobic metabolism is not needed; thus, it is logical that the changes observed related to *yedF* are restricted to transcription. Dahl *et al.* [23] proposed a model in which interaction of the IscS protein with TusA decreases the pool of available IscS needed for FeS cluster biogenesis and thus, activation of FNR. As YedF also contains the same conserved cysteine, the catalytic residue involved in the interaction with IscS [52], we suggest that a similar situation may have occurred in the present study. According to the information available together with the results obtained in this work, we suggest that *yedF* affects gene activation by influencing FeS cluster biosynthesis in the cell in the same way as the model previously proposed for TusA [23]. Nevertheless, we have no explanation for the mechanism by which *yedF* activates protein production of the formate dehydrogenase subunit alpha, ornithine decarboxylase and glycyl radical cofactor, all of which are also involved in anaerobic processes. Involvement of the oxygen-sensor-related gene *aer* in the positive regulation of anaerobic enzymes has been previously reported, being this gene activated by the FlhD/FlhC proteins [53,54]. Expression levels of *aer* and the *flhDC* operon were not considered in our transcriptomic results as the differences were not significant; for this reason, in our case, regulation of the anaerobic respiration related to *yedF* seems to be independent from the aer-mediated pathway.

As mentioned previously, TusA has been reported to be involved in bacterial growth as an *E. coli* mutant lacking TusA presented growth defects [48,49]. This function could not be attributed to *yedF*, at least in the case of *Salmonella*, as no differences in the growth rate and morphology of the cells was observed between the *ΔyedF* mutant and the wild-type strain (data not shown).

In addition to the involvement of *yedF* in anaerobic metabolism in *Salmonella*, we propose that it may have a role in virulence. A decrease in the *in vitro* invasion ability of eukaryotic cells was observed in the strain lacking *yedF*, being this ability restored when the gene was reintroduced. Moreover, the absence of *yedF* caused a decrease in virulence-related determinants at both gene and protein levels in correlation with the *in vitro* invasion results. A decrease in the transcription levels by means of RNAseq analysis of the global regulators *hilA*, *hilC* and *hilD*, which control the expression of the genes involved in the invasion process encoded in SPI-1, 2, 4 and 5 [3], was observed in the *yedF*-deficient mutant compared to the wild-type strain and was further verified by RT-PCR for *hilA* and *hilD.* Moreover, the *rtsAB* operon, which is activated by *hilD* and in turn, activates the expression of *hilA* by means of RtsA [55], was also significantly downregulated in the deficient strain [-4.74-fold (*rtsA*) and -6.16-fold (*rtsB*)], as reported in **Supplementary data S1**. Consequently, the expression of most of the genes located in these SPIs was also repressed in the strain lacking *yedF* compared to SL1344. In accordance with these results, the proteomic analysis revealed two proteins with a significantly decreased production in the *ΔyedF* mutant compared to the wild-type strain: the SPI-1 encoded protein SipC (-5.7-fold change) and SigD, located in the SPI-5 (-2.1-fold change). In addition, genes involved in motility, chemotaxis and attachment (e.g. *fliC* and *fljB*) were also affected at both the transcriptional and proteomic levels as shown in **Table 3** and **Table 5**. Transcription of phase 1 flagellin FliC was repressed and also less produced in the absence of *yedF* despite presenting repression of *rtsB*, reported to inhibit the flagellar gene [55]. It has been well established that *Salmonella* possesses two flagellin proteins, called phase 1 and phase 2, encoded by FliC and FljB, respectively, which cannot be expressed simultaneously. During

flagellar phase variation, the inversion of the genetic region called H segment occurs allowing transcription of one of the two phases. When the H segment is in the "on" state, transcription of both *fljB* and *fljA* takes place and thus, phase 2 flagellin FljB and inhibition of phase 1 flagellin FliC is induced posttranscriptionally by FljA. In the "off" state, *fljB* and *fljA* are not transcribed, and thus, FliC is produced [31,32]. Taking this regulation into consideration, in the present work an inconsistent result was observed: decreased expression (-2.17) and production (-5.44) of *fliC* were seen in addition to lower mRNA levels for *fljA* (-11.48 fold change) and *fljB* (-5.97) in the absence of *yedF* compared to the wild-type strain. Nevertheless, the FljA protein was not detected in the proteomic approach suggesting that translation did not occur probably due to a post-transcriptional modification.

Virulence regulation is complex and many factors interplay in this network leading to gene expression in particular conditions; thus, interpretation of the specific role of *yedF* in this system needs to be further studied. Nevertheless, our results reveal that this gene is involved in the regulation of virulence as the absence of *yedF* causes significant changes. Taking into account this multifactorial regulation, alteration of this system could, in some cases, lead to paradoxal situations such as the unexpected gene expression profile of determinants involved in the flagellin biosynthesis pathway. Another surprising result was the important repression of the fimbrial gene *fimA* in the complemented mutant (-10-fold) compared to the parental strain, being even below the levels reported in the knock-out strain. We suggest that a possible explanation for this situation could be that *yedF* regulates *fimA* transcription, but in the presence of excessive amounts of the regulator it provokes the opposite effect.

Another of the virulence aspects affected by *yedF* was LPS biogenesis. A decrease was observed in the expression of the genes involved in O-antigen biosynthesis as well as in the composition of the core-oligosaccharide in the *yedF*-deficient mutant. Expression of *rfb* genes which encode various sugar synthases and transferases needed for the assembly of the O-antigen polysaccharide, as well as genes which introduce the inner glucose and galactose residues in the core-oligosaccharide of the LPS [34,35] was also repressed in the mutant lacking *yedF* compared to the wild-type strain. In the *ΔyedF* mutant, a decrease was observed in the concentration of the global LPS in the endotoxin assay, but this was almost restored when the strain was complemented with the intact gene. Kong *et al.* [56] reported that alteration of the composition of LPS in *S*. Typhimurium led to a decrease in virulence in an *in vivo* murine model. In this previous study, mutants obtained by the deletion of genes involved in the O-antigen and core biosynthesis of LPS were administered orally in mice and revealed that intact LPS was required for optimal invasion and colonization of host tissues.

The transcriptional regulation network underlying the pathogenesis of *Salmonella* is a complex system involving a large number of intracellular factors and environmental inputs as yet not completely understood. Taking into consideration this complicated situation we have identified a transcriptional regulator, *yedF*, that activates many genes, as demonstrated by both transcriptomic and proteomic analysis, which are involved in the host-pathogen interaction. Thus, we suggest that *yedF* contributes to the activation of the virulence machinery. The pleitotropic functions of *yedF* seen in the present work suggest its involvement in different aspects of the invasion process. We hypothesize that in the very initial steps of infection, *yedF* is activated and therefore contributes to positive transcription of the genes involved in motility and

attachment facilitating the arrival of bacterium to the host cell, together with the activation of genes encoded in SPIs needed for its internalization. When oxygen is available, *yedF* also contributes to keeping the anaerobic machinery repressed, and when bacteria encounter conditions in which oxygen is absent, such as in the intracellular state, transcription of *yedF* is repressed allowing the activation of the anaerobic metabolism pathways. Nevertheless, in order to validate this theory, further characterization of this factor needs to be undertaken.

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Transparency declarations

None to declare.

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Figures.

Figure 1. Relative differences in the *in vitro* invasion ability due to the presence/absence of the genes selected (*ynaJ*, *STM1441*, *ydiY* and *ybdJ*).

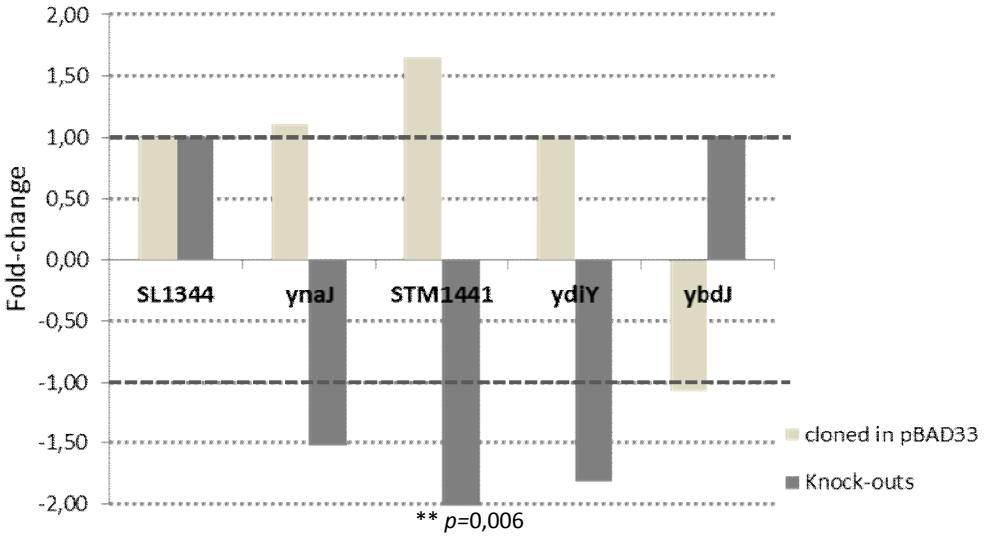


Figure 2. Percentage of invasion in HeLa cells in the wild-type (SL1344), strain lacking *yedF* (Δ*yedF)* and the complemented strain (Δ*yedF* _p9817*yedF*).

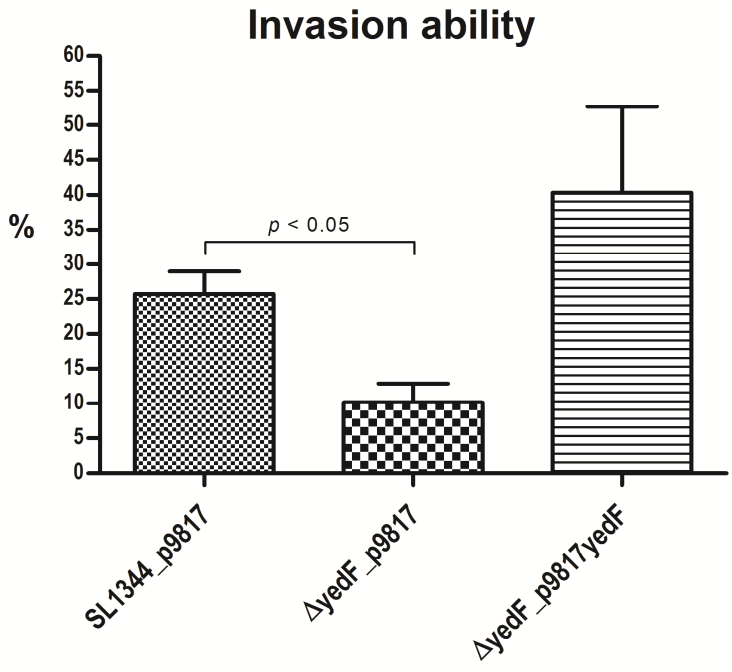


Figure 3. Hierarchical clustering of Euclidean distances of the genes differentially expressed between SL1344 and the Δ*yedF* mutant was obtained by RNASeq analysis using the pheatmap package in R. The levels of expression are color coded from green to red, representing the lowest to highest expression levels in the comparison.

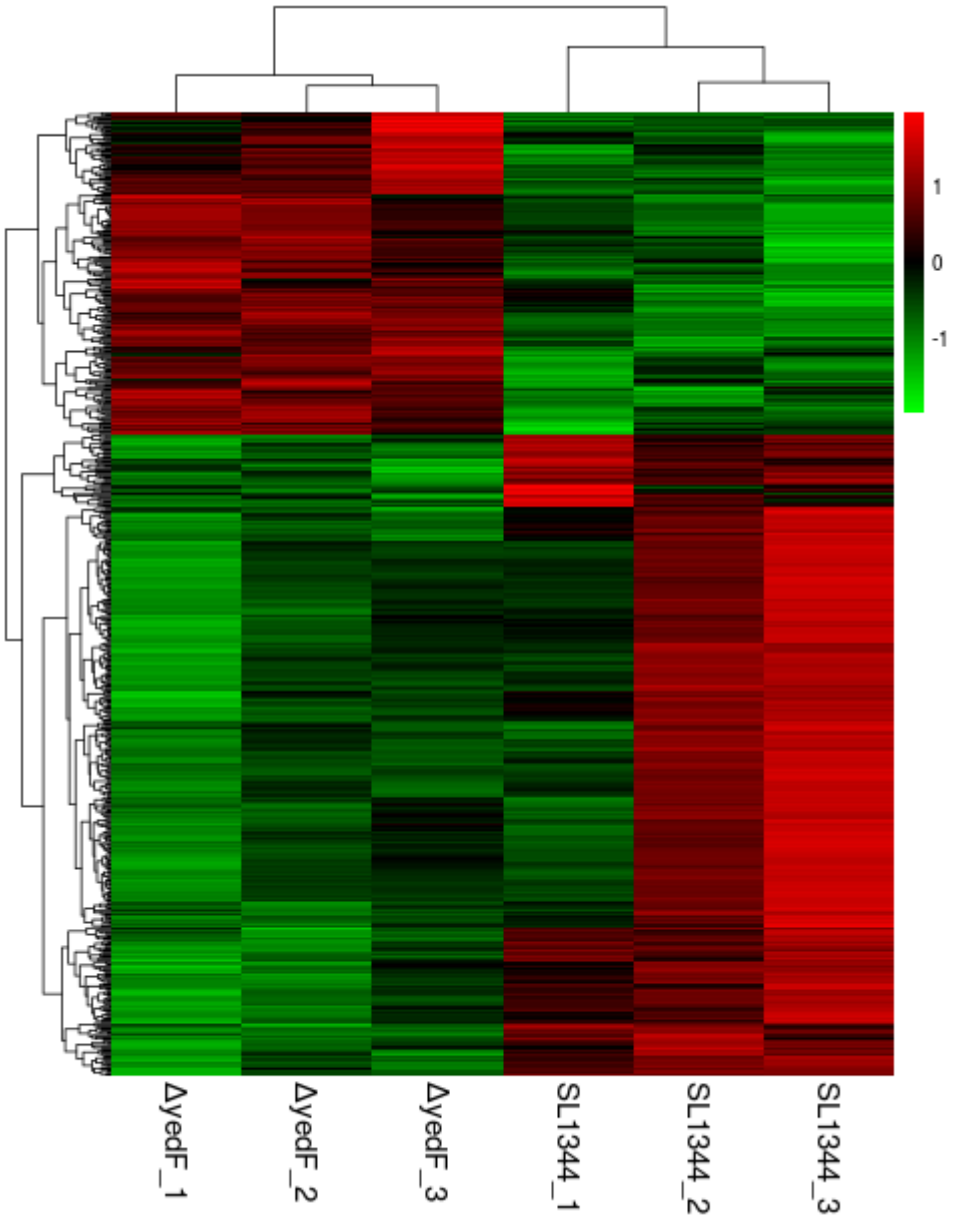
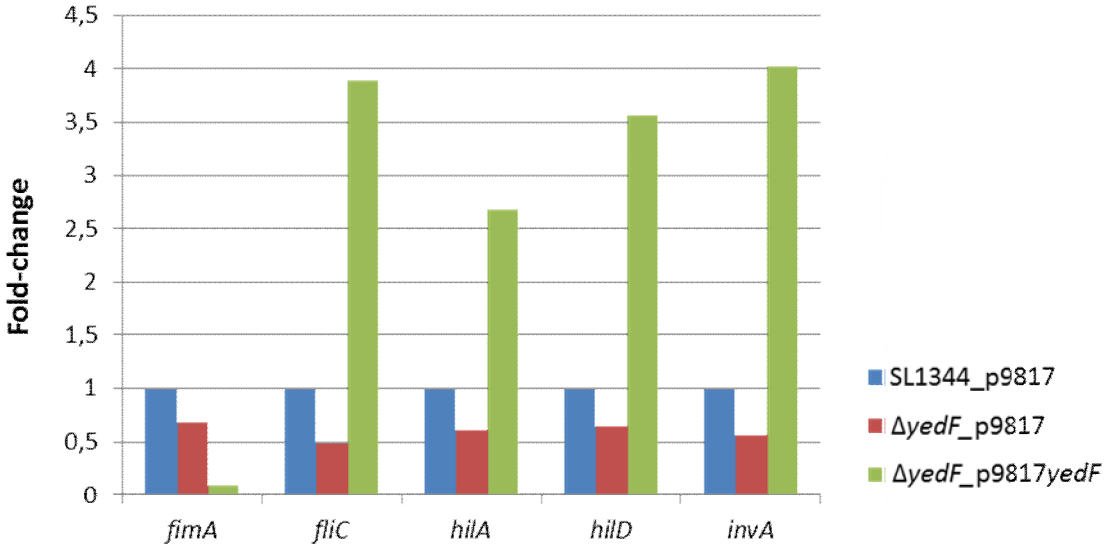


Figure S1. Expression levels of the genes involved in virulence in the wild-type (SL1344), strain lacking *yedF* (Δ*yedF)* and the complemented strain (Δ*yedF* _p9817*yedF*).



Tables.

Table 1. Primers used for cloning and disrupting genes. The annealing temperature used for knock-out obtention was 55ºC in all cases.

Genes	Primers for clonation (5' to 3')	Annealing Temp (°C)	Primers for knock-out (5' to 3')
STM1441	1441.BADF ATATATAGAGCTCAAGGAGAGCGGTAATGAAACTCTC 1441.BADR TTTTTTTCTAGAGCGGAATGCGTTAC	56	1441.KOF ACCGTAGGCGGCGTAATCAGTAAAAGTCTGGGGCGTATTGGTGTAGGCTGGAGCTGCTTCG 1441.KOR AGCGCATCCTGACTATTGCTGAGCTGACTGACGTGATGATCATATGAATATCCTCCTTAG
ybdJ	ybdJ.BADF ATATATAGAGCTCAAGGAGACAAAGCCATGAAACAC ybdJ.BADR TTTTTTTCTAGACGCATTGAAATGTTTA	53	ybdJ.KOF TTGAATCATTGATGACCGCTGCCGGTATTTTGCTGATGGCGTGTGTAGGCTGGAGCTGCTTCG ybdJ.KOR AATCGGCCAGCGAAAACCAGCGCCGCCAGATAAAGCCATATGAATATCCTCCTTAG
ydiY	ydiY.BADF ATATATAGAGCTCAAGGAGATATATAAATGAAGCTTT ydiY.BADR TTTTTTTCTAGACCGGTACAATATTACATCT	51	ydiY.KOF CAGTACCCGCTGTAGTTATGCTGGCGGGTGGCGTGTTCGCGTGTAGGCTGGAGCTGCTTCG ydiY.KOR TTACATCTTATAGCCCAGCGTTACCGTAGTGCGACGATCGCATATGAATATCCTCCTTAG
yedF	yedf.BADF AAATATATGAGCTCCTAAGGAATCTGTATGA (pBAD33) yedf.BADR TTTTTTTCTAGATGCGCTTATTTTTGAA (pBAD33) yedF.CF AAATATATCATATGAAAAATATCGTCCCT (p9817) yedF.CR TTTTTTGGATCCTGCGCTTATTTTTGAATC (p9817)	53 57	yedF.KOF ATGAAAAATATCGTCCCTGATTACCGTCTGGATATGGTTGGTGTAGGCTGGAGCTGCTTCG yedF.KOR TTATTTTTGAATCAGATAACGAATTGTCGGGCCATCCTGTTCATATGAATATCCTCCTTAG
ynaJ	ynaJ.BADF AAATATATGAGCTCAAAGGACAATACTATGAT ynaJ.BADRôTTTTTTTCTAGACCCTATTCCTGTTACT	51	ynaJ.KOF GGTGTGATTGAGCAGTATCATATTCCACTGTCCGAGTGTAGGCTGGAGCTGCTTCG ynaJ.KOR TTACTCATCTCCGCCGAGGAAATAGATCCCCAAAGCATATGAATATCCTCCTTAG

Table 2. Primers used for RT-PCR.

Genes	Primers for RT-PCR (5' to 3')	Reference
STM1441	STM1441.RT1 TCCCTGCCGGCCCTGCGAAATAC STM1441.RT2 CGATCCAGGCGGCCATGCTAAATA	this study
ybdJ	ybdj.RT1 TGAAACACCCGCTTGAATCA ybdj.RT2 AGCAACGCCATCAGCAAAAT	this study
ydiY	ydiY.RT1 GTATGCCGCCGCCGATGATT ydiY.RT2 TAAGGAACTGCCGCCCGTAAC	this study
yedF	yedF.RT1 CACTGGATGCGCGCAAT yedF.RT2 CGGGCCATCCTGTTGGA	this study
ynaJ	ynaJ.RT1 TATGCCTGCTGGCAGTGTTTA ynaJ.RT2 TCGCGCGCGTTACCA	this study
Virulence-related genes		
hilA	hilA_RT_F TTGCTGACTCAATGCGTTAATATG hilA_RT_R TGCCAGCGCACAGTAAGG	this study
hilD	hilD_RT_F TGGCCACATGGATTTCGATA hilD_RT_R GTGCATAGAGAGCGCCAAGTC	this study
invA	invA_RT_F TTGGCGATCTCGATAAAGTCTCT invA_RT_R CGGCTCTTCGGCACAAGTAA	this study
fliC	fliC_RT_F GCCCGTAGCCGTATCGAA fliC_RT_R GCGCGCGAGACATGTTG	this study
fimA	fimA_RT_F CGCGCAGGTGCCTTTCT fimA_RT_R GGCCGCCACTTTTGGAT	this study
Reference gene		
16S rRNA	16S_RT_F GCGGCAGGCCTAACACAT 16S_RT_R GCAAGAGGCCCGAACGTC	18

Table 3. **Transcriptional values of representative genes repressed in the *ΔyedF* mutant compared to the wild-type strain SL1344.** Fold change indicate the ratio between the expression levels in SL1344 and *ΔyedF.* For RNA-Seq, only statistically significant results ($p < 0.05$) are represented except for *hilD* ($p = 0.07$)

	Locus tag	Gene	Description	Fold change (SL1344/*ΔyedF*)	
				RNA-Seq	RT-PCR
Approach: motility and chemotaxis					
	SL1344_4464	-	methyl-accepting chemotaxis protein	-2,52	
	SL1344_3189	*cheM*	methyl-accepting chemotaxis protein	-2,45	
	SL1344_1588	-	putative chemo-receptor protein	-2,45	
	SL1344_3126	-	methyl-accepting chemotaxis protein	-2,55	
	SL1344_4464	-	methyl-accepting chemotaxis protein	-2,52	
	SL1344_1110	*flgA*	flagellar basal body P-ring protein	-2,41	
	SL1344_1849	*flhB*	flagellar biosynthetic protein FlhB	2,64	
	SL1344_1857	*motB*	motility protein B	1,75	
	SL1344_1888	*fliC*	flagellin	-2,17	-1,99**
	SL1344_1910	*fliR*	flagellar biosynthetic protein FliR	-2,77	
	SL1344_2755	*fljA*	repressor of phase 1 flagellin gene	-11,48	
	SL1344_2756	*fljB*	phase 2 flagellin gene	-5.97	
Attachment					
	SL1344_0536	*fimA*	type-1 fimbrial protein, a chain	-5,10	-1,55
	SL1344_0542	*fimZ*	transcriptional regulator (FimXZ protein)	-3,07	
Invasion					
SPI-1					
	SL1344_0776	*slrP*	leucine rich repeat	-2,01	
	SL1344_1784	*sopE2*	invasion-associated secreted effector protein (sopE2)	-3,56	
	SL1344_2674	*sopE*	invasion-associated secreted protein	-3,03	
	SL1344_2841	*sitA*	Iron transport protein, periplasmic-binding protein	-2,28	
	SL1344_2842	*sitB*	Iron transport protein, ATP-binding component	-2,03	
	SL1344_2845	*avrA*	Avirulence determinant of plant pathogenic bacteria	-2,96	
	SL1344_2846	*sprB*	AraC family transcriptional regulator	-3,05	
	SL1344_2847	*hilC*	AraC family transcriptional regulator	-3,57	

	SL1344_2850	*orgAa*	oxygen-regulated invasion protein	-2,77	
	SL1344_2851	*prgK*	type III secretion system apparatus	-2,10	
	SL1344_2852	*prgJ*	type III secretion system apparatus	-2,17	
	SL1344_2855	*hilD*[a]	invasion protein regulator	-1,89	-1,64*
	SL1344_2856	*hilA*	invasion protein regulator	-3,10	-1,68
	SL1344_2857	*iagB*	cell invasion protein	-3,17	
	SL1344_2858	*stpA*	tyrosine phosphatase	-3,44	
	SL1344_2859	*sicP*	chaperone	-4,38	
	SL1344_2860	*sipF*	acyl carrier protein	-5,22	
	SL1344_2861	*sipA*	pathogenicity island 1 effector protein	-2,54	
	SL1344_2862	*sipD*	pathogenicity island 1 effector protein	-2,40	
	SL1344_2863	*sipC*	pathogenicity island 1 effector protein	-2,68	
	SL1344_2865	*spaT*	type III secretion-associated chaperone	-3,44	
	SL1344_2866	*spaS*	type III secretion system secretory apparatus	-2,75	
	SL1344_2869	*spaP*	type III secretion system secretory apparatus	-2,42	
	SL1344_2870	*spaO*	associated with type III secretion and virulence	-3,14	
	SL1344_2871	*invJ*	associated with type III secretion and virulence)	-2,16	
	SL1344_2872	*invI*	type III secretion system secretory apparatus	-3,31	
	SL1344_2873	*invC*	secretory apparatus ATP synthase	-2,66	
	SL1344_2874	*invB*	chaperone protein for type III secretion system effectors	-3,31	
	SL1344_2875	*invA*	secretory apparatus of type III secretion system	-3,07	-1,9*
	SL1344_2876	*invE*	cell invasion protein	-2,98	
	SL1344_2879	*invH*	outer membrane lipoprotein	-2,37	
SPI-2	SL1344_1325	*ssrB*	putative two-component response regulator	-2,62	
	SL1344_1326	*ssrA*	putative two-component sensor kinase	-2,44	
	SL1344_1327	*ssaB/spiC*	putative pathogenicity island 2 secreted effector protein	-3,34	
	SL1344_1328	*ssaC/spiA*	putative outer membrane secretory protein	-3,35	
	SL1344_1329	*ssaD*	putative pathogenicity island protein	-2,93	
	SL1344_1330	*ssaE*	type three secretion system protein	-2,75	
	SL1344_1331	*sseA*	T3SS chaperone	-4,41	

	SL1344_1332	*sseB*	putative pathogenicity island effector protein	-2,61
	SL1344_1334	*sseC*	putative pathogenicity island effector protein	-3,22
	SL1344_1335	*sseD*	putative pathogenicity island effector protein	-2,07
	SL1344_1337	*sscB*	type III secretion system chaperone protein	-3,21
	SL1344_1338	*sseF*	putative pathogenicity island effector protein	-3,10
	SL1344_1340	*ssaG*	putative pathogenicity island protein	-3,68
	SL1344_1341	*ssaH*	type three secretion system apparatus	-2,51
	SL1344_1342	*ssaI*	putative pathogenicity island protein	-3,11
	SL1344_1343	*ssaJ*	putative pathogenicity island lipoprotein	-2,22
	SL1344_1345	*ssaK*	putative pathogenicity island protein	-2,62
	SL1344_1346	*ssaL*	putative secretion system protein	-2,14
	SL1344_1347	*ssaM*	putative pathogenicity island protein	-3,46
	SL1344_1348	*ssaV*	putative type III secretion protein	-2,46
	SL1344_1349	*ssaN*	type III secretion ATP synthase	-2,67
	SL1344_1350	*ssaO*	putative type III secretion protein	-3,08
	SL1344_1351	*ssaP*	putative type III secretion protein	-2,38
	SL1344_1353	*yscR*	putative type III secretion protein	-3,06
	SL1344_1354	*ssaS*	putative type III secretion protein	-2,07
	SL1344_1355	*ssaT*	putative type III secretion protein	-3,55
SPI-4				
	SL1344_4193	*siiA*	type I secretion-related protein	-5,46
	SL1344_4194	*siiB*	hypothetical protein	-5,38
	SL1344_4195	*siiC*	putative type-I secretion protein	-4,63
	SL1344_4196	*siiD*	putative type-I secretion protein	-4,46
	SL1344_4197	*siiE*	adhesin	-2,20
	SL1344_4198	*siiF*	putative type-1 secretion protein	-4,05
SPI-5				
	SL1344_1026	*pipA*	hypothetical protein	-2,74
	SL1344_1027	*pipB*	hypothetical protein	-4,01
	SL1344_1029	*sigE/pipC*	cell invasion protein	-4,11
	SL1344_1030	*sigD*	cell invasion protein	-3,76
LPS				
	SL1344_2059	*rfbP*	undecaprenyl-phosphate galactosephosphotransferase	-4,86 -3,85

SL1344_2060	*rfbK*	phosphomannomutase	
SL1344_2061	*rfbM/cpsB2*	mannose-1-phosphate guanylyltransferase	-4,15
SL1344_2062	*rfbN*	putative rhamnosyltransferase	-5,01
SL1344_2063	*rfbU*	putative glycosyltransferase	-5,58
SL1344_2064	*rfbV*	putative glycosyltransferase	-5,41
SL1344_2065	*rfbX*	putative O-antigen transporter	-6,79
SL1344_2066	*rfbJ*	CDP-abequose synthase	-5,72
SL1344_2067	*rfbH*	putative dehydratase RfbH	-3,04
SL1344_2068	*rfbG*	CDP-glucose 4,6-dehydratase	-2,89
SL1344_2069	*rfbF*	glucose-1-phosphate cytidylyltransferase	-4,20
SL1344_2070	*rfbI*	putative reductase RfbI	-4,56
SL1344_2071	*rfbC*	dTDP-4-dehydrorhamnose 3,5-epimerase	-5,72
SL1344_2072	*rfbA*	TDP-glucose pyrophosphorylase	-3,71
SL1344_2073	*rfbD*	dTDP-4-dehydrorhamnose reductase	-3,05
SL1344_2074	*rfbB*	dTDP-glucose 4,6-dehydratase	-3,27
SL1344_2077	*wcaL*	putative glycosyltransferase	-2,20
SL1344_2078	*wcaK*	colanic acid biosynthesis protein	-3,22
SL1344_2079	*wzx*	putative transmembrane transport protein	-3,68
SL1344_2080	*wcaJ*	putative extracellular polysaccharide biosynthesis protein	-2,07
SL1344_2208	-	putative lipopolysaccharide modification acyltransferase	-5,56
SL1344_3679	*rfaL*	O-antigen ligase	-4,21
SL1344_3680	*waaK*	lipopolysaccharide 1,2-N-acetylglucosaminetransferase	-2,67
SL1344_3681	*rfaZ*	lipopolysaccharide core biosynthesis protein	-2,96
SL1344_3682	*rfaY*	lipopolysaccharide core biosynthesis protein	-4,26
SL1344_3683	*rfaJ*	lipopolysaccharide 1,2-glucosyltransferase	-5,14
SL1344_3684	*rfaI*	lipopolysaccharide 1,3-galactosyltransferase	-4,55
SL1344_3685	*rfaB*	lipopolysaccharide 1,6-galactosyltransferase	-4,90
SL1344_3687	*rfaP*	lipopolysaccharide core biosynthesis protein	-2,63
SL1344_3688	*rfaG*	lipopolysaccharide core biosynthesis protein	-2,72
SL1344_3689	*rfaQ*	lipopolysaccharide core biosynthesis protein	-2,03

* $p < 0.05$; ** $p < 0.01$

Table 4. **Transcriptional values of representative genes overexpressed in the *ΔyedF* mutant compared to the wild-type SL1344.** Fold change indicate the ratio between the expression levels in SL1344 and *ΔyedF.* For RNA-Seq, only statistically significant results ($p < 0.05$) are represented.

Enzymes and related genes	Locus tag	Gene	Description	Fold change (SL1344/*ΔyedF*)
Nitrate/nitrite reductase				
	SL1344_1689	*narI*	respiratory nitrate reductase 1 subunit gamma	8,18
	SL1344_1690	*narJ*	respiratory nitrate reductase 1 subunit delta	12,10
	SL1344_1691	*narH*	respiratory nitrate reductase 1 subunit beta	21,68
	SL1344_1692	*narG*	respiratory nitrate reductase 1 subunit alpha	30,19
	SL1344_1693	*narK*	nitrite extrusion protein	12,21
	SL1344_1694	*narX*	nitrate/nitrite sensor protein NarX	2,12
	SL1344_1695	*narL*	nitrate/nitrite response regulator protein NarL	2,07
	SL1344_2225	*napC*	cytochrome c-type protein NapC	5,61
	SL1344_2226	*napB*	cytochrome c-type protein NapB	3,46
	SL1344_2227	*napH*	ferredoxin-type protein NapH	6,15
	SL1344_2228	*napG*	ferredoxin-type protein NapG	5,20
	SL1344_2229	*napA*	nitrate reductase	8,42
	SL1344_2230	*napD*	putative napAB assembly protein	12,09
	SL1344_2231	*napF*	ferredoxin-type protein NapF	5,80
	SL1344_2443	*narQ*	nitrate/nitrite sensor protein NarQ	4,08
	SL1344_3441	*nirB*	nitrite reductase large subunit	8,98
	SL1344_3442	*nirD*	nitrite reductase (NAD(P)H) small subunit	26,92
	SL1344_3443	*nirC*	putative nitrite transporter	38,90
	SL1344_4213	*nrfA*	cytochrome c552 (subunit of nitrite reductase complex)	3,51
	SL1344_4214	*nrfB*	penta-heme cytochrome c (subunit of nitrite reductase complex)	5,16
	SL1344_4215	*nrfC*	4Fe-4S subunit (subunit of nitrite reductase complex)	1,55
	SL1344_4216	*nrfD*	subunit of nitrite reductase complex	2,28
	SL1344_4217	*nrfE*	formate-dependent nitrite reductase	1,34
Fumarate reductase				
	SL1344_4277	*frdd*	involved in the anchoring of the catalytic components of the fumarate reductase complex to the cytoplasmic membrane	3,40
	SL1344_4278	*frdc*	-	3,50
	SL1344_4279	*frdB*	fumarate reductase	4,76

SL1344_4280	*frdA*	fumarate reductase flavoprotein subunit	4,43
Anaerobic dimethyl sulfoxide reductase			
SL1344_0902	*dmsA*	anaerobic dimethyl sulfoxide reductase subunit A	16,37
SL1344_1428	*dmsA2*	putative dimethyl sulfoxide reductase subunit	14,79
SL1344_4242	-	anaerobic dimethyl sulfoxide reductase chain	7,42
Anaerobic ribonucleoside reductase			
SL1344_4381	*nrdG*	anaerobic ribonucleoside-triphosphate reductase activating protein	3,21
SL1344_4382	*nrdD*	anaerobic ribonucleoside-triphosphate reductase	12,49
Anaerobic glycerol-3-phosphate dehydrogenase			
SL1344_2253	*glpA*	anaerobic glycerol-3-phosphate dehydrogenase subunit A	4,64
SL1344_2254	*glpB*	anaerobic glycerol-3-phosphate dehydrogenase subunit B	5,28
SL1344_2255	*glpC*	anaerobic glycerol-3-phosphate dehydrogenase subunit C	2,56
Hydrogenase			
SL1344_2833	*hycA*	formate hydrogenlyase regulatory protein	4,82
SL1344_2834	*hypA*	hydrogenase nickel incorporation protein HypA	6,81
SL1344_2835	*hypB*	hydrogenase isoenzymes formation protein HypB	10,38
SL1344_2836	*hypC*	hydrogenase isoenzymes formation protein HypC	2,82
SL1344_2837	*hypD*	hydrogenase expression/formation protein HypD	5,27
SL1344_2838	*hypE*	hydrogenase isoenzymes formation protein HypE	2,67
SL1344_2839	*fhlA*	transcriptional activator of the formate hydrogenlyase system	5,33
SL1344_3118	*hybF*	hydrogenase-2 component protein	2,13
SL1344_3119	*hybE*	hydrogenase-2 component protein	3,13
SL1344_3120	*hybD*	hydrogenase-2 component protein	2,04
SL1344_3121	*hybC*	hydrogenase-2 large subunit	2,70
SL1344_3122	*hybB*	hydrogenase-2 cytochrome b subunit	2,10
SL1344_3123	*hybA*	hydrogenase-2 small subunit	6,49
SL1344_3124	*hyb0*	hydrogenase-2 small chain protein	3,63
SL1344_4221	*fdhF*	putative formate dehydrogenase H	4,16

Table 5. Proteins with decreased production levels in the *ΔyedF* mutant compared to the wild-type strain SL1344.

	Locus tag	Protein	Description	Fold change (SL1344/*ΔyedF*)
Flagella				
	SL1344_1114	FlgE	flagellar hook protein FlgE	-2,40
	SL1344_1888	FliC	flagellin	-5,44
	SL1344_1899	FliG	flagellar motor switch protein FliG	-2,03
Chemotaxis				
	SL1344_1856	CheA	chemotaxis protein	-2,52
	SL1344_1855	CheW	purine binding chemotaxis protein	-2,24
	SL1344_3189	-	chemotaxis protein	-2,03
	SL1344_1850	CheZ	chemotaxis protein	-2,01
	SL1344_2283	CheV	chemotaxis protein	-4,78
	SL1344_4464	-	methyl-accepting chemotaxis protein	-3,54
Fimbriae				
	SL1344_0536	FimA	type-1 fimbrial protein, a chain	-4,70
	SL1344_0538	FimC	fimbrial chaperone protein	-4,26
Invasion processes				
	SL1344_1030	SigD	cell invasion protein	-2,12
	SL1344_2863	SipC	pathogenicity island 1 effector protein	-5,69
Anaerobic processes				
	SL1344_2610	-	glycyl radical cofactor	-5,93
	SL1344_0683	SpeF	ornithine decarboxylase	-2,63
	SL1344_1500	FdNG	formate dehydrogenase subunit alpha	-2,50

4. Complete description and identification of the outer membrane proteins of the virulent reference strain *S.* Typhimurium SL1344

Paper II

Characterization of the outer membrane subproteome of the virulent strain *Salmonella* Typhimurium SL1344

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Invasive nontyphoidal salmonellosis is considered an important infectious disease leading to a major public health issue especially in low- and middle-income countries. Poor sanitation conditions together with the spread of antimicrobial resistant isolates contribute to the dissemination of this infectious disease. Among non-Typhi *Salmonella*, serovar Typhimurium is commonly found to be responsible for this disease, but unfortunately no vaccine to prevent this syndrome is currently available. Thus, the lack of profilaxis together with the increase in treatment failure caused by the emergence of multidrug-resistant isolates account for this worrisome situation.

The aim of the present study was to characterize the outer membrane protein (OMP) profile of the virulent strain *S.* Typhimurium SL1344 by means of the sarkosyl extraction method in order to describe the subproteome of this strain.

Upon OMP extraction of two biological replicates of SL1344 cultures grown to the exponential phase, proteins were analyzed by SDS-PAGE and tryptic digestion of cut-gel bands was subsequently performed. Then, a GeLC-MS/MS approach was used to detect the peptides and protein identification was achieved using Mascot (Matrix Science). Localization of the proteins was predicted by means of the PSORTb v3.0 software.

The number of peptides obtained from both replicas consisted of 34,812 peptides for replica 1 while 28,571 peptides were obtained for replica 2, showing 78% of coincidence in the peptides identified. Among these, only peptides with a p-value lower than 0.05 were further studied, corresponding to a total of 1,191. These peptides were then grouped into 181 different sequences potentially coding for proteins, called open reading frames (ORFs). Among the 181 proteins, 160 were classified according to their major or putative function whereas 21 were annotated as hypothetical proteins. For these latter proteins, this is the first biological evidence of their existence as they were only previously identified by an *in silico* approach. Regarding the other proteins, almost 27% were attributed to be involved in transport, followed by 18% with a virulence-related function and 14.4% classified as ribosomal proteins.

Prediction of the subcellular location of the proteins identified was possible using the PSORTb tool and revealed that 63.7% of the proteins were located in the inner (n=66; 36.26%) and the outer membrane (n=50; 27.47%), and an additional 5.52% (n=10) were extracellular. Two proteins were periplasmically located and 30 were cytoplasmic, whereas the remaining 23 (12.63%) had an unknown location. It is worth mentioning that 8 of the proteins belonging to the latter group were predicted to be non-cytoplasmic but no subcellular location was determined. A possible explanation for the presence of cytoplasmic proteins may be due to the fact that outer membrane vesicles (OMVs) containing cytoplasmic proteins had not been totally removed during the protein extraction step.

In addition to these results, we compared the protein predictions obtained from the PSORTb approach and the Uniprot database. Using this latter database, a lower number of proteins (n=141) could be classified in a particular subcellular location compared to the 158 proteins identified with PSORTb, as mentioned above.

Among the 66 inner membrane proteins predicted with PSORTb, only 15 were equally classified with Uniprot; however, among the remaining 51 proteins, 38 were annotated as being membrane-located, with no distinction between the inner and outer membrane. The other 13 proteins were classified into distinct groups, 2 were flagellar, 1 was part of the nitrate reductase complex, and 10 had an unspecified location. More balanced results were

seen for the outer membrane-located proteins: 50 proteins were classified in this way using the PSORTb approach whereas 40 proteins were likewise annotated with Uniprot. Moreover, 5 of these latter proteins had an unknown location according to PSORTb. Regarding the cytoplasmic proteins, huge differences were seen according to the two approaches: 30 proteins were predicted to be in this category according to PSORTb and only 3 using the Uniprot approach. However, according to the Uniprot database, 23 proteins predicted to belong to the cytoplasmic group in PSORTb classification were categorized as ribosomal proteins. The remaining 4 proteins corresponded to two integral membrane components and the two others had an unknown location. An extracellular location was attributed to 10 proteins according to the PSORT database, with only 4 being detected with the other approach; the 6 remaining ones were seen to be flagellar proteins. Lastly, the 2 proteins classified as periplasmically located according to PSORTb could not be classified into any category using the Uniprot database.

Despite the variations seen in the bacterial location of the proteins obtained depending on the database, the differences observed are partially due to the specific nomenclature of the subgroups used by the two approaches. If we simplify the groups in more basic categories such as membrane, cytoplasmic and others, most of the proteins would coincide in the same category regardless of the method used.

The results of this study increase the information of the SL1344 proteome, particularly regarding the cell envelope, and thus will be useful to identify potential new targets for the development of vaccines. Furthermore, they also contribute to reinforce the existence of putative proteins enlarging the knowledge of the biology of *Salmonella*.

Characterization of the outer membrane subproteome of the virulent strain *Salmonella* Typhimurium SL1344.

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Keywords: *Salmonella* Typhimurium, outer membrane, OMP subproteome, LC-MS/MS.

Abstract

Outer membrane proteins (OMPs) play an important role in the interaction of bacterial pathogens with host cells. Indeed, some OMPs from different Gram-negative bacteria have been recognized as important virulence factors for host immune recognition. This scenario has led to the study of the outer membrane proteome of pathogenic bacteria as an essential step for gaining insight into the mechanisms of pathogenesis and for the identification of virulence factors. Although progress in the characterization of the outer membrane has recently been reported, detailed protein composition of this subcellular localization has not been clearly defined for most pathogens. *Salmonella enterica* serovar Typhimurium is not only a leading cause of human gastroenteritis in high-income countries but is also one of the main causes of invasive non-typhoidal salmonellosis (iNTS) in middle- and low-income countries. The incidence of non-typhoidal salmonellosis is increasing worldwide, causing millions of infections and deaths among humans each year. Regrettably, antimicrobial resistance to a broad spectrum of antibiotics is common among non-Typhi *Salmonella* strains. Therefore, the development of vaccines targeting this leading invasive pathogen is warranted. In the present study we have identified the outer membrane protein profile of the virulent *S.* Typhimurium strain SL1344 by means of sarkosyl extraction.

Introduction

Salmonella enterica serovar Typhimurium is a common facultative intracellular pathogen that causes food-borne gastroenteritis around the world. In high-income countries such gastrointestinal disease is rarely life-threatening and is normally self-limiting. Nausea, vomiting, profuse watery diarrhea, and abdominal pain are the usual clinical manifestations in immune-competent individuals [1]. According to the 2013 annual report of the Rapid Alert System for Food and Feed (RASFF) of the European Union, there were about 120 alerts for *Salmonella* contaminations in food other than poultry in Europe during 2013, while in poultry meat the number of alerts had tripled compared to the previous year [2]. In addition, according to the European Food Safety Authority (EFSA), over 100,000 human cases are reported only in the European Union each year and it has been estimated that the overall economic burden of human salmonellosis could be as high as 3 billion euros a year [3]. In low- and middle-income countries, however, *S.* Typhimurium*,* together with *Salmonella* Enteritidis, are responsible for a more serious manifestation of the disease through a form of invasive illness, invasive non-typhoidal *Salmonella* (iNTS) disease, which is considered a major public health problem in these countries [4]. iNTS disease is a neglected disease that is endemic in sub-Saharan Africa, but is also significantly present in Asia. Data of the global burden of disease estimates are not currently available, although associated case fatality rates range from 20-25 % [5]. Clinical manifestations are diverse including fever, hepatosplenomegaly and respiratory symptoms as the most common, whereas typical features of enterocolitis are often absent [6]. In sub-Saharan African countries non-typhoidal *Salmonella* are either the leading or next most common pathogen isolated from blood after pneumococcus [7], for which vaccines are available and currently implemented in the region. Unfortunately no vaccines against iNTS are currently available, although some attempts are ongoing [4]. Moreover, among the most worrisome facts is the increasing resistance to antimicrobials which notably limits the clinical success of the present therapeutic options [8].

S. Typhimurium has evolved to survive in adverse environments. After ingestion of this bacterium by a mammalian host through contaminated food, or other vehicles, it progresses through the diverse environments of the gastrointestinal tract until reaching the intestine. Here it interacts with the wall of the intestine, and the invasion process takes place through expression of specific proteins involved in the translocation of *S.* Typhimurium across the epithelial cell barrier [9]. Once these bacteria reach the basolateral membrane, they are engulfed by phagocytes [10]. In order for the infection to extend beyond the intestinal mucosa,

S. Typhimurium survives and replicates inside macrophages, a privileged niche that allows this pathogen to elude the adaptive immune response thereby facilitating dissemination throughout the body, and reaching strategic organs like the liver or spleen [11]. During this process, infected individuals and animals expel this pathogen through their feces. In regions with poor sanitation systems, these bacteria can then contaminate water sources and food, thus spreading and infecting larger populations. From the point of view of induction of antibody production, the ability of *S.* Typhimurium to survive and replicate inside macrophages represents a challenge for vaccinology, since humoral immunity plays a key role in dealing with extracellular bacteria [12, 13].

Based on the observation that most bacterial vaccines inducing protective antibodies are mainly constituted by highly expressed, surface-exposed antigens and/or secreted toxins [14], the identification of such components in a bacteria can provide invaluable information. Thus, outer-membrane proteins (OMPs) are among the most obvious targets for protective immune response, particularly for subunit vaccine research, primarily because they are surface exposed and can therefore be recognized by the host immune system. It has been estimated that about 2-3 % of the genomes of Gram-negative bacteria encode integral OMPs, and a significant proportion of these are expressed ubiquitously [15].

In 2001 Molloy *et al.* performed an analysis of the OMPs of *S*. Typhimurium by means of carbonate extraction, followed by two-dimensional electrophoresis (2DE) and identification of proteins by PMF [16]. In their work the authors used the *S*. Typhimurium LT2 strain and were able to identify 24 different spots out of 37, corresponding to 23 different ORFs. The LT2 strain is a non-pathogenic reference strain commonly used in most laboratories. In fact, most information on genetic and phenotypic variation in *Salmonella* has derived from studies conducted in this strain, as only a small number of other laboratory strains has been referred elsewhere [17]. Since then, only another study performed by Coldham and Woodward in 2004 analyzed the proteome of the virulent *S*. Typhimurium SL1344 strain [18]. However, to date no exhaustive analysis of the outer membrane (OM) subproteome of *S.* Typhimurium has been done, particularly using the most appropriate protocols for the extraction of OMPs. In order to address this gap of knowledge and take advantage of the recently published genome of *S.* Typhimurium SL1344 in 2012 [19], in the present study we have analyzed the OM subproteome of the *S*. Typhimurium SL1344 strain by means of the sarkosyl extraction protocol in combination with MS/MS approaches.

Materials and Methods.

Cultures and media

The *S.*Typhimurium strain ATCC SL1344 was grown in Luria-Bertani (LB) medium at 37 °C with shaking until it reached the exponential phase at an OD_{600} of 0.6. Two independent cultures were obtained and further processed for protein extraction.

Preparation of outer membrane protein extracts

OMPs extraction was performed by means of the N- lauroyl sarcosinate (also known as sarkosyl) method [20]. Briefly, *Salmonella* cells were harvested from 200 mL of exponential cultures (O.D. = 0.6) by centrifugation at 4 °C and 3500 x g. Cells were cleaned twice in PBS in order to remove any medium residue. Afterwards, cells were resuspended in 6 mL of 10mM Tris, pH 8.0 and NaCl 1%. At this point the cells were disrupted by sonication during 15 minutes (in cycles of 59 seconds on and 59 seconds off). After cell disruption the samples were centrifuged at 4 °C and 3500 x g in order to remove any cell debris. The supernatants were collected and transferred into ultra-centrifugation tubes and samples were centrifuged at 100.000 x g for one hour at 4 °C in a Sorvall MS-150 micro-ultracentrifuge (Thermo Scientific) using a S50-ST rotor. After this centrifugation step, the supernatants were discarded and the pellets were resuspended in 1% of freshly prepared sarkosyl solution and incubated during 60 minutes at room temperature with gentle agitation. After incubation, the samples were again centrifuged at 100.000 x g for one hour at 4 °C, and the resulting pellets were cleaned twice with 10mM Tris, pH 8.0 and NaCl 1%. Lastly, after the final centrifugation step the pellets were carefully resuspended in 500 μL of milliQ water. The protein concentration was estimated with the 2D-Quant Kit from GE Healthcare (Fairfield, Connecticut, USA).

SDS-PAGE

Salmonella OMP preparations were analyzed by SDS-PAGE using a gel casting system (Bio-Rad tetra cell) and 12.5% isocratic Laemmli gels. Approximately 75 μg of protein were loaded in each lane. Gels were run at constant amperage (20mA) until the bromophenol blue tracking front had run off the end of the gel. Gels were stained with 0.1% Coomassie blue R-250 dye at room temperature for 30 minutes, and then distained overnight with 10% acetic acid in distilled water. The range of OMP molecular weights was estimated from a standard size marker (Benchmark™ protein ladder, Invitrogen (Chicago, USA)).

In-Gel Tryptic Digestion

The Coomassie-stained lanes were cut into 10 equal bands, and immediately distained and digested as described elsewhere [21]. Briefly, the Coomassie-stained lanes were washed twice with water for 20 min and distained with 200 µL of 50 mM ammonium bicarbonate/50% acetonitrile. Before tryptic digestion, reduction and alkylation with DTT/IAA was performed by incubating the samples with 200 µL of 10 mM DTT in 50 mM ammonium bicarbonate for 1 h at 56°C, followed by alkylation with 200 µL of 55 mM IAA in 50 mM ammonium bicarbonate for 30 min at room temperature, protected from light.

Gel pieces were digested overnight with 6 ng/µL trypsin at 37 °C. The peptide extraction was carried out with three consecutive washes with 1% formic acid for ESI analysis. The eluted peptides were dried in a SpeedVac and stored at −20 °C until analysis by mass spectrometry.

Mass Spectrometry (MS) Analysis

For GeLC–MS/MS analysis, the digests of the SDS-PAGE lanes were analyzed on an AmaZon ETD Ion Trap mass spectrometer (Bruker Daltonics), coupled to a nano-HPLC system (Proxeon). Peptide mixtures were first concentrated on a 300 mm i.d., 1 mm PepMap nanotrapping column and then loaded onto a 75 mm i.d., 15 cm PepMap nanoseparation column (LC Packings). Peptides were then eluted by a 0.1% formic acid/acetonitrile gradient (0–40% in 120 min; flow rate ca. 300 nL/min) through a nanoflow ESI Sprayer (Bruker Daltonics) onto the nanospray ionization source of the Ion Trap mass spectrometer. MS/MS fragmentation (3 × 0.3 s, 100–2800 *m*/*z*) was performed on three of the most intense ions, as determined from a 0.8 s MS survey scan (310–1500 *m*/*z*), using a dynamic exclusion time of 1 min for precursor selection and excluding single-charged ions. An automated optimization of MS/MS fragmentation amplitude, starting from 0.60 V, was used.

Proteins were identified using Mascot (Matrix Science) to search in the NCBInr (July 2014). First, common contaminants (tryptic autolytic fragment-, keratin-, and matrix-derived peaks) were removed using the contaminants database available in the Mascot search engine. MS/MS spectra were searched with a precursor mass tolerance of 0.4 Da, fragment tolerance of 0.7 Da, trypsin specificity with a maximum of one missed cleavage and methionine oxidation was set as variable modification. Replicate analyses of all the LC-MS/MS analysis, using independent biological replicas, showed ≈ 80% coincidence indicating a high level of reproducibility. In order to ensure that the data are reliable a decoy database was used. In this

sense the searches were repeated using identical search parameters against a database in which the sequences had been reversed or randomized [22] Every time a protein sequence from the target database is tested during the search in the Mascot search engine a decoy sequence of the same length is automatically generated and tested. The average amino acid composition of the decoy sequences is the same as the average composition of the target database. Protein localization was predicted using the PSORTb v3.0[23]

Complete information on all peptide and protein identifications, including identification probabilities and sequences can be found in Supporting information Tables S1 and S2, respectively.

Results and discussion

The cell envelope of *S.* Typhimurium and other Gram-negative enteric bacteria is a complex structure composed of 3 morphologically distinct layers [24, 25]: a cytoplasmic membrane, a rigid peptidoglycan layer external to the cytoplasmic membrane and a second membranous structure, the OM or L-layer, at the outer surface of the cell. The OM contains substantial amounts of proteins and phospholipids and, in addition, most or all of the lipopolysaccharide of the cell envelope. Osborn *et al.* estimated that the OM of bacteria contains approximately 60 % of all the proteins present in the two membranes [26]. However, although some OMPs from *S.* Typhimurium have been reported and characterized, no extensive analysis of the OM subproteome has been done using the most appropriate methodology.

The SL1344 genome contains 4742 protein-coding genes. A total of 4530 of these genes are present on the chromosome, and 212 genes are encoded by three plasmids. Currently, proteomes inferred from genome sequence data are extremely accessible but often remain unverified [27]. Only proteomics can unambiguously determine if the gene expressed is translated into a protein. High-throughput LC-MS/MS-based proteomics approaches measure protein fragments directly, and the resulting peptide sequences confirm the existence of a protein from a specific genome. Peptides that map to genomic regions outside the boundaries of previously annotated genes represent evidence of novel genes or extensions of their predicted termini.

In the present study we used the GeLC-MS/MS approach, widely used in shotgun proteomics, in order to identify the OM subproteome of *S.* Typhimurium SL1344 obtained through the sarkosyl extraction method. In terms of protein analysis, several reports have proven and identified the sarkosyl protocol as the best methodology for OMP extraction in

order to obtain the purest samples. Hobb *et al.* [28] analyzed a total of nine methodologies and concluded that glycine extraction, differential detergent extraction using Triton X-100, serial extraction using 1M Tris pH 7, spheroplasting by lysozyme and sonication, and carbonate extraction did not produce pure OM preparations[28]. According to the authors, the extraction of OMPs using sarkosyl produced the purest samples leading to the most reproducible results. In addition, Cao *et al.* extracted the OMPs from the Gram-negative bacterium *Caulobacter crescentus* using the carbonate method and the sarkosyl extraction protocol. Similarly, they also concluded that the sarkosyl protocol gave the purest OMP preparations[29].

After OMP extraction, proteins were separated by SDS-PAGE. Two biological replicas were processed and each was loaded into a different lane, which was cut into 10 pieces and analyzed by LC-MS/MS. Using this approach about 42315 MS/MS spectra were acquired for replica 1 while 45331 MS/MS spectra were acquired for replica 2. Searches were performed with MASCOT using the *Salmonella* taxonomy entry from the NCBInr database. However, since not all the entries had their corresponding entry to the SL1344 strain, a BLAST search was carried out each time against the SL1344 strain in order to generate a list with the SL1344 entries.

The results delivered 34812 peptides for replica 1 while 28571 peptides were obtained for replica 2. The two independent biological replicas showed approximately a 78% of coincidence in the peptides identified. In order to obtain reliable results we used a quite strict cut-off in order to accept positive identifications. Proteins were accepted only if at least two different peptides had been identified and the p-values were lower than 0.05. After removing duplicates, peptides identified with p-values higher than 0.05 and single peptides representing a protein, a total of 1191 different peptides were identified (Supporting Information Table S1). Then, we grouped together the peptides into proteins and a total of up to 181 different ORFs were identified (Supporting Information Table S2). As exceptions, two proteins were accepted with the identification of only one peptide, these were the Small protein A and Entericidin B (gi numbers: 378700596 and 378702179 respectively). These proteins were accepted because the peptide identified represented 18 % (Small protein A) and 40 % (Entericidin B) of the sequence coverage with very good p-values (6,5E-5 and 7E-12, respectively). The Small protein A is a small outer-membrane lipoprotein that is a component of the essential YaeT outer-membrane protein assembly complex [30]. Entericidin B is a small cell-envelope bacteriolytic lipoprotein that can maintain plasmids in bacterial populations by means of post-segregational killing [31]. Spectra of these two proteins are provided as Supporting Information Figure 1 and Figure 2.

The first and most notable result was the experimental evidence of proteins the existence of which had not previously been demonstrated. Indeed, 21 of the total proteins identified were annotated as hypothetical proteins (Supporting Information Table S2). As the existence of these proteins had been predicted only by means of *in silico* methods, herein we provide the first experimental evidence of their synthesis. Concerning the remaining 160 proteins, these were classified according to their major or putative function. The resulting classification is detailed in Supporting Information Table S1 but Table 1 summarizes the results of the proteins belonging to each category. Moreover, Figure 1 shows a schematic representation of this distribution. Most of the proteins (43) were related to transport functions, whereas the second and third most representative groups were composed of proteins related to virulence (29) and ribosomal proteins (23), respectively.

On comparing our results with those reported in previous studies several considerations need to be taken into account. In the work conducted in 2001 by Molloy *et al.* using a carbonate extraction method and 2DE analysis, the authors only identified 23 different ORFs in the non-pathogenic LT2 strain [16]. It is well known that one of the limitations of 2DE analysis is the under-representation of membrane proteins due to poor solubility in the buffer required for the isoelectrofocusing separation procedure. This limitation could lead to the limited results obtained. Almost all of the proteins identified by Molloy *et al.*, were also identified in the present study. The proteins not identified in our study were the molecular chaperone DnaK, the 30S ribosomal protein subunit S1, a phosphoglycerate kinase, an enolase and the heat shock protein Hsp90, most of which are known to have an intracellular function. Thus, these differential identifications are likely to be the result of the different methodological approaches used in the two studies. Molloy *et al.* used the carbonate method which, as mentioned previously [28, 29] does not produce pure OM preparations.

On the contrary, we used the sarkosyl extraction method, described as the purest and most reproducible methodology, and yet we also identified proteins whose function is known to be intracellular. Although we cannot completely exclude the possibility of cytoplasmic protein contamination, a feasible explanation of the presence of these cytoplasmic proteins in both OMP extractions can be the formation of budding vesicles. It is well known that bacteria constitutively secrete native outer membrane vesicles (OMVs) into the extracellular milieu containing cytoplasmic proteins [32]. It has also been demonstrated that such vesicles carry DNA and RNA molecules. Since translation of OMPs may occur simultaneously with their integration into the membrane, the presence of ribosomal proteins, chaperones or other

cytoplasmic proteins may not be surprising, suggesting that they may play a role in OMVs hence justifying their presence in OMP extraction [33-36].

Moreover, two different studies have analyzed the proteome of the pathogenic strain *S.* Typhimurium SL1344. In 1996 Qi *et al.* analyzed by 2DE the phosphate buffer-insoluble proteome in the absence of a specific OMP extraction method [37]. Proteins were electroblotted from the gel onto a PVDF membrane and then, N-terminal sequences were determined by sequential Edman degradation. However, in addition to the handicap of the solubilization of membrane proteins in 2DE analysis, Edman degradation presents limitations in terms of sensitivity as well as the problem of identifying the blocked N- terminal residues. Thus, the well-known under-representation of membrane proteins when using this methodology may explain why only 6 out of the 53 proteins identified by these authors were also found in the present study [37]. Later, in 2004 Coldham and Woodward performed an in-depth analysis of the proteome of *S.* Typhimurium SL1344 [18]. The authors considered the insoluble fraction of a buffer containing urea/thiourea, triton X-100/CHAPS as the OM subproteome. It was then expected that these sample preparations contained all kinds of membrane proteins as well as macromolecular complexes. They identified 34 OMPs from a total of 816 proteins identified. Among these 34 proteins, 24 were also found in the present work. Thus, neither of these studies used specific and accurate methods for OMP extraction hence justifying the important differences observed between their findings and our results. Moreover, the data of these previous studies could not be checked with the genome of *S.* Typhimurium SL1344 since it had not been published until 2012 [19].

To further complement the information obtained in the present study, we also investigated the subcellular location of all the 181 proteins identified by two different approaches: we used the PSORTb v3.0 prediction server and, next, we also searched for the annotated subcellular location, if available, from the Uniprot database (Table 2). A total of 158 proteins were classified in a particular bacterial location using the PSORTb prediction software *versus* the 141 classified according to the information reported in the Uniprot database. Details of the predictive results obtained from the analysis of the PSORTb software can be found in Supplementary Information Table S2. According to the prediction, 65 proteins (36.26 %) were located in the inner membrane, 50 (27.47 %) in the OM, 30 (16.48 %) were cytoplasmic, 10 (5.52 %) extracellular, 2 (1.09 %) periplasmic and the remaining 23 (12.63 %) proteins had an unknown location. Among the latter group 8 proteins were predicted to be non-cytoplasmic, although PSORTb was unable to specify the subcellular location.

Thereafter, we aimed to compare the results obtained from the predictive software with the information published in the Uniprot database in order to determine their potential contribution (Table 2). In terms of the 66 inner membrane proteins predicted by PSORTb, only 15 were equally annotated in their corresponding entries in the Uniprot database. Of the remaining 51 proteins, 38 showed membrane location although no distinction between inner or outer membrane was stated, 2 were related to the flagellum basal body, 1 corresponded to the nitrate reductase complex, and 10 did not have any specified location. The PSORTb database was able to classify 50 proteins as being located in the OM *versus* 40 proteins by Uniprot (35 were equally assigned as OMPs whereas the other 5 were of unknown location by PSORTb). For the 30 proteins predicted by PSORTb to be located in the cytoplasm, according to Uniprot 3 they were similarly classified, and 23 corresponded to ribosomal proteins. The other 4 proteins were two integral components of the membrane (Uniprot entries: A0A0H3NL53 and A0A0H3NVV1) and two proteins with no information about subcellular location in the Uniprot database (Uniprot entries: A0A0H3NX93 and A0A0H3NUC4). Additionally, according to the PSORTb annotation, 10 proteins were classified as being extracellularly located whereas only 4 were so classified by the Uniprot database. The remaining 6 proteins were assigned in the bacterial-type flagellum subgroup. Concerning the two proteins predicted to be located in the periplasm by PSORTb, one was a protein involved in the formation of diffusion channels in the OM during phage adsorption (Uniprot entry A0A0H3NIT6) and the other was an OMP with unknown function (Uniprot entry A0A0H3NM65). Neither of these two proteins could be classified in any subgroup in the Uniprot database.

The comparison between the two different databases used (PSORTb and Uniprot) for classification into distinct bacterial locations showed that PSORTb was able to assign a greater number of proteins in a specific subgroup (87.3% with PSORTb *vs* 77.9% with Uniprot). Moreover, proteins located in the membrane, without further identification of inner or outer membrane, according to Uniprot, showed a more precise location by means of the PSORTb software. The number of proteins assigned in the different categories did not fully coincide between the two databases, being the proteins assigned in the inner membrane subgroup the least similar. Only 22.7% of the proteins identified as being located in the inner membrane with PSORTb were equally grouped by Uniprot. However, it is of note that both databases have different categories of bacterial location, therefore the differences seen between the classification of the proteins obtained in the present study could, in part, be due to this fact.

In conclusion, in the present work we describe for the first time a detailed analysis of the OM subproteome of the pathogenic strain *S*. Typhimurium SL1344 using the most appropriate methodology. We report the extraordinary efficiency of the sarkosyl extraction method in characterizing a large number of proteins of this bacterial compartment. This methodology together with the GeLC-MS/MS approach allowed the identification of up to 181 proteins whereas previous studies have reported less than 53 proteins. Moreover, further information regarding the intracellular location of these proteins has also been considered. In view of these results, this study provides new information about the proteome of SL1344. According to the current clinical situation of increasing trends of antibiotic resistance and the lack of an effective vaccine, this new insight will be very helpful for future studies on *S*. Typhimurium, particularly those focused on identifying new targets for the development of novel tools to fight against this important pathogen, such as subunit vaccines or inhibitory drugs.

Supporting information available: This material is available free of charge via the Internet at http://pubs.acs.org

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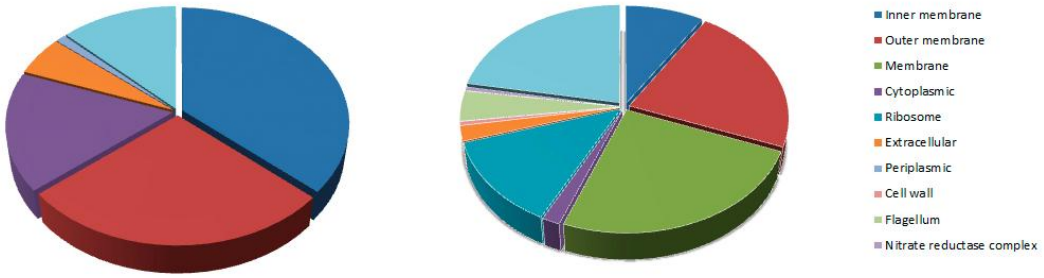
467 Figure 1. Distribution of the OMPs characterized in the present study according to several
468 categories. Left: Distribution according to P-SORT 3.0 prediction; Right: Distribution according
469 to subcellular location obtained from Uniprot.



470

471 **Table 1.** Classification of all the proteins characterized into functional categories.

Functional categories (Nº)	**Proteins**						
Transport (43)							
	AcrA	AcrB	BtuB	CirA	CycA	ExbB	FadL
	FeoB	FepA	FhuA	FocA	FruA	GlpF	GlpT
	GltS	HemM	LysP	ManZ	MetQ	MsbA	MtlA
	NagE	NupC	PotE	PtsG	SdaC	SecD	SecF
	SecY	TamA	TolC	Tsx	UlaA	VacJ	YajC
	YajR	YbjY	YdjN	YedE	YeeF	YrbD	YrbK
	N.A.[a]						
Porins (9)							
	OmpA	OmpC	OmpD	OmpF	OmpN	OmpS	OmpW
	KdgM	LamB					
Cell envelope integrity and biogenesis (16)							
	BamA	BamB	BamC	BamD	BamE	FtsH	Lpp
	LppB	MipA	NlpD	Pal	SlyB	YbhC	YbjR
	YiaD	YidC					
LPS (5)							
	LptD	LptE	RfaL	WzzB	WzzE		
Electron transport (14)							
	AtpF	CydA	CydB	CyoA	CyoB	DmsA	DmsB
	NarG	NuoA	NuoH	NuoL	PntA	PntB	TrxA
Virulence (29)							
	FimD	FlgE	FlgG	FlgH	FlgK	FliC	FliD
	FliF	FliL	FljB	InvA	InvG	Mce	OmpX
	PagC	PagN	PagP	PagP	PrgH	PrgK	RcK
	SafC	SiiB	SiiC	SipA	SipB	SipC	SopB
	SopE						
Cellular responses (7)							
	CstAa	CstAb	Dps	EcnB	GroEL	RcsF	YeaY
Metabolism (7)							
	ApeE	Cdh	DgkA	FrdC	Gcd	PldA	Psd
Ribosome (23)							
	RplA	RplB	RplE	RplF	RplJ	RplM	RplN
	RplO	RplP	RplQ	RplR	RplT	RplU	RplV
	RplX	RpsB	RpsC	RpsD	RpsE	RpsI	RpsK
	RpsP	RpsS					
Other (13)							
	DcrB	Ftn	HflC	HflK	HybC	OsmE	PqiB
	RlpA	SppA	TraF	TraI	TraT	YeiU	
Unknown function (15)							
	LpxR	YajG	YbjP	YdgA	YdgH	YdiY	YecR-like
	YfaZ	YfeY	YgiB	YhcB	YibN	YijP	YnfB
	YraP						

472 [a] N.A., Not Available.

473

474 **Table 2.** Comparison of the predicted location by the PSORTb software and the information
475 published in the Uniprot database.

Bacterial location[a]	PSORTb v3.0		Uniprot		Common results[b]	
	Nº proteins	%	Nº proteins	%	Nº proteins	%
Inner membrane	66	36.3	16	8.8	15	8.3
Outer membrane	50	27.5	40	22.1	35	19.3
Membrane			45	24.9		
Cytoplasmic	30	16.5	3	1.7	26	14.4
Ribosome			23	12.7		
Extracellular	10	5.5	4	2.2	4	2.2
Periplasmic	2	1.1				
Cell wall			1	0.6		
Flagellum			8	4.4		
Nitrate reductase complex			1	0.6		
Unknown (N.A.)[c]	23	12.6	40	22.1	16	8.8

476

477 [a] Different bacterial locations are referred for each classification according to the information
478 supplied by each approach.

479 [b] Common results have only been specified for those common locations. Cytoplasmic and
480 ribosome categories have been unified in the comparison.

481 [c] N.A., Not Available.

482

5. Genomic and phenotypic comparison of *S.* Typhimurium isolates belonging to the sequence types ST19 and ST313

Additional Results

Brief introduction

Invasive nontyphoidal salmonellosis (iNTS) is the major cause of bacteremia in children from sub-Saharan Africa [20] and is associated to a case fatality of 20–25% in both children and adults [18]. Children with malnutrition, severe anemia, malaria or HIV are the population most susceptible to this bloodstream infection, in addition to HIV-infected adults [76]. The clinical manifestations involve a febrile systemic illness that may be indistinguishable from malaria or pneumonia, especially in young children, and is rarely accompanied by diarrhea [18].

A study conducted in 2009 by Kingsley *et al.* described that the isolates responsible for this invasive disease in Africa belonged to a new sequence type, named ST313 [76]. Genome sequencing revealed that isolates belonging to this newly described lineage shared particular features, such as the presence of pseudogenes, involved in host pathogen interaction. Interestingly, this genome degradation coincides with what is observed in the Typhi or Paratyphi A serovars suggesting adaptation to the human niche. Moreover, it was also seen that ST313 isolates carried a virulence plasmid derived from pSLT, called pSLT-BT, harboring a Tn*21*-like transposon encoding multiple antibiotic resistance genes [76]. Thereafter, a study was published by Okoro *et al.* in 2012 in which whole-genome sequencing of 179 S. Typhimurium isolates collected from different areas of the world distinguished a predominance of the sequence type ST313 in African isolates (93%) whereas ST19 was the most prevalent elsewhere (82%) [92].

The aim of the present study was to compare clinical isolates belonging to ST19 and ST313 in terms of adaptation to antimicrobial pressure. Moreover, our objective was also to identify specific genetic features related to the type of infection they caused (systemic

versus gastrointestinal disease), the sequence type to which they belonged and the geographical area where they were isolated.

Materials and Methods

Strains. Ten clinical strains of *S*. Typhimurium belonging to ST19 (n=5) and ST313 (n=5) from the Manhiça District Hospital (Manhiça, Mozambique) were studied. Among them, 7 caused iNTS disease as they were isolated from blood and belonged to the ST19 (B78, B52, B69 and B10) and ST313 (B65, B17 and B9) sequence types. The remaining 3 strains causing gastroenteritis were recovered from stool and were classified as ST19 (S15) and ST313 (S1941 and S1949). Two ST19 clinical strains isolated from stools of patients from the Hospital Clínic (Barcelona, Spain) named A7 and 50wt were also studied. The mutants B17-2, B9-4, S15-0.125, S1941-0.5 and A7-0.5 were generated from a multi-step selection process by exposure of the bacteria to doubling concentrations of ciprofloxacin (Fluka), starting at half of the MIC for each isolate (B17, S15 and S1941 at 0.016 mg/L; B9 at 0.25 mg/L and A7 at 0.008 mg/L), as described previously [210].

Antimicrobial susceptibility. Antimicrobial susceptibility to nalidixic acid, ciprofloxacin, ampicillin, ceftriaxone, chloramphenicol, tetracycline, trimethoprim and kanamycin was assessed using Etests (Biomérieux) following the manufacturer's recommendations. At least 3 replicates of each susceptibility test were performed.

Screening of mutations in the quinolone resistance-determining region (QRDR) of the target genes. Amplification of the QRDR of the target genes *gyrA*, *gyrB*, *parC* and *parE* was done by PCR using the previously reported primers [210]. Sequencing was then performed by Beckman Coulter Genomics (Essex, UK) and analyzed using the BioEdit® software (Ibis Biosciences, Carlsbad, CA) through alignment with the template sequence of *S*. Typhimurium LT2 (RefSeq NC_011294.1).

Real-time PCR. The relative expression of efflux components (*tolC* and *acrB*) and virulence-related genes (*invA*, *hilA* and *hilD*) was determined by RT-PCR as previously described [218]. The primers used have also been previously reported by our group [218].

Genome and plasmids sequencing. The Nextera XT DNA Library Prep Kit (Illumina) was used to generate 150 nt paired-end reads following the manufacturer's procedures. QC on raw sequences was checked using FASTQC (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc) and the samples were trimmed to a minimum phred-quality score of 20 and minimum read size of 10. Sequence alignment was made by BWA [219] using the following reference sequences: *S*. Typhimurium strain SL1344 (RefSeq NC_011294.1) and D23580 (RefSeq NC_016854.1) for ST19 and ST313 sequence types, respectively. Plasmid sequencing was also performed using the reference sequences for pSLT (NC_003277.1) and pSLT-BT (FN432031). Picard tools were used to identify and remove duplicate reads and for realignments. Samtools and VarScan 2.3.9 [220] were used for variant detections and Snpeff [221] for annotation. Visualization of segmental losses in the three samples against the SL1344 reference strain was obtained by alignment using the progressiveMauve software [222]. Home-made scripts were also used for the analysis.

Statistical analysis. The one-way ANOVA test was used for comparisons as data was normally distributed and the software IBM SPSS Statistics 20 was used.

Results

Characterization of the clinical strains. A collection of 10 clinical strains of *S*. Typhimurium isolated from Manhiça (Mozambique) and recovered from blood and stool samples of infected patients were first studied in terms of antimicrobial susceptibility. As shown in **Table 1**, among the strains from Mozambique no differences were detected regarding the sample origin (blood or stool) but a distinct antimicrobial susceptibility pattern was observed according to the sequence type.

Table 1. Antimicrobial susceptibility testing of the strains from Mozambique and the mutants derived.

ST Origin	Strain[c]	mg/L NAL[a]	CIP[a]	AMP[a]	CRO[a]	CHL[a]	TET[a]	TMP[a]	KAN[a]
ST19									
blood	B78	4	0.023	1	0.032	2	6	0.19	1.5
blood	B52	3	0.023	1.5	0.047	3	4	0.25	1.5
blood	B69	4	0.023	1	0.032	4	4	0.19	1.5
blood	B10	4	0.047	1	0.032	6	4	0.19	1.5
stool	S15	3	0.023	1	0.047	4	3	0.25	1.5
	S15-0.125	24	0.094	4	0.094	12	8	1.5	0.75
stool[b]	A7	4	0.016	>256	0.047	3	3	>32	1.5
	A7-0.5	24	0.19	>256	0.5	12	8	>32	1.5
ST313									
blood	B65	4	0.032	>256	0.047	>256	4	>32	2
blood	B17	3	0.032	>256	0.047	>256	4	>32	2
blood	B9	>256	0.5	>256	0.047	>256	3	>32	1.5
	B9-4	>256	3	>256	0.125	>256	8	>32	0.38
stool	S1941	3	0.032	>256	0.047	>256	4	>32	4
	S1941-0.5	>256	1.5	>256	0.19	>256	16	>32	1
stool	S1949	3	0.047	>256	0.047	>256	4	>32	2

[a]: NAL: nalidixic acid; CIP: ciprofloxacin; AMP: ampicillin; CRO: ceftriaxone; CHL: chloramphenicol; TET: tetracycline; TMP: trimpetoprim; KAN: kanamycin. / [b]: strain isolated at Hospital Clínic (Barcelona, Spain). / [c]: Strains in italics correspond to the *in vitro* mutants obtained.

Full susceptibility to all the antibiotics tested was observed for the strains belonging to the ST19 group, except for ampicillin (>256 mg/L) and trimethoprim (>32 mg/L) in the case of A7 (isolate from Barcelona). Similar MICs were reported for quinolones, ceftriaxone, tetracycline and kanamycin in the ST313 group, except for the strain B9, which was resistant to nalidixic acid (>256 mg/L). In this strain, sequencing of the QRDR region of the target genes reported an amino acid substitution at position 83 of GyrA (Ser83Tyr), explaining the nalidixic acid resistance. However, resistance to ampicillin (>256 mg/L), chloramphenicol (>256 mg/L) and trimethoprim (>32 mg/L) was seen for all ST313 isolates. Sequencing data revealed that strains S1941 and B17 harbored the MDR plasmid pSLT-BT described by Kingsley *et al.* [76] both presenting a fragment deletion of 1398 and 1253 nucleotides compared to the reference sequence (FN432031), respectively. These deletions caused the

loss of a putative integrase *rlgA* gene (SLT_BT091) and the truncation of another one (SLT_BT0281) but did not affect the antibiotic resistance genes. This plasmid contains a Tn*21*-like region that encodes, among others, the *dhfrI* gene, conferring trimethoprim resistance, the chloramphenicol acetyltransferase (*cat*) gene (chloramphenicol resistance) and the β-lactamase encoded by $bla_{TEM\text{-}50}$ (ampicillin resistance). This pattern of resistance goes along with previous results showing that a particular lineage of multidrug resistance (MDR) ST313 carrying an MDR plasmid is currently being disseminated throughout sub-Saharan Africa [92].

In vitro-selected mutants upon ciprofloxacin exposure. All the clinical strains were exposed to antibiotic pressure in order to identify potential differences regarding the virulence-related features of the different type of isolates. However, among the 10 clinical isolates, *in vitro* resistant mutants were only obtained from two ST19 *Salmonella* isolates causing gastroenteritis, one from Mozambique (S15) and one from Spain (A7); and two ST313 isolates from Mozambique recovered from blood (B9) and stool (S1941). Unfortunately, no mutants belonging to ST19 and causing invasive disease could be obtained. The isolates were exposed to increasing concentrations of ciprofloxacin and the mutants selected for the study corresponded to those able to grow at the highest concentration tested of this drug. Antimicrobial susceptibility was assessed and revealed an increase in the MIC values between the original strains and the mutants, as shown in **Table 1**. Resistance to nalidixic acid was almost achieved by all four mutants (≥ 24 mg/L) and corresponded to an increase of 6-8-fold for A7-0.5 and S15-0.125, belonging to sequence type ST19, and a >85-fold increase for the ST313 mutant S1941-0.5. In the case of B9-4, no changes were recorded for this drug as the parental strain was already resistant to nalidixic acid. Accordingly, the MIC of ciprofloxacin increased from 4-12-fold (S15-0.125, B9-4 and A7-0.5) to almost 47-fold in S1941-0.5. Screening of mutations in the quinolone target genes revealed the acquisition of an amino acid change Asp87Tyr in GyrA in the mutant S1941-0.5 and in Glu466Asp in GyrB for the A7-0.5 strain. On the contrary, no change was detected in the target genes of the mutants B9-4 and S15-0.125 compared to their parental strains.

Regarding the remaining antibiotics tested, a similar pattern was found in strains belonging to both ST19 and ST313 sequence types for ceftriaxone and tetracycline. Despite remaining susceptible, an increase in the susceptibility of these antibiotics ranging from 2- to 4-fold was detected for all the mutants except for A7-0.5, for which the MIC of ceftriaxone increased almost 10-fold reaching 0.5 mg/L. In the case of ampicillin, chloramphenicol and trimethoprim, no differences were reported in the ST313 isolates as the original strains were already resistant to these antibiotics. Among ST19 isolates, only the S15-0.125 mutant reported an increase in trimethoprim (6-fold) and in ampicillin (4-fold), as the clinical strain A7 was also resistant to these drugs; whereas the MIC of chloramphenicol increased 3- and 4-fold for S15-0.125 and A7-0.5, respectively, reaching 12 mg/L in both cases. Regarding kanamycin, no major increase in the MICs were reported in any case.

In order to evaluate the contribution of the major efflux system AcrAB/TolC in the reduction of antimicrobial susceptibility of all the paired isolates (clinical isolate *versus in vitro*-selected mutant), we evaluated the gene expression of *acrB* and *tolC*, as shown in **Table 2**.

Table 2. Relative gene expression (RQ) of the efflux components and virulence genes of the isolates and their derived mutants.

ST and sample origin	Strain	Relative gene expression (RQ)				
		Efflux-related genes		Virulence-related genes		
		acrB	*tolC*	*invA*	*hilA*	*hilD*
ST19 stool						
	S15	1	1	1	1	1
	S15-0.125	3,26**	2,93	-1,60*	-1,2	1,13
	A7	1	1	1	1	1
	A7-0.5	1,79	-1,43*	-2,22*	2,69*	-1,47
ST313 stool						
	S1941	1	1	1	1	1
	S1941-0.5	4,75	3,27*	-1,92	-1,47	-1,6
ST313 blood						
	B9	1	1	1	1	1
	B9-4	3,77	3,97*	2,01	4,23	1,56

*$p < 0.5$; **$p < 0.01$

An enhancement of the transcription levels of *acrB* and *tolC* was observed for all the mutants compared to their susceptible counterpart, ranging from 2.9- to almost 5-fold overexpression except for A7-0.5. In this strain, a modest 1.79-fold increase was found in the gene expression of *acrB* and a slight, albeit significant decrease was reported in the expression of *tolC* (RQ=-1.43).

Impact of quinolone resistance acquisition on virulence. In *Salmonella*, the acquisition of quinolone resistance has frequently been associated with a decrease in virulence features [189,210–212,223,224]. To evaluate if the decrease in quinolone susceptibility reported in the mutants had a differential effect on virulence depending on the origin and sequence type to which they belonged, expression of the SPI-1 encoded key virulence genes (*hilD*, *hilA* and *invA*) was determined by RT-PCR. In the present work, however, no clear pattern was observed, as shown in **Table 2**. A significant 2.69-fold overexpression was reported for *hilA* in the A7-0.5 mutant, although *invA* and *hilD* were both repressed by 2.22-fold and 1.47-fold ($p>0.05$), respectively. A different situation was seen for B9-4 for which transcription was activated in the three genes; the highest expression levels were seen for *hilA* (RQ=4.23) followed by *invA* (RQ=2.01), and a modest 1.56-fold increase was reported for *hilD*. This strain was derived from B9, an isolate belonging to the ST313 sequence type and having caused an invasive salmonellosis. In the case of S1941-0.5, a modest repression of these genes was observed, ranging from -1.47-fold repression for *hilA* to -1.92-fold for *invA* compared to the parental strain. Lastly, inconclusive results were obtained for the S15-0.125 mutant, as only *invA* showed a modest but significant reduction in the expression levels (RQ:-1.60) compared to the wild-type strain.

Genomic comparison between different sequence types, sample origin and geographic area. A genome sequencing approach was also performed with the aim of identifying common patterns that could provide new information to understand the specific features between sequence types as well as the pathogenesis and geographical origin. To do so, genome sequencing of a selection of 6 strains from the initial collection was performed. The strains chosen were B52 (ST19) and B17 (ST313), both isolated in Mozambique from blood, strains S15 (ST19) and S1941 (ST313) recovered from stools in the same clinical setting, and

the Spanish isolate 50wt (ST19), causing gastroenteritis. Our analysis was focused on the identification of genes that were annotated as having important modifications in the nucleotide sequence leading to the alteration of the gene, such as premature stop codons or frameshift mutations presumably causing the loss of the gene function. Thus, we considered these genes as putative pseudogenes. Moreover, identification of deleted/inserted regions along the genome was also investigated.

The results obtained from the comparison of the gene sequences did not identify any important differential alteration when comparing strains recovered from blood and stool belonging to the ST313 sequence type. However, this was not the case for the strains belonging to ST19. As shown in **Table 3**, great variability was observed in terms of gene integrity among this group.

Table 3. Classification of the genes found to be importantly altered among the different groups of strains belonging to the sequence type ST19.

Groups	Gene ID	Description
ST19-specific		
	leuC2	isopropylmalate isomerase
	dacC	D-alanyl-D-alanine carboxypeptidase
	fimH	Type 1 Fimbrial Adhesin
	glpT	glycerol-3-phosphate transporter
	katE	Catalase HPII
	rhaS	L-rhamnose operon regulatory protein
	SL1344_0703	galactosyltransferase
	SL1344_0708	putative glycosyl transferase
	SL1344_0741	LysR family transcriptional regulator
	SL1344_0967	putative bacteriophage lysozyme
	SL1344_1528	gamma-aminobutyraldehyde dehydrogenase
	SL1344_2174	putative hydrolase
	SL1344_2556	bacteriophage tail protein
	SL1344_3663	glucarate transporter
	SL1344_4051	inner membrane protein
	SL1344_4465	transcriptional regulator

Stool-specific		
	*avrA**	avirulence determinant of plant pathogenic bacteria
	lpfC	outer membrane usher protein
50wt-specific [a]		
	atpI	ATP synthase protein I
	creC	two-component sensor kinase
	fimC	fimbrial chaperone protein
	gabP	GabA permease (4-amino butyrate transport carrier)
	nfnB	oxygen-insensitive NAD(P)H nitroreductase
	nupG	nucleoside permease nupg (nucleoside-transport system protein)
	ordL	putative oxidoreductase
	phnV	membrane component of 2-aminoethylphosphonate transporter
	rhtC	threonine efflux protein
	siiC	putative type-I secretion protein
	SL1344_2152	putative n-hydroxybenzoate hydroxylase
	SL1344_2587	bacteriophage protein
	SL1344_4023	putative ABC transporter ATP-binding protein
	SL1344_4418	superfamily I DNA helicase
	sspH2	secreted effector protein
	yhjN	putative polysaccharide biosynthesis protein subunit B
S15-specific [b]		
	glpT	glycerol-3-phosphate transporter
	sgaT	putative transport protein SgaT
	SL1344_1938	bacteriophage tape-measure protein
	ttrB	tetrathionate reductase subunit B
	xylB	xylulose kinase

[a] Isolate recovered from stool (Barcelona, Spain); [b] Isolate recovered from stool (Manhiça, Mozambique); *gene sequence different from the ST19 reference strain SL1344 but identical to the ST313 reference strain D23580.

Sixteen putative pseudogenes were identified as being commonly found in all isolates from ST19 sequence type, as they were detected in both stool and blood isolates regardless of their origin. However, the only gene directly linked to virulence was *fimH*, encoding for the adhesive subunit of the type-1 fimbria [34,46]. In this gene, a single nucleotide

substitution at position 238 (according to the reference sequence of SL1344) resulted in a premature stop codon, and thus the truncation of the gene. Interestingly, the genes *lpfC* and *avrA*, related to virulence in *S*. Typhimurium, were altered in S15 and 50wt, both of which were recovered from stool, and caused a frameshift variant, with the disruption of the translational reading frame due to the alteration of the number of nucleotides. In the case of *lpfC*, a single nucleotide insertion (+T) at position 2,297 was reported for S15 and a single deletion (-T) at the same position was seen for 50wt; regarding *avrA*, an insertion of three nucleotides (+AAA) was detected at position 460 in both strains.

Furthermore, differences were also reported depending on the geographical area in which they were isolated. Fifteen genes were only found to be altered in the 50wt isolate from Spain and corresponded to the insertion or deletion of a single nucleotide within the encoding region. Among these genes, we found the chaperone-encoding gene *fimC* which is part of the operon *fimAICDHF*, responsible for the formation of the type-1 pilus system that mediates adhesion to the host cell [34,46]. Additionally, the *sspH2* gene, a SPI-2 effector protein involved in modulation of the innate immune response [225] and the *siiC* gene, encoded in the SPI-4, were also altered in this strain. This latter gene was reported to be part of the type I secretory system, allowing the secretion of the adhesin SiiE for the attachment to epithelial cells [226]. Lastly, the nucleoside permease *nupG* was also highlighted, as it has been reported in *S.* Typhi to be involved in the transport of nucleosides that serve as precursors for nucleic acid synthesis during the intracellular state of the pathogen [227]. According to the isolate S15, from Mozambique, only 4 genes were seen to be putatively altered in this particular strain. They consisted in the tetrathionate reductase subunit B (*ttrB*), encoded in SPI-2 and involved in anaerobic respiration [228], the xylulose kinase (*xylB*) involved in the xylose metabolism [229], and two other genes, *sgaT* [230] and SL1344_1938 for which no information was available.

To further analyze possible differences in terms of sequence integrity across the genome, strains from each sequence type were aligned with their reference strain and deleted and inserted regions were screened. Among ST313 isolates, no differences were seen compared

with their reference strain D23580; however, in strains belonging to ST19, differences were observed depending on the origin of the sample as shown in the following figure:

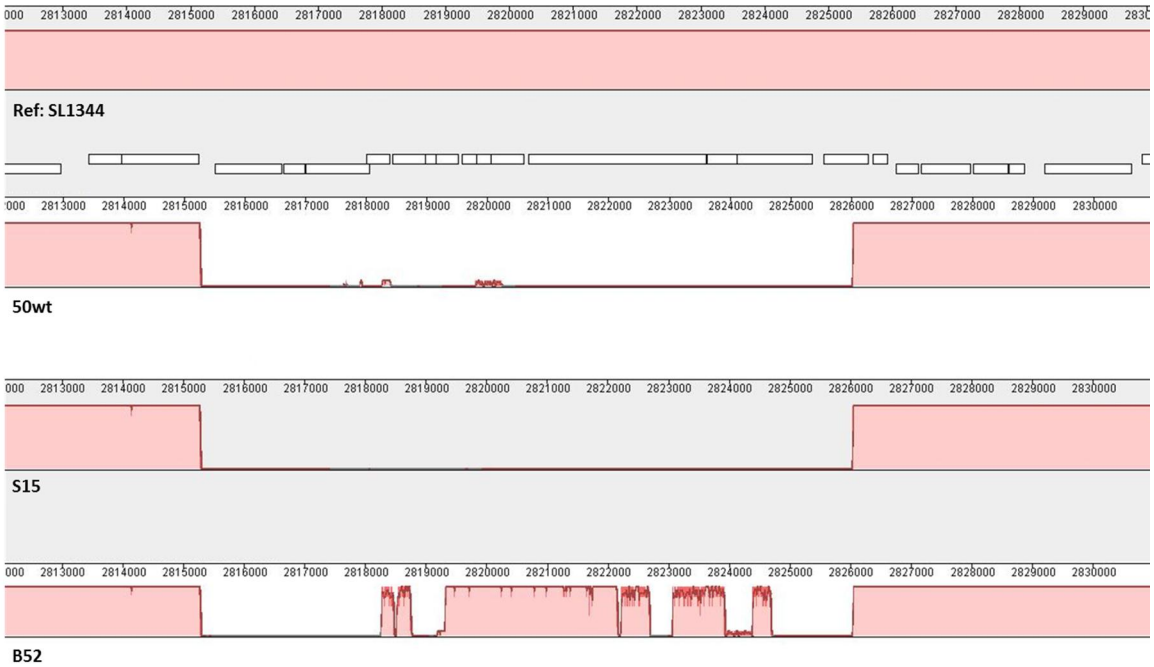


Figure 1. Amplification of the genome region where differences were detected between the two ST19 strains recovered from stool (50wt and S15) and from blood (B52). The first sample corresponds to the ST19 reference strain SL1344. Numbers indicate the nucleotide positions across the genome and pink regions represent alignment of the sequence with the reference strain.

In isolates from stool (50wt and S15), the region including the genes from position 2819480 to 2823573 from SL1344, was deleted, whereas a perfect alignment was observed in the case of the blood derived isolate B52. Among the 4 genes deleted, 3 were unknown (annotated as coding for “hypothetical proteins” in 2 cases, and one “replication protein”) and the other encoded for a “phage tail assembly protein”. Information regarding these genes is scarce and thus, we cannot link the presence/absence of these genes with the differences in pathogenicity they were found to cause.

Altogether, the results obtained in this work suggest the presence of particularities in terms of gene integrity and virulence features among isolates depending on their sequence type (ST19 and ST313), the type of disease they cause and the geographical region in which they were isolated. However, further studies including a larger number of strains are needed

in order to confirm these findings and allow better understanding of the differences in the pathogenesis across the *S*. Typhimurium serovar.

DISCUSSION

V. DISCUSSION

In the last part of this PhD thesis the results have been discussed considering the following sections:

5.1 Incidence of quinolone resistance

Salmonellosis is an important cause of gastrointestinal disease worldwide for which the major etiological agents are *S*. Enteritidis followed by *S*. Typhimurium [16,17]. Antimicrobial resistance, mainly regarding fluoroquinolones and cephalosporins, is of special concern as an increase has been described in the incidence of isolates causing antibiotic treatment failure in the last years [231,232]. To date, *S.* Typhimurium has been reported to be more prone to the acquisition of antimicrobial resistance than *S.* Enteritidis, as demonstrated by the emergence of MDR clones [132,233]. However, *S*. Enteritidis isolates showing antibiotic resistance, mostly to quinolones, have recently been reported [234–237]. Resistance to the quinolone nalidixic acid is associated with a decreased susceptibility to fluoroquinolones, including ciprofloxacin, giving rise to strains with a DCS (decreased ciprofloxacin susceptibility) phenotype. Percentages ranging from 10-13% of *S*. Typhimurium clinical isolates reporting ciprofloxacin resistance or DCS have been published in several studies [132,233,238]. In the case of *S*. Entertidis, a lower proportion of ciprofloxacin resistance has been reported, ranging from < 1% to 8% [132,238].

In the collections of clinical isolates studied in Papers I and III we report that all the isolates belonging to either of these two serovars (*S*. Typhimurium or *S.* Enteritidis) were susceptible to ciprofloxacin (MIC ≤0.75 μg/mL). On the contrary, strains resistant to nalidixic acid (MIC ≥32 μg/mL) were only seen among the Enteritidis serovar. The proportion of *S.* Enteritidis resistant isolates ranged from 38.6% (39 out of 101 isolates, in Paper III) to 84.2% (16 out of 19 isolates, in Paper I). In spite of reports in the literature describing that *S*. Typhimurium is more frequently found as being resistant than *S.* Enteritidis, as above mentioned,these two papers indicate the opposite situation. A possible explanation could be related to the geographical area of study; as *S*. Enteritidis is the major cause of salmonellosis in Europe [239] and the strains studied in Papers I and III were isolated in Spain. Moreover,

regardless of the serovars, our findings support the current scenario observed in Europe in which resistance to nalidixic acid in *Salmonella* has been increasing over time. As stated in previous reports, the percentage of resistance to nalidixic acid has risen from 0.8% in 1995 to 8.5% in 2000 in Denmark [237], and from 14% in 2000 to 20% in 2004 in Europe [129]. Outside Europe, however, a general increase in nalidixic acid resistance has also been reported in different studies, indicating a global trend. A study from Brazil revealed an increase from 19% to 24.3% of nalidixic acid-resistant strains of *S*. Enteritidis isolates involved in foodborne salmonellosis outbreaks during 2001 and 2002 [240], and 18.5% of the clinical isolates from a study of Korea were also resistant to this drug [236].

In *S*. Typhi the percentage of isolates resistant to nalidixic acid is also of great concern: values ranging from 44-59% have been reported in Vietnam, India and Pakistan in the period of time from 2002-2004 [129]. Moreover, the incidence of strains showing a DCS phenotype has reached extremely high values (>90% in India) as has the emergence of MDR strains (87% in Nigeria, 74% in Vietnam and 65% in Pakistan) in a similar period of time (2002-2006) [129]. Thus, it is not surprising that the *S*. Typhi clinical isolate studied in Paper IV already presented nalidixic acid resistance.

5.2 Mechanisms of quinolone resistance: target gene mutations

Concerning the acquisition of quinolone resistance, the principal mechanism of resistance in Enterobacteriaceae is through point mutations in the QRDR (quinolone resistance-determining region) of the target genes *gyrA*, *gyrB*, *parC* and *parE*. These genes encode for the A and B subunits of the DNA gyrase (*gyrA* and *gyrB*) and DNA topoisomerase IV (*parC* and *parE*) [115]. Screening of these regions was carried out in the collection of *S*. Enteritidis isolates studied in Paper I and the results revealed a single mutation in *gyrA* in all nalidixic acid-resistant isolates. This mutation consisted of an amino acid substitution at position 87 (D87Y) in all but one strain, for which the position affected was 83 (S83F). This latter mutation was also seen in the *S*. Typhi clinical isolate studied in Paper IV. These results are in accordance with the information mainly reported in the literature: mutations in *gyrA* are usually the first type of target gene mutations acquired (either in residue S83 or D87) conferring resistance to nalidixic acid and a DCS phenotype in clinical isolates [70,164]. The

results obtained from the clinical study of Paper I, showing the predominance of the amino acid substitution D87Y in GyrA, suggest that the dissemination of a particular clone could have occurred. In order to investigate this possibility and to evaluate the genetic relationship among the isolates belonging to *S*. Enteritidis and *S*. Typhimurium serovars, two different approaches were used: MLST and PFGE. Even though we performed a PFGE with 2 different restriction enzymes (XbaI and BlnI), no differences were seen in the restriction band pattern obtained of the *S*. Enteritidis isolates, and no better results were detected in this serovar with the MLST technique. Hence, these methods did not allow to confirmation that a predominant clone harboring the *gyrA* mutation was circulating in our clinical setting. These results are in accordance with previous studies in which *S*. Enteritidis is seen to be much more clonal than *S*. Typhimurium [100]. Thus, this work demonstrates the need to implement new tools with a higher discriminatory power to differentiate homogenous populations, as is the case for *S*. Enteritidis. Additionally, in our study we demonstrated that PFGE is a much more discriminatory tool than MLST concerning *S*. Typhimurium.

According to the type of QRDR mutations and the order in which they were acquired, differences were seen between clinical isolates compared to *in vitro*-selected mutants. Although mutations are usually first acquired in *gyrA,* in the *S*. Typhimurium mutant 59-0.03 from Paper III as well as in mutant A7-0.5 from Additional Results, the first change was found in the GyrB subunit affecting residue 466 (E466D). In both strains, this change conferred the same 6-fold increase in the MIC value of nalidixic acid whereas a 4- and 11.8-fold increase was observed in the MIC of ciprofloxacin for 59-0.03 and A7-0.5, respectively. Although this amino acid change has previously been reported by O'Regan *et al.*[186] in *S*. Enteritidis, in their study the change occurred together with modifications in other residues in *gyrA* or *parE* or in both of them, contrary to what was seen in our strains. However, in another study reported by Song *et al.* this modification was also seen alone in a clinical isolate of *S*. Typhi [241].

Two additional changes were acquired in 59-2, one in GyrA (S83Y) and one in ParC (S80R), reflecting a 64-fold increase in the MIC of naldixic acid and a >15-fold increase for ciprofloxacin. Finally, the most resistant 59-64 mutant gained a second amino acid

substitution in both GyrA (D87G) and ParC (F115S). In this strain, the MIC of nalidixic acid did not increase above the levels of 59-2 (MIC: 8,192 μg/mL) probably because of having attained a plateau. Amino acid changes within GyrA (S83Y and D87G) and ParC (S80R) were found at subsequent steps in the resistance process and are frequently detected in the literature [164]. Regarding the mutation affecting codon 115 of ParC which was first reported in this study, its contribution to the resistance phenotype could not be completely elucidated since it was acquired concomitantly with the second amino acid change in GyrA.

Considering the results of both studies, a possible explanation for the non-classical acquisition of mutations could be attributed to a random selection of the mutants, suggesting that at the initial steps of selection other mutants probably acquired these commonly described mutations, such as in *gyrA*, despite not being selected. However, this hypothesis is not supported by the results obtained from the alternative mutants selected and studied in Paper V (59-mut1 and 59-mut2) which derived from the same 59-wt strain. In this case, both 59-mut1 and 59-mut2 also harbored a single same amino acid substitution, the same as that reported in GyrB for 59-0.03 from Paper III and A7-0.5 from Additional Results (E466D).

However, although the role of target gene mutations has been extensively demonstrated in the literature, it is not the only mechanism conferring quinolone resistance. In the Results section different situations have been described for the *S.* Typhi *in vitro*-selected mutants (Ty_c1 and Ty_c2, in Paper IV), the *S*. Typhimurium 60-wt derivatives (60-mut1 and 60-mut2, in Paper V) and strains B9-4 and S15-0.125 from Additional Results. None of these mutants acquired any mutation in the QRDRs, although they did present an increase in the MICs of the antibiotics tested, including quinolones (nalidixic acid, ciprofloxacin and norfloxacin) and other unrelated compounds (chloramphenicol, tetracycline, erythromycin, amoxicillin, ceftriaxone, cefoxitin, ampicillin and trimethoprim). In these cases, the contribution of efflux in the acquisition of resistance seemed to be evident.

The situation observed in these mutants is in accordance with the idea that the first step in quinolone resistance acquisition among *S. enterica* is due to efflux overexpression [179,242].

5.3 Mechanisms of quinolone resistance: increased efflux and decreased membrane permeability

The first step in the quinolone resistance acquisition process among *S. enterica* has been reported to be due to efflux enhancement. The resulting antibiotic extrusion can also be further increased at subsequent steps in the resistance process to trigger a MDR phenotype. As a result, the contribution of efflux can be found in the absence of target gene mutations or in combination, showing then a synergic contribution of both mechanisms as reported in several studies [179,242]. Involvement of efflux can be evaluated through several approaches, such as the determination of the MICs of several unrelated antibiotics since most are substrates for efflux pumps [243], the assessment of the same MICs in the presence of the efflux pump inhibitor PAβN [164], and analysis of the intracellular accumulation of an exportable drug, such as ciprofloxacin [173].

The effect of PAβN was studied in the analysis of the MIC of nalidixic acid in the clinical isolates of Paper I. The results showed a decrease ranging from 1.5- to 10-fold among nalidixic acid-susceptible strains and a greater decrease attaining 64-fold in the resistant strains. However, determination of ciprofloxacin accumulation did not reveal any differences due perhaps to the high susceptibility to ciprofloxacin even for the nalidixic acid-resistant strains. In the case of the study of the 59-wt and its derivative mutants of Paper V, in comparison with 59-mut1, 59-mut2 had the same QRDR profile but showed higher MIC values for most of the compounds. In the analysis of the derivative mutants of Paper III, all three approaches were carried out and indicated that increased efflux occurred particularly in those isolates which did not acquire target gene mutations (e.g. 59-0.06, 50-0.25 and 59-16). Our results are in accordance with previous reports in which this situation was also observed. Clinical strains showing nalidixic acid resistance and DCS, both accompanied or not by the mutations in the target genes, as well as decreases in the susceptibility of unrelated compounds, were demonstrated to have increased efflux [186,242]. Moreover, in these studies as well as in our results, efflux was also present in susceptible isolates.

The efflux system most widely found to be related to antibiotic resistance is AcrAB/TolC [173,242,244]. Nonetheless, several studies have reported that strains from *E. coli* and *S.*

Typhimurium with an inactivated *acrB* compensate for this by overexpressing *acrEF* [245–247]. Likewise, efflux systems such as AcrEF and also EmrAB have been reported to contribute to antibiotic extrusion [179,247]. Thus, these efflux systems were investigated across the different studies discussed in this chapter.

Surprisingly, although the contribution of efflux was phenotypically demonstrated in Papers I, III, IV and V as mentioned previously, this could not be confirmed in the first two papers (I and III) and only in part in Papers IV and V. Gene expression of the efflux components *acrB*, *tolC*, *emrB* and *acrF* was tested for the collection of clinical strains of Paper I; however, all these genes were found to be unchanged among the isolates. In view of these results, we suggest that unknown efflux systems likely exist and play a role in drug resistance in *Salmonella*. Regarding the 59-wt parental strain (results presented in Papers III and V), AcrA could not be the efflux component responsible for antibiotic extrusion in any of its derivative mutants as a deletion within the *acrA* gene was deduced to trigger a truncated protein. On the contrary, *tolC* was clearly overexpressed (>2.3-fold) not only in 59-64 (also supported at the protein level), but also in the other set of *in vitro*-selected mutants, 59-mut1, 59-mut3 and 59-mut2, the values of which were particularly relevant (5.4-fold) (Paper V). As mentioned previously, it has been reported that other efflux components can compensate the role of *acrB* in *S*. Typhimurium mutants lacking this gene [180,247]. Thus, we next assessed the *acrF* and *emrB* expression values of the alternative efflux components in our strains. In the first case, *acrF* remained virtually unchanged in 59-64 (Paper III), 59-mut1 and 59-mut2, whereas it was suggested to play a role in 59-mut2, as expression increased 6-fold compared to the parental strain (Paper III). In the case of *emrB* expression, it was not affected in the three mutants of Paper V but was not screened in the 59-64 mutant (Paper III), and hence, its contribution cannot be ruled out.

On the other hand, *acrB* overexpression was positively identified in the 60-wt derivatives also analyzed in Paper V. Both *acrB* and *tolC* were consistently overexpressed, with an increase globally ranging from 5.2- to 9.5-fold. In addition, *acrF* was tested and seemed to contribute to efflux in 60-mut2, since there was an increase in its expression of almost 5-fold. The mutants S15-0.125, B9-4 and S1941-0.5, presented as Additional Results, also

showed increased transcriptional levels of both *acrB* and *tolC* along with an increase in the MICs to different antibiotics.

Regarding our study carried out in *S*. Typhi (Paper IV), outer membrane protein analysis revealed overexpression of AcrB as well as TolC in Ty_c2, which was also seen at a transcriptional level. To our knowledge, this is the first demonstration of the contribution of AcrAB/TolC in this serovar. However, *emrB* and *acrF* were repressed in this mutant. This repression could be due to a compensatory regulation: when overexpression of AcrB takes place, other redundant efflux components such as *emrB* and *acrF* are not needed, and thus, remain transcriptionally inactive. This has been demonstrated by Blair *et al.* for the RND efflux pump genes [180]; in this study a deletion of *acrB* was linked to the overexpression of *acrF*, demonstrating the interregulation of these efflux components. Unfortunately, the contribution of efflux in Ty_c1 could not be demonstrated as the levels of all the above-mentioned efflux components remained unchanged; however, the increased MICs values seen for a great part of the antibiotics tested suggest the contribution of unknown extrusion systems.

Thus, altogether these results indicate that in all the studies in which AcrAB was not seen to contribute to efflux, the transcriptional levels, and in some cases protein production, of *tolC* were increased, except in Ty_c1 from Paper IV and the clinical collection of nalidixic acid resistant isolates from Paper I. These latter results are in agreement with data by O'Regan *et al.* [186], in which the MIC of a strain resistant to nalidixic acid decreased in the presence of PAβN but remained unchanged when *acrB* was deleted suggesting the possible contribution of undisclosed efflux pumps. Moreover, only in the 59-mut2 and 60-mut2 mutants was the alternative AcrEF pump system likely to be involved. In the remaining isolates characterized, the efflux partner of TolC was not identified, suggesting that unraveled proteins implicated in this resistance mechanism need to be discovered.

In an attempt to identify new proteins involved in the extrusion of antibiotics, a putative outer (*ydiY)* and three inner (*ybdJ*, *STM1441* and *ynaJ)* membrane proteins were studied in the Manuscript. Unfortunately, the results were not conclusive and their role could not be elucidated.

Possible reasons to explain the lack of differences regarding antimicrobial susceptibility may be that they play a minor role in efflux, and thus, alterations of these proteins may not be sufficient to observe a relevant phenotype; or they may not be related to virulence and/or antimicrobial resistance, but are rather a collateral effect derived from adaptation of the bacteria to antibiotic pressure.

Although investigation in this field is currently ongoing, additional efforts are needed in order to increase the knowledge of quinolone resistance mechanisms and to anticipate the emergence of *Salmonella* strains with novel resistance mechanisms.

The contribution of membrane permeability to the multidrug resistance profile was also evaluated in Papers IV and V. In Paper IV, the mutant Ty_c2 presented alterations of the porin content, as reduced protein levels of OmpC and a downregulation of the *ompF* gene. In Paper V, decreased *ompF* expression was also observed for all the mutants obtained. Although alterations in the membrane permeability through a decrease in porin expression has been previously reported in *E. coli* and non-Typhi *Salmonella* isolates [186,210,248,249], to our knowledge this is the first demonstration in the Typhi serovar.

5.4 Mutations in the regulatory genes related to multidrug resistance

In an attempt to investigate the regulation of quinolone resistance, the global regulators (MarA, SoxS and RamA) that activate transcription of AcrAB/TolC as well as the local repressor of the *acrAB* genes (AcrR) [115,174,183,184] were examined. Taking into account all the results obtained in this chapter, the most significant changes were found to affect the levels of *ramA* (in the case of *S*. Typhimurium, Papers III and V) and *marA* (in the case of *S*. Typhi, Paper IV).

Our findings concerning the regulation of quinolone resistance in *S*. Typhimurium are defined and compared in Paper V. Sequencing of both the *ramR* gene and the *ramA* promoter showed that all 59-wt derivatives acquired different changes in this region and, consequently, overexpression of the *ramA* gene was always seen in these mutants. Deletions and point mutations within *ramR* (59-mut1, 59-mut3 and 60-mut1) were associated with intermediate levels of *ramA* (13.4 to 19.6-fold) as well as a point mutation in the *ramA*

promoter (59-64); however, important deletions in the promoter region of *ramA*, seen in 59-mut2 and 60-mut2, led to the highest levels of *ramA* (66- and 74.2-fold, respectively). As reported previously, RamR exerts its repressive effect through binding as a homodimer to two RamR binding sites located in the *ramA* promoter region [250]. Thus, modification of these regions, as is the case in 59-mut2 and 60-mut2, may avoid this interaction and consequently a constitutive expression of RamA may occur. Previous reports are in agreement with our findings, in which mutations or gene interruptions within *ramR* or in the *ramA* promoter, in both antibiotic resistant clinical and *in vitro*-mutants have also been detected [186,188,190].

The results obtained in these two latter mutants together with their above-mentioned levels of *acrEF* are in accordance with previous studies. Bailey *et al.* observed that overexpression of *acrEF* was linked to high levels of *ramA* transcription [251]; furthermore, another study showed that deletion in the *ramA* promoter was associated with *acrEF* overexpression [252].These observations suggest that this effect only occurs in a concentration-dependent manner, as a similar response dependent on the concentration of the activator has also been previously reported for MarA in *E. coli* [251,253]. This would mean that only important levels of expression (>65-fold), such as those seen in 59-mut2 and 60-mut2 strains are able to enhance AcrEF expression, whereas intermediate values (13- to 20-fold), would not have the same effect.

Our results indicate that a similar situation may occur regarding the link between *ramA* and *marA,* suggesting that a certain concentration of *ramA* is needed to activate *marA* transcription. It has been stated that *ramA* binds the marbox located in the *marA* promoter region, thus triggering activation of this regulator [254]; however, it has been previously reported that an *in vitro* ciprofloxacin-resistant *S*. Enteritidis isolate showing increased levels of *ramA* (33.7-fold compared to the parental strain) presented unchanged *marA* levels [186]. Our results obtained in Paper V are in accordance with the concentration-dependent hypothesis as mutants showing a >65-fold increased expression of *ramA* (59-mut2 and 60-mut2) also presented increased levels of *marA* (4.3- and 3.6-fold, respectively).

Similar to the above-mentioned link between RamA and MarA, the transcriptional activator SoxS has also been reported to bind to the marbox located in the *marRAB* promoter region [185]. Thus, our data support this statement since *soxS* overexpression (>4-fold) was seen in the 59-64 mutant in Paper V along with increased levels of *marA*.

The regulatory mechanisms of quinolone resistance were also investigated in the case of *S*. Typhi (Paper IV) and revealed an important deletion in the Ty_c2 mutant, affecting the local MarA repressor (*marR*) and its upstream region. The presence of two binding sites (Site I and Site II) for MarR has been previously reported in the *marO* region in *E. coli* [255]. In Ty_c2, most of the *marR* gene was deleted as were 34 nucleotides upstream of the gene, affecting the MarR binding site II but not the binding site I and the -35 and -10 regions of the promoter. Subsequently, a >100-fold overexpression of *marA* was also observed. These results are in agreement with previous observations in which mutations within *marR* caused overexpression of *marA* in *E. coli* isolates [191]. However, this has never been demonstrated before in *Salmonella*. Hence, as far as we know, the present study demonstrates the local regulation of *marA* for the first time in this genus. Besides, *marA* overexpression was associated with overproduction of the AcrAB/TolC efflux system and repression of the expression levels of *ompF* in the same MDR mutant. Although both overexpression and deletion of *marA* have been reported to affect the antimicrobial susceptibility profile in *S*. Typhimurium [179] and *S*. Enteritidis [186], this has not yet been shown in *S*. Typhi. Thus, Paper IV describes this situation for the first time.

The results discussed in this chapter allow a better understanding of the regulatory mechanisms related to the MDR phenotype as well as the reinforcement of previous findings. In this context, the demonstration of the regulation of *tolC* and *acrF* mediated by *ramA* in Paper V confirms previous data [251,252]. Moreover, the investigations performed in *S*. Typhi increase the current knowledge associated with MDR in this clinically relevant serovar.

Nevertheless, more studies are needed in order to clarify the interrelations present between this complex regulatory network.

5.5 Bacterial growth and quinolone resistance

Another interesting feature studied in this block of results has been the fitness cost associated with the acquisition of antibiotic resistance. In Paper III, strains 59-16 and 59-64 (the most resistant mutants) had significant growth impairment compared to the previous strains, showing a longer lag phase before exponential growth. These findings are in accordance with previous results in which a correlation has been seen between the selection of fluoroquinolone resistance and impaired fitness [210,211,223,256]. Different theories have been proposed to support this fact: i) acquisition of certain mutations in the topoisomerase genes alters their activity affecting replication and thus, bacterial growth [257,258]; ii) overexpression of MDR efflux pumps also extrudes the nutrients and metabolic intermediates needed for adequate growth [259]. In the case of Paper III, fitness alteration could not be attributed to mutations in the target genes as 59-16 and its previous mutant (59-2), which showed a normal growth rate, showed the same genotype. However, an increase in the efflux activity was seen in 59-16 compared to the previous mutant, suggesting that this could be the reason for the growth defect seen in the two more resistant isolates.

In contrast with these results, the *S*. Typhi resistant isolates, Ty_c1 and Ty_c2, studied in Paper IV did not show this feature, as both strains presented the same growth rate as the parental strains. The moderate levels of resistance of Ty_c1 could explain the unaltered fitness in this strain; however, Ty_c2 showed increased efflux activity and an MDR phenotype unrelated to fitness impairment, hence contradicting the above-mentioned theories. On the contrary, a recent study performed in *S.* Typhi considered the amino acid change S83F in the QRDR of GyrA as an advantageous change leading to fitness benefits [260]. The presence of this change in the three strains studied in Paper IV may explain the unchanged growth observed. It is noteworthy to highlight that *S*. Typhi mutants showing the highest MICs for the antibiotics tested did show fitness impairment; which is why these mutants were not studied further. Considering these results, we suggest that a certain degree of efflux activity, at least above the levels seen in Ty_c2, are needed in order to affect the growth rate by extrusion of a certain concentration of molecules required for this process.

In spite of presenting a growth defect, 59-64 was grown in medium without ciprofloxacin and the strain recovered after 50 passages did not present any compensatory mutation and had the same MDR profile as 59-64; this was also the case for Ty_c2 for which 35 consecutive passages were performed without success. The inability to select for reversion has also been reported in a previous study in which no revertants were obtained from a highly ciprofloxacin-resistant mutant (MIC>32 μg/mL) after 200 passages in an antibiotic-free medium [211]. These strains should have selected a stable genetic background thereby preventing changes in the genome that would revert the phenotype.

5.6 Virulence properties and acquisition of quinolone resistance

In *S. enterica*, ciprofloxacin resistance acquisition has been previously related to a decreased expression of virulence factors and decreased virulence properties, including *in vitro* invasion ability [210–212] and to a decreased ability to form biofilm in non-*Salmonella* isolates [213,214]. Accordingly, this phenomenon was observed in Papers III and IV. First, virulence was shown to be compromised in both *S.* Typhi mutants in Paper IV as the invasion ability of eukaryotic cells and internalization and survival inside human macrophages was decreased compared to the wild-type strain. Besides, expression of the SPI-1 encoding genes was also reduced in the resistant mutants. Regarding the ability to cause immunogenic response, they were also less able to promote cytokine production in infected host cells than the parental strain. Secondly, strains 59-16 and 59-64 in Paper III showed the highest levels of ciprofloxacin resistance, were both unable to form biofilm, did not show the rdar morphotype (which reflects the production of curli fibers) and presented diminished expression levels of the *csgB* gene, encoding for the latter mentioned biofilm-component, compared to the previous mutants. In addition, in this study a gradual decrease in the rdar morphotype was already seen between 59-wt and 59-2.

All of this information together support the hypothesis that efflux pump activity, which contributes at different levels to drug resistance in mutants with decreased virulence, also contributes to this phenotype as previously suggested [211]. It has been reported that quorum-sensing molecules are needed for the activation of virulence genes [261], and that extrusion of such molecules through efflux pumps may disrupt quorum-sensing homeostasis

and thus, lead to impaired gene transcription [262]. Furthermore, Bailey *et al.* showed that overexpression of *ramA* led to an increased expression of efflux pumps as well as a decrease in the expression of virulence genes which was reflected by impaired host-pathogen interactions [251]. In contrast with these results, a lack of efflux activity (caused by both the addition of efflux pump inhibitors and gene inactivation of efflux components in *S.* Typhimurium) has been associated with the production of a defective biofilm due to the repression of all curli biosynthetic genes [263,264]. Exceptionally, the *acrA* mutant was shown to be a biofilm-producer [264] in accordance with the ability to form biofilm of the 59-wt strain in Paper III, which presented a truncated AcrA. These previously mentioned studies suggest that the decrease in curli gene transcription is due to the sensitivity of the two-component regulatory system that respond to membrane stress (*cpxRA*, *rcsCB* and *envZ*/*ompR*). Taking this hypothesis into consideration, the lack of TolC and AcrB, proteins embedded in the outer and inner membranes, respectively, may have a greater impact on membrane composition than the lipoprotein AcrA, which is located in the periplasmic space.

A global overview of the results compiled in this block highlights the need for further studies dedicated to investigate new efflux systems involved in antibiotic resistance as well as to increase the knowledge of the regulation network. Moreover, it is demonstrated that acquisition of resistance sometimes has a fitness cost which probably depends on the levels of resistance, and always negatively affects the virulence of the strain.

Besides, we suggest that, although fluoroquinolones are being increasingly used to treat salmonellosis, the low prevalence of *S. enterica* clinical isolates (serovars Typhimurium and Typhi) with high levels of resistance to ciprofloxacin may be due to the concomitantly reduced virulence phenotype that may result in impaired dissemination of the resistant pathogen.

5.7 Differential virulence features related to the *S.* Typhimurium sequence type and geographical area

According to comparison of the genomes of the ST19 and ST313 isolates presented as Additional Results, a common feature was seen among the 3 strains (B52, S15 and 50wt) sequenced belonging to the ST19 sequence type. All strains presented truncation of the

fimbrial encoding gene *fimH*. Besides, similarities among strains having caused gastroenteritis (S15 and 50wt) revealed that two genes, *lpfC* and *avrA*, were both altered compared to the reference strain SL1344. The *lpfC* gene encodes for the fimbrial outer membrane usher and a previous study reported that the lack of *lpfC* led to a decrease in the organ dissemination ability compared to the wild-type when administered orally in mice [265]. Considering this fimbrial deficiency together with the *fimH* truncation seen for all the ST19 strains above-mentioned, we propose that this additional loss may influence in the limited ability of this isolates to cause an infection other than the gut restricted disease, and supports the invasive phenotype of ST313 *versus* ST19 Nevertheless, a different situation was seen for the acetyltransferase *avrA.* This effector protein has been reported to be involved in systemic dissemination in a mice model, as well as it has been postulated to have a role in survival inside macrophages [266]. Despite this information, our results showed the insertion of a Leucine in this gen in both ST19 strains isolated from stool (S15 and 50wt) leading to a form equivalent to that of the reference strain ST313 D23580. For this reason, we cannot attribute the modification of this gene as relevant for pathogenicity but we rather suggest that this amino acid insertion has no or limited impact on virulence. Additionally, the strain having caused gastroenteritis at the Hospital Clínic (Barcelona), 50wt, showed additional defects in virulence-related genes to the homologs from Mozambique. These genes were involved in attachment (*fimC*, *siiC*) [34,46]; the *nupG* gene was needed for survival inside the host cell [226], and *sspH2* was involved in inducing innate immune response [225]. These results may insinuate that ST19 *S*. Typhimurium isolates circulating in Europe are less pathogenic than the African isolates, although more isolates need to be sequenced in order to confirm this hypothesis.

Altogether, the results obtained in this work suggest that particularities in terms of gene integrity and virulence features exist among isolates depending on their sequence type (ST19 and ST313), the type of disease they caused and the geographical isolation. However, a greater number of strains need to be analyzed in order to confirm these findings that will allow a better understanding of the differences in the pathogenesis across the *S*. Typhimurium serovar.

5.8 Identification of *yedF*, a gene potentially involved in virulence in *S*. Typhimurium

The study of *yedF* in *S.* Typhimurium, conducted in the Manuscript, revealed the potential role of this gene in the regulation of different processes. A previous study suggested that *yedF* could have similar functions as the sulfur transferase TusA by structure prediction from sequence homology with the *E. coli tusA* gene [267]. TusA function has been reported to consist in sulfur transport for the formation of 2-thiouridine in tRNA and for molybdenum cofactor biosynthesis [268,269]. Although scarce information is available about *yedF,* Dahl *et al.* reported that this gene did not present a redundant role of *tusA* in *E. coli* [268]. Despite this previous report, our data revealed that some of the functions of *yedF* in S. Typhimurium seemed to be shared with *tusA,* although another TusA-like gene with a protein sequence identity of 90% is present in this serovar (GI:486186069). We show that deletion of *yedF* triggers an increase in the gene expression of enzymes involved in anaerobic respiration, similar to what was demonstrated by Dahl *et al.* in a *tusA*-deleted mutant of *E. coli* [268]. Interestingly, we observed that all the genes showing transcriptional activation in the *ΔyedF* mutant compared to the wild-type strain, hence genes which are repressed by YedF, are regulated by FNR, the main transcriptional regulator of the adaptive response of *S. enterica* to the lack of oxygen [268,270–276]. Expression of this transcriptional regulator was not affected by the presence/absence of YedF; however, our results indicate that even when oxygen is available, when most of the FNR is inactive, an important activation of the genes encoding for the anaerobic enzymes occurs in the absence of *yedF.* Thus, we suggest that *yedF* contributes to the repression of the genes involved in anaerobic metabolism. As expected, these anaerobic enzymes were not detected at a protein level since in aerobic conditions these proteins are not needed; thus, it is logical that the changes related to *yedF* observed were restricted to transcription. Dahl *et al.* [268] proposed a model in which interaction of the IscS protein with TusA decreases the pool of available IscS, a protein needed for the FeS cluster biogenesis which is required for the activation of FNR. As YedF also contains the same conserved cysteine as TusA, the catalytic residue involved in the interaction with IscS [277], we suggest that *yedF* affects gene activation by influencing FeS cluster biosynthesis in the same way as the model previously proposed for TusA [268].

Nevertheless, we cannot provide any explanation as to the mechanism by which *yedF* activates protein production of the formate dehydrogenase subunit alpha, ornithine decarboxylase and glycyl radical cofactor, all of which are also involved in anaerobic processes.

TusA has also been suggested to be involved in the general physiology of *E. coli*, as a mutant lacking TusA was shown to present severe growth defects [278,279]. Contrary to this observation, this was not observed in the case of our *yedF*-deficient mutant ruling out this role in *S*. Typhimurium.

In addition to the involvement of *yedF* in anaerobic metabolism in *Salmonella*, we propose a possible role of this gene in virulence. A decrease of more than 3-fold in the *in vitro* invasion ability of eukaryotic cells was seen in the strain lacking *yedF* compared to the wild-type strain, being this ability restored when the gene was reintroduced. Moreover, the absence of *yedF* caused a decrease in virulence-related determinants at both gene and protein levels, correlating with the *in vitro* invasion results. A decrease in the transcription levels of the global regulators *hilA*, *hilC* and *hilD*, which control the expression of genes involved in the invasion process encoded in SPI-1, 2, 4 and 5 [34], was seen in the *yedF*-deficient mutant compared to the wild-type strain. Moreover, the *rtsAB* operon, which is activated by *hilD* and in turns, activates expression of *hilA* by means of RtsA [280], was also significantly downregulated in the wild-type strain. Consequently, expression of most of the genes located in these SPIs was also repressed in the strain lacking *yedF* compared to SL1344 thus suggesting YedF to activate such virulence-related genes. In accordance with these results, proteomic analysis revealed a <-2-fold decreased production of the SPI-1 and SPI-5 encoded proteins SipC and SigD, respectively, in the *yedF*-deficient mutant.

Moreover, the genes involved in motility, chemotaxis, attachment and internalization in the host cell were also affected at both transcriptional and proteomic levels. According to flagellin, both the phase 1 and phase 2 flagellin encoding genes, *fliC* and *fljB*, were transcriptionally repressed; however, it is well defined that during flagellar phase variation, inversion of the genetic region called H segment occurs allowing transcription of one of the two phases. When the H segment is in the “on” state, transcription of both *fljB* and *fljA* takes place and thus, phase 2 flagellin FljB and inhibition of the phase 1 flagellin FliC is induced

posttranscriptionally by FljA. In the "off" state, *fljB* and *fljA* are not transcribed and thus FliC is produced [215,216]. Our results indicate lower mRNA levels for *fljA* (-11.48 fold change) in the absence of *yedF* compared to the wild-type strain, although this was not observed in the proteomic approach. Virulence regulation is complex and many factors interplay in this network leading to gene expression in particular conditions; thus, interpretation of the specific role of *yedF* in this system needs to be further studied. Taking account this multifactorial regulation, the alteration of this system could lead, in some cases, to paradoxal situations as the unexpected gene expression profile of determinants involved in the flagellin biosynthesis pathway. Another surprising result was the important repression of the fimbrial gene *fimA* in the complemented mutant (-10-fold) compared to the parental strain, even below the levels reported in the knock-out strain (-1.46-fold). We suggest that a possible explanation for this situation could be that *yedF* regulates *fimA* transcription but in the presence of excessive amounts of the regulator it provoques the opposite effect.

Another of the virulence aspects affected by *yedF* was LPS biogenesis, as in the absence of *yedF* a decrease was observed in the expression of the genes involved in O-antigen biosynthesis as well as in the composition of the core-oligosaccharide. Moreover, lesser amounts of LPS were also detected in the *ΔyedF* mutant compared to the wild-type strain. Therefore, YedF seems to activate LPS biogenesis. In agreement with a previous study in which an intact LPS in *S*. Typhimurium was required for optimal invasion and colonization of host tissues [281], we suggest that *yedF* enhances virulence in S. Typhimurium.

Altogether, we propose that *yedF* is involved in the complex regulation related to the pathogenesis of *Salmonella* and contributes to the activation of the virulence machinery. The pleiotropic functions of *yedF* suggest its involvement in different aspects of the invasion process. We hypothesize that in the very initial steps of infection, *yedF* is activated and therefore contributes to the positive transcription of genes involved in motility and attachment allowing the bacteria to reach the host cell. When oxygen is available, *yedF* contributes to keeping the anaerobic machinery repressed, and when bacteria encounter conditions in which oxygen is absent, such as in the intracellular state, transcription of *yedF* is repressed allowing the activation of the anaerobic metabolism pathways, together with a

downregulation of the genes involved in the invaison machinery. Nevertheless, in order to validate this theory, further characterization of this factor is required.

5.9 Identification and description of the protein composition of the outer membrane of *S.* Typhimurium SL1344

Bacterial proteins located in the outer membrane play an important role in the pathogenesis and host immune recognition. Based on this fact, outer membrane proteins of Gram-negative bacteria have been focused on as targets for the development of vaccines [282]. Although progress in the characterization of the outer membrane has recently been reported, an in depth description of the protein composition of this subcellular localization has not been clearly defined for most pathogens, including *S*. Typhimurium.

In Paper II, the sarkosyl extraction method was performed to separate outer membrane proteins in combination with MS/MS approaches. Other methods have previously been used for the analysis of OMPs in *S*. Typhimurium. Carbonate extraction followed by two-dimensional electrophoresis (2DE) and identification of proteins by peptide mass fingerprinting (PMF) was reported by Molloy *et al.* [283] using the non-virulent strain LT2.

Although most of the information regarding genetic and phenotypic variation have been conducted in non-virulent strains of *Salmonella* [284], in Paper II the pathogenic SL1344 strain was used, as this bacteria was considered to be more interesting in terms of clinical relevance. As far as we know, only two studies have analyzed the proteome of this latter strain using other methodologies [285,286]. In the study conducted by Qi *et al.* the phosphate buffer-insoluble proteome was analyzed by 2D electrophoresis in the absence of a specific OMP extraction method and protein identification was performed by sequential Edman degradation [285]. This procedure presents several limitations such as solubilization of membrane proteins in 2D electrophoresis as well as sensitivity restrictions of the Edman degradation approach. According to another study [286], the outer membrane subproteome of SL1344 was considered to correspond to the insoluble fraction of a buffer containing urea/thiourea and triton X-100/CHAPS. However, this sample preparation is expected to contain a mixture of different membrane proteins in addition to the outer membrane located, as well as macromolecular complexes.

Taking into account this information and considering the different methods reported in the literature to isolate OMPs, it has been suggested that the sarkosyl protocol provides the most reproducible results and the purest preparations [287,288]. This was concluded upon comparing nine different extraction methodologies in *Campylobacter jejuni* [287] and two different protocols (the carbonate and the sarkosyl methods) in the Gram-negative bacterium *Caulobacter crescentus* reported in another study [288].

In Paper II, 50 proteins were classified as OMPs considering the PSORTb database and 40 considering the Uniprot approach. Considering previous results in *S*. Typhimurium, and as far as we know, this is the study in which the greatest number of OMPs have been identified to date. According to Coldham *et al.* 34 proteins belonging to this category were isolated from SL1344 [286], among which 24 were also identified in Paper II. Regarding LT2, Molloy *et al.* identified 23 different ORFs [283], almost all also being identified in Paper II. Only five proteins were not isolated in our study and corresponded to proteins having mostly intracellular functions: the molecular chaperone DnaK, the 30S ribosomal protein subunit S1, a phosphoglycerate kinase, an enolase and the heat shock protein Hsp90. In the other work in which SL1344 was also studied, only 6 out of the 53 proteins identified were also found in Paper II [285]; this under-representation of OMPs is most likely due to the methodology used. Another difference concerning the data analysis of Paper II compared to other studies based on SL1344 is that the present work is the only one in which the results have been checked using the genome of SL1344 as a reference as it was published in 2012 [289].

In addition to OMPs, 16% of the proteins were identified as being cytoplasmic in Paper II suggesting that contamination with intracellular proteins may have occurred during the sarkosyl extraction method. Since this method is considered the most reproducible and allows recovery of the purest samples, another feasible explanation could be that these cytoplasmic proteins were located inside outer membrane vesicles (OMVs). These structures are budding vesicles that are constitutively released to the extracellular space and contain a diversity of cytoplasmic proteins, including virulence determinants, endotoxins, RNA and DNA molecules, among others [290]. Although the aim of this study was to characterize the outer membrane proteome of SL1344, it has been reported that several compounds present

inside OMVs are recognized by host microbial PPRs (pattern-recognition receptors), thus being relevant in order to identify novel antigens for vaccine development [291].

Comparison of the results obtained from the two different databases according to protein location (PSORTb and Uniprot) revealed that a greater number of proteins were assigned to a specific group using PSORTb than Uniprot (87.3% *vs* 77.9%, respectively). Moreover, differences were also found in the protein location of the outer and inner membrane groups, with the latter group showing more considerable differences (36% with the PSORTb approach vs. 8.8% using Uniprot). However, interpretation of these results is not evident as the two databases considered different subgroup classifications. For example, the Uniprot database included an additional bacterial group of membrane-located proteins. In this category 45 proteins were assigned among which 38 belonged to the inner membrane location group of the PSORTb approach.

The results obtained in Paper II provide greater knowledge concerning the proteome landscape of *Salmonella*, specifically of SL1344, and demonstrate the presence of proteins only having previously been identified by means of *in silico* approaches. Moreover, the novel identification of proteins with a potential involvement in host recognition is of great importance in order to encourage the development of NTS vaccines.

CONCLUSIONS

VI. CONCLUSIONS

1. The high clonality among *S*. Enteritidis isolates makes the distinction of clones in epidemiological studies difficult despite the use of different approaches. A combination of improved techniques and the development of new tools are needed for appropriate surveillance as well as for the identification and characterization of the circulating clones.
2. The PFGE technique has a higher discriminatory power than MLST to differentiate *S*. Typhimurium clones; hence PFGE is a more appropriate tool to study the genetic diversity of *S*. Typhimurium.
3. The increase in nalidixic acid resistance among *S*. Enteritidis isolates observed is in agreement with the global trend and indicates that the use of fluoroquinolones should be limited.
4. Fluoroquinolone resistance in *S. enterica* is mainly due to the presence of mutations in the QRDR of the target genes. The absence of mutations detected in *parE* supports its minor role in fluoroquinolone resistance acquisition both *in vitro* and through natural selection.
5. The contribution of efflux in antimicrobial resistance is evident and is a first step to antibiotic pressure in *S. enterica*. Although the major efflux system AcrAB/TolC is commonly found, the presence of other efflux components unraveled till now should also be considered.
6. The MDR regulatory gene *marA* is repressed by *marR* and contributes to the overexpression of the efflux system AcrAB/TolC in *S*. Typhi.
7. The MDR regulatory loci *ramRA* is the most susceptible region for the acquisition of mutations related to MDR in *S*. Typhimurium. Alterations of the RamR binding sites in the *ramA* promoter lead to higher *ramA* expression associated with the highest expression levels of the efflux components *acrB*, *tolC*, and *acrF*, as well as *marA* in a concentration-dependent manner.
8. RamA and SoxS activate *marA* transcription in *S*. Typhimurium probably by binding the marbox/rambox located in the *marA* promoter.

9. Quinolone resistance acquisition in *S*. Typhimurium is associated with a decrease in biofilm production due to a gene repression of c*sgB,* which encodes for curli fiber.
10. An association between efflux activity, decreased bacterial growth and biofilm production is seen in *S*. Typhimurium.
11. Acquisition of antimicrobial resistance is related to a loss of virulence in *S*. Typhi as demonstrated by a decrease in the invasion ability and survival inside the host cell as well as reduced activation of innate immune response.
12. The low prevalence of *S*. *enterica* isolates highly resistant to ciprofloxacin in the clinical setting may be related to a decrease in their virulence features.
13. The novel *yedF* gene may play a role in the repression of genes involved in anaerobic metabolism in *S*. Typhimurium
14. The novel *yedF* gene is potentially involved in the transcriptional activation of the invasion genes in *S*. Typhimurium.
15. In comparison with isolates belonging to ST313 sequence type, isolates belonging to ST19 putatively lack several genes, among which a functional fimbrial encoding gene *fimH*, involved in host cell attachment.
16. The ST19 isolate obtained from a Spanish clinical setting as having caused gastroenteritis lacks several virulence determinants involved in the invasion process compared to the homolog African isolates.
17. The identification of novel outer membrane proteins of *S*. Typhimurium SL1344 provides greater knowledge about the cell envelope composition of this pathogen. Moreover, it contributes to the identification of exposed antigens potentially used as targets in vaccine development.

VII. LIST OF ACRONYMS

CDC: Center for Disease Control and Prevention of the United States of America

DCS: Decreased ciprofloxacin susceptibility

ECDC: European Centre for Disease Prevention and Control

EFSA: European Food Safety Authority

iNTS: Invasive Nontyphoidal *Salmonella*e

LPS: Lipopolysaccharide

MDR: Multidrug resistance

MIC: Minimum inhibitory concentration

MLST: Multilocus sequence typing

NTS: Nontyphoidal *Salmonellae*

OM: Outer membrane

OMP: Outer membrane protein

PAMPs: Pathogen-associated molecular patterns

PAβN: Phenyl-arginine-β-naphthylamide

PFGE: Pulsed-field gel electrophoresis

PMQR: Plasmid-mediated quinolone resistance

PRRs: Pattern recognition receptors

QRDR: Quinolone resistance-determining regions

RQ: Relative gene expression

SCVs: Salmonella-containing vacuoles

SIFs: Salmonella-induced filaments

SPIs: Salmonella pathogenicity islands

ST: Sequence type

TLR: Toll-like receptor

T3SS: Type III secretion system

WHO: World Health Organization

RESUM DE LA TESI

1. Justificació del treball

Les infeccions degudes a *Salmonella enterica* son de gran rellevància clínica ja que representen una important causa de morbiditat i mortalitat arreu del món.

Degut a l'aparició de resistència als antibiòtics que són utilitzats per al tractament de la salmonel·losi, la teràpia d'elecció ha anat canviat al llarg del temps per tal d'adaptar-se a les noves característiques dels patògens circulants responsables de causar la malaltia. Actualment, les fluoroquinolones, representades majoritàriament per la ciprofloxacina i l'ofloxacina, són àmpliament utilitzades per tractar aquest tipus d'infeccions tot i que ocasionalment cal recórrer a altres classes d'antibiòtics així com fluoroquinolones d'última generació en els casos en què falla el tractament. Aquesta situació es deu a l'increment en el nombre de soques resistents a l'àcid nalidíxic associades a un descens en la sensibilitat a les fluoroquinolones, com la ciprofloxacina. A més, l'aparició d'aïllats multiresistents, amb la presència de determinants de resistència plasmídics o localitzat al cromosoma, expliquen parcialment la disminució en l'eficàcia dels tractaments de primera línia, la qual cosa representa un important problema.

Malgrat aquesta tendència, el nombre de casos reportats d'aïllats de *Salmonella enterica* resistents a fluoroquinolones es manté baix. L'estudi d'aquesta inesperada situació és una de les línies en què treballa el nostre grup de recerca. Per tal d'explicar aquest escenari, partim de la hipòtesi que podria existir un vincle entre l'adquisició de resistència a quinolones i la disminució en les característiques lligades a la virulència en aquesta espècie.

Aquesta tesi doctoral aborda el context de la *Salmonella* des de diferents perspectives, incloent una aproximació epidemiològica així com models *in vitro* per tal d'entendre la biologia d'aquest patogen i la seva relació amb la resistència a quinolones.

Els objectius que s'han establert per l'assoliment d'aquest treball de recerca es descriuen en la següent secció.

2. Objectius

Per tal de desenvolupar la nostra hipòtesi, s'han definit els següents objectius específics:

1. Avaluació de la relació clonal entre aïllats clínics de *S.* Enteritidis i *S.* Typhimurium mitjançant l'ús de dues tècniques diferents
2. Anàlisi del subproteoma de la membrana externa de *S.* Typhimurium SL1344
3. Investigació dels mecanismes moleculars de resistència a quinolones i de la seva regulació
4. Avaluació de les propietats relacionades amb la virulència en aïllats clínics així com en mutants seleccionats *in vitro* de *Salmonella enterica* amb diferents nivells de sensibilitat/resistència a quinolones.
5. Identificació de nous gens potencialment implicats en la resistència a quinolones i/o virulència en *S.* Typhimurium.
6. Comparativa dels genomes d'aïllats clínics de *S.* Typhimurium causants de salmonel·losi invasiva *versus* no-invasiva provinents de diferents àrees geogràfiques.

Els **Objectius 1** i **3** s'han assolit en l'**Article I** mentre que l'**Objectiu 2** s'ha completat en l'**Article II**. L'**Objectiu 3** s'ha pogut aconseguir en l'**Article V** així com en els **Articles III** i **IV**. Aquests dos últims articles també han contribuït a assolir l'**Objectiu 4**. Finalment, l'**Objectiu 5** s'ha completat en el **Manuscrit**, mentre que **els Resultats Addicionals** han afectat els **Objectius 4** i **6**.

3. Resultats

Tenint en compte els objectius principals assolits en els articles, manuscrit i resultats addicionals que composen aquesta tesi doctoral, els resultats obtinguts s'han englobat en cinc blocs per tal de facilitar-ne la interpretació:

3.1 Caracterització molecular dels mecanismes de resistència a quinolones i la seva regulació en *Salmonella enterica.*

Article I

Títol: Molecular study of quinolone resistance mechanisms and clonal relationship of *Salmonella enterica* clinical isolates.

Autors: Clara Ballesté-Delpierre, Mar Solé, Òscar Domènech, Jordi Borrell, Jordi Vila and Anna Fàbrega

Revista: *International Journal of Antimicrobial Agents* 43 (2014) 121– 125

Resum: En els últims anys, ha augmentat el nombre de soques de *Salmonella enterica* resistents a l'àcid nalidíxic. En un estudi previ es va analitzar la sensibilitat a quinolones des d'un punt de vista fenotípic i genotípic de 23 aïllats clínics de *S. enterica* (19 *S. enterica* serovar Typhimurium i 19 *S. enterica* serovar Enteritidis). Un 42% dels aïllats era resistent a l'àcid nalidíxic, associat a una mutació en *gyrA* junt amb una suposada sobreexpressió de bombes d'expulsió. En el present estudi es van analitzar les mutacions en QRDR de *parE* i dels reguladors de AcrAB (*acrR*, *marRAB*, *soxRS*, *ramR)*. També es va determinar l'acumulació intracel·lular de ciprofloxacina i àcid nalidíxic. A més, es va dur a terme un estudi epidemiològic dels aïllats mitjançant la tècnica de "MultiLocus Sequence Typing" (MLST) i "Pulsed Field Gel Electrophoresis" (PFGE). No es va detectar cap mutació en *parE*, però sí que es van identificar dues substitucions aminoacídiques en MarR (I84L) i AcrR (N214T) en dues soques sensibles, de manera que es van considerar polimorfismes. No es van detectar canvis en l'expressió dels gens *acrB*, *tolC*, a*crF* i *emrB* entre soques resistents i

sensibles a l'àcid nalidíxic. La tècnica de l'acumulació intracel·lular no va servir per detectar diferències entre aïllats. L'anàlisi epidemiològic va demostrar una important relació clonal entre els aïllats de *S*. Enteritidis mentre que les soques de *S*. Typhimurium van presentar una major divergència. Els resultats obtinguts en aquest estudi suggereixen la presència de bombes d'expulsió d'antibiòtics no conegudes fins ara i confirmen l'alta clonalitat de *S*. Enteritidis i la divergència genètica de *S*. Typhimurium.

Article V

Títol: Differential impact of *ramRA* mutations on both *ramA* transcription and decreased antimicrobial susceptibility

Autors: Anna Fàbrega, Clara Ballesté-Delpierre, Jordi Vila

Revista: *Journal of Antimicrobial Chemotherapy* (under review)

Resum: En aquest estudi es va analitzar l'heterogeneïtat de les mutacions que es donen en els gens reguladors de la multiresistència a antibiòtics en *Salmonella* Typhimurium. A més, també es va estudiar l'efecte d'aquestes mutacions en la transcripció de *ramA*, *acrB*, *tolC* i *acrF* així com en el fenotip de multiresistència. Les soques es van seleccionar per exposició a concentracions creixents de ciprofloxacina. La determinació de la concentració mínima inhibitòria (CMI) a antibiòtics pertanyents a famílies diferents es va dur a terme a través de la tècnica de l'Etest i per microdilució en medi líquid. A més, també es va analitzar la presència de mutacions en els gens diana de les quinolones i en els reguladors de la multiresistència. Els nivells d'expressió de *acrB*, *tolC*, *ompF*, *acrF*, *emrB*, *acrR*, *ramA*, *soxS* i *marA* es van avaluar per RT-PCR. Tots els mutants estudiats van presentar un increment en la CMI de la majoria d'antibiòtics testats, excepte de la kanamicina. Tot i que no es van identificar mutacions en els gens diana de les quinolones en totes les soques, totes elles presentaven mutacions en la regió reguladora de ramRA. Tots els mutants sobreexpressaven *ramA*, *tolC* i *acrB* (en els casos en què aquest estava actiu) mentre que en la resta de gens es van trobar resultats diferencials. Les mutacions en la regió *ramRA* són predominants en *Salmonella* pel que fa a la multiresistència. Existeix heterogeneïtat pel que fa al tipus de

mutacions, sent les delecions que afecten el lloc d'unió a RamR les que tenen un major impacte en l'expressió de *ramA* i el fenotip de multiresistència.

3.2 Relació entre l'adquisició de resistència a quinolones i la disminució en la virulència.

Article III

Títol: Impact of quinolone-resistance acquisition on biofilm production and fitness in *Salmonella enterica*

Autors: Anna Fàbrega, Sara M. Soto, Clara Ballesté-Delpierre, Dietmar Fernández-Orth, M. Teresa Jiménez de Anta and Jordi Vila

Revista: *Journal of Antimicrobial Chemotherapy* 69 (2014) 1815-1824

Resum: L'objectiu principal d'aquest estudi va consistir en investigar la potencial relació entre resistència a quinolones i producció de biofilm en una col·lecció d'aïllats clínics de *Salmonella enterica* i en mutants de *S. enterica* serovar Typhimurium amb nivells creixents de resistència a ciprofloxacina. La sensibilitat a l'àcid nalidíxic i la formació de biofilm es va avaluar en una col·lecció de 122 aïllats clínics de *S. enterica.* A partir d'un aïllat clínic sensible a quinolones i formador de biofilm (59-wt) es va obtenir una soca mutant resistent a quinolones (59-64) en un procés de selecció per exposició a concentracions creixents de ciprofloxacina. Es van caracteritzar els mecanismes de resistència a quinolones [mutacions en gens diana i en gens reguladors de la multiresistència, CMIs a diferents antibiòtics, anàlisi de proteïnes de l'envolta cel·lular, RT-PCR i acumulació de ciprofloxacina] de les soques mutants. A més, també es va avaluar la taxa de creixement bacterià, la formació de biofilm, el morfotip rdar i l'expressió de gens relacionats amb el biofilm per RT-PCR. Els resultats obtinguts van demostrar que una major proporció de les soques de *S. enterica* sensibles a l'àcid nalidíxic eren productores de biofilm comparat amb les seves parelles resistents. La soca 59-64 va adquirir mutacions en gens diana i presentava un fenotip de multiresistència. La sobreexpressió d'AcrAB i *acrF* es va excloure mentre que sí que es va detectar un

augment en els nivells d'expressió de TolC en la soca 59-64, que a més acumulava menys ciprofloxacina. En aquesta mateixa soca, es va observar un augment en els nivells d'expressió de *ramA* atribuïda a una mutació en la regió promotora del gen. També, aquest mutant va presentar una disminució en la producció de biofilm relacionada amb una descens en l'expressió de *csgB* així com en una reducció de la taxa de creixement, a més de no formar el morfotip rdar. L'adquisició de la resistència a quinolones pot estar associada a un descens en la producció de biofilm degut a una disminució en l'expressió de *csgB*. Existeix una possible interrelació entre expulsió activa, producció de biofilm i taxa de creixement.

Article IV

Títol : Attenuation of *in vitro* host-pathogen interactions in quinolone-resistant *Salmonella* Typhi mutants

Autors : Clara Ballesté-Delpierre, Anna Fàbrega, Mario Ferrer-Navarro, Ramkumar Mathur, Sankar Ghosh and Jordi Vila

Revista: *Journal of Antimicrobial Chemotherapy* (In press)

Resum: La relació entre adquisició de resistència a quinolones i afectació en la capacitat d'invasió s'ha estudiat en alguns serovars de *Salmonella enterica.* No obstant això, es disposa de poca informació al respecte pel que fa a *Salmonella* Typhi, un patogen invasiu que només causa malaltia en l'ésser humà. L'objectiu d'aquest estudi va consistir en investigar, en aquest serovar, els mecanismes moleculars d'adquisició de resistència a quinolones i el seu impacte en la virulència. Per això, es van generar dos mutants resistents a antibiòtics (Ty_c1 i Ty_c2) a partir d'un aïllat clínic de *S.* Typhi (Ty_wt). Aquestes 3 soques es van comparar pel que fa a la sensibilitat antibiòtica, els mecanismes moleculars de resistència, expressió gènica de factors relacionats amb la virulència, capacitat invasiva en cèl·lules eucariotes (cèl·lules epitelials humanes i macròfags) i la producció de citoquines. El perfil de multiresistència en Ty_c2 es va atribuir a l'augment en la producció d'AcrAB/TolC i en el descens en OmpF (ambdós mediada pel reguló *mar*) i en el descens de la producció d'OmpC. Els 2 mutants van presentar una reducció gradual en l'expressió dels gens

relacionats amb la virulència (*invA*, *hilA*, *hilD*, *fliC*, *fimA*) la qual es correlacionava amb una reducció de la motilitat, de la capacitat d'infectar cèl·lules HeLa i de ser fagocitades i sobreviure dins de macròfags humans. A més, el mutant Ty_c2 presentava una reducció en l'expressió de *tviA.* Els resultats obtinguts també van demostrar que aquesta soca induïa una menor producció de TNFα i IL-1β així com una menor activació de NF-κB. Aquest estudi proporciona una caracterització exhaustiva dels mecanismes moleculars de resistència a antibiòtics en el serovar Typhi i evidencia que l'adquisició de la resistència a antibiòtics està relacionada amb una disminució de la virulència (invasió de cèl·lules epitelials, fagocitosi en macròfags i producció de citoquines). Suggerim que la baixa prevalença d'aïllats clínics de *S*. Typhi altament resistent a ciprofloxacina és deguda a la baixa capacitat d'inducció de la resposta immune i a l'escassa capacitat de disseminació d'aquests aïllats.

3.3 Estudi de gens potencialment relacionats amb l'adquisició de resistència a quinolones i/o virulència. Identificació de *yedF*, un nou gen potencialment relacionat amb la virulència.

Manuscrit

Títol: Evaluation of the pleiotropic role of *yedF*, a novel gene of *S.* Typhimurium

Autors: Clara Ballesté-Delpierre, Dietmar Fernandez-Orth, Mario Ferrer-Navarro, Anna Fàbrega1 and Jordi Vila

Resum: *Salmonella* posseeix un gran nombre de factors de virulència que permeten al bacteri replicar-se en condicions extremes i envair la cèl·lula hoste per tal de causar la malaltia. L'objectiu d'aquest estudi va consistir en identificar nous gens implicats en la virulència i/o resistència a antibiòtics. Amb aquesta finalitat, es van seleccionar gens a partir d'un estudi de microarrays dut a terme prèviament en el nostre grup en què un aïllat clínic de *S*. Typhimurium sensible a quinolones (50-wt) i un mutant multiresistent (50-64), que també presentava una capacitat reduïda d'invasió *in vitro*, van ser comparats. D'entre els gens que presentaven un perfil de transcripció diferencial (tant sobreexpressats com reprimits en la soca resistent), 4 d'entre ells van ser predits com a proteïnes de membrana externa (*ydiY)* o interna (*ybdJ*, *STM1441* i *ynaJ).* A més, també es va escollir *yedF*, un possible factor de transcripció. Per a cada gen, i a partir de la soca de referència SL1344, es van obtenir mutants que sobreexpressaven i que tenien el gen interromput i se'n va avaluar el perfil de sensibilitat a antibiòtics. Els resultats van demostrar que cap dels gens seleccionats estava implicat en l'adquisició de resistència ja que no es van veure diferències en la CMI dels diferents antibiòtics testats. A continuació, es va avaluar la contribució dels diferents gens en el fenotip de virulència pel que fa a la capacitat d'invasió *in vitro*, i es va veure que tant *ydiY* com *yedF* presentaven resultats consistents: l'absència dels gens comportava un descens en la capacitat invasiva, com en el cas de 50-64, mentre que només en el cas de *yedF*, aquest fenotip es revertia quan el gen salvatge es tornava a expressar. Per tal d'elucidar el rol de *yedF* en *Salmonella*, es van utilitzar aproximacions tant transcriptòmiques com proteòmiques les quals van demostrar que l'absència de *yedF* causava una disminució

en l'expressió de factors de virulència, representats per gens implicats en la motilitat, quimiotaxi i adherència a la cèl·lula hoste. A més, també es va observar una disminució en l'expressió gènica dels reguladors globals implicats en el procés invasiu (*hilA*, *hilC* i *hilD*). La biosíntesi de LPS també es va veure compromesa en la soca amb el gen delecionat tant a nivell de transcripció com fenotípicament. Finalment, també es va detectar un increment en l'expressió de gens implicats en la ruta del metabolisme anaeròbic en la soca mutant. En vista dels resultats obtinguts en aquest estudi proposem que *yedF* està implicat en la complexa regulació de la patogènia de *Salmonella*, contribuint a l'activació de la maquinària de virulència. A més, suggerim que en presència d'oxigen, *yedF* contribueix a mantenir reprimida la ruta del metabolisme anaeròbic.

3.4 Descripció i identificació de proteïnes de la membrana externa de la soca virulenta de referència *S.* Typhimurium SL1344.

Article II

Títol: Characterization of the OMP subproteome of the virulent strain *Salmonella* Typhimurium SL1344

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Resum: Les proteïnes de membrana externa (en anglès, OMPs) juguen un paper important en la interacció entre patògens i cèl·lula hoste. De fet, algunes OMPs de bacteris Gram-negatius són considerats importants factors de virulència i dianes per al reconeixement immunitari de l'hoste. Per aquesta raó, l'estudi proteòmic de la membrana externa de bacteris causants d'infeccions és un pas essencial en l'estudi dels mecanismes de patogènesi i per la identificació de factors de virulència. Tot i que recentment s'han publicat estudis centrats en la caracterització de la membrana externa, en la majoria de patògens no existeixen estudis en què la composició de les proteïnes d'aquest compartiment subcel·lular s'hagi detallat clarament. Les infeccions causades per *Salmonella enterica* serovar Typhimurium no només donen lloc a gastroenteritis en països de renda alta, sino que també són una de les majors causes de salmonel·losi invasiva no-tifoidea en països de renda mitjana i baixa. La incidència d'aquesta malaltia invasiva està incrementant arreu del món i causa anualment milions d'infeccions i morts en la població humana. Malauradament, la gran proporció de soques de *Salmonella* no-Typhi resistents a un gran nombre d'antibiòtics és una realitat preocupant. Per aquesta raó, és necessari el desenvolupament de vacunes contra aquests patògens invasius. En aquest estudi s'ha identificat el perfil proteic de la membrana externa de la soca virulenta de *S*. Typhimurium SL1344 a través del mètode d'extracció amb sarkosyl.

3.5 Comparativa genòmica i fenotípica d'aïllats de *S.* Typhimurium pertanyent als patotips ST19 i ST313.

Resultats Addicionals

Resum: La salmonel·losi invasiva no-tifoidea és la major causa de bacterièmia en la població infantil de l'Àfrica sub-sahariana, i està associada a una mortalitat del 20-25%. A l'Àfrica, la major part dels aïllats de *S.* Typhimurium responsables de la salmonel·losi invasiva pertanyen al patotip ST313 mentre que el més prevalent fora del continent africà és el ST19. L'objectiu d'aquest estudi va ser, per una banda, estudiar el comportament d'aïllats clínics pertanyent tant a ST19 com a ST313 enfront a la pressió antibiòtica, i per l'altra, identificar característiques genètiques específiques relacionades amb el tipus d'infecció que van causar (malaltia sistèmica *versus* gastrointestinal), al patotip al qual pertanyien i àrea geogràfica on van ser aïllades (Espanya *versus* Moçambic). En la primera part de l'estudi, l'adquisició de resistència a quinolones i a altres antibiòtics es va associar a la presència de mutacions en els gens diana de les quinolones i/o a l'augment en l'expressió de la principal bomba d'expulsió AcrAB/TolC. Pel que fa a l'expressió de gens de virulència (*hilA*, *hilD* i *invA*), tots els mutants resistents van presentar una disminució en l'expressió respecte la soca sensible, excepte pel mutant resistent derivat de sang i pertanyent a ST313, pel qual es va observar un increment en l'expressió d'aquests gens. Referent a la segona part de l'estudi, l'anàlisi genòmic va demostrar que totes les soques del patotip ST19 presentaven una fimbria (*fimH*) no funcional comparat amb les soques ST313. A més, l'aïllat 50-wt, provinent de l'Hospital Clínic de Barcelona i pertanyent al patotip ST19, presentava alteracions addicionals a les soques homòlogues de Moçambic en gens relacionats amb la virulència. L'absència d'una fímbria funcional en les soques del patotip ST19 comparat amb les ST313, recolza estudis previs que demostren una major capacitat invasiva de ST313 respecte ST19. A més, la pèrdua d'altres factors de virulència en la soca 50wt suggereix una major virulència dels aïllats circulant a l'Àfrica tot i pertànyer al mateix patotip. Aquests resultats indiquen que existeixen particularitats pel que fa a la integritat i característiques de virulència associades al patotip al que pertanyen, el tipus de malaltia que causen i a la localització geogràfica.

4. Conclusions

1. L'elevada clonalitat entre aïllats de *S*. Enteritidis dificulta la distinció entre clons en estudis epidemiològics tot i que es van utilitzar diferents metodologies. La combinació de la millora en les tècniques actualment utilitzades junt amb el desenvolupament de noves eines és necessària per tal de portar a terme una vigilància adequada així com per la identificació i caracterització dels clons circulants.
2. La tècnica del PFGE posseeix un poder de discriminació superior al MLST per diferenciar clons pertanyent a *S*. Typhimurium; per aquesta raó, el PFGE és una eina més apropiada per a l'estudi de la diversitat genètica de *S*. Typhimurium.
3. L'increment en la resistència a l'àcid nalidíxic observat en aïllats clínics de *S*. Enteritidis va en consonància amb la tendència global i indica que l'ús de les fluoroquinolones hauria de limitar-se.
4. La resistència a fluoroquinolones en *S. enterica* es deu principalment a mutacions en la regió QRDR del gens diana. L'absència de mutacions detectades en *parE* sosté el seu rol minoritari en l'adquisició de la resistència a quinolones tant *in vitro* com per selecció natural.
5. La contribució de l'expulsió activa en la resistència a antibiòtics és evident i es dóna com a primer pas d'adaptació a la pressió antibiòtica en *S. enterica*. Tot i que el sistema d'expulsió trobat majoritàriament és AcrAB/TolC, la presència d'altres components de bombes d'expulsió desconeguts fins ara s'ha de tenir en compte.
6. El gen implicat en la regulació de la multiresistència antibiòtica *marA* és reprimit per *marR* la qual cosa contribueix a la sobreexpressió del sistema d'expulsió AcrAB/TolC en *S*. Typhi.
7. El loci implicat en la regulació de la multiresistència antibiòtica *ramRA* és la regió en la qual s'adquireixen més fàcilment mutacions relacionades amb la multiresistència en *S*. Typhimurium. Alteracions en els llocs d'unió de RamR en la regió promotora de *ramA* donen lloc a un augment en l'expressió de *ramA*, i s'associen a un increment dels nivells d'expressió dels components de bombes d'expulsió *acrB*, *tolC* i *acrF*, així com de *marA* d'un mode depenent de concentració.

8. RamA i SoxS activen la transcripció de *marA* en *S*. Typhimurium probablement per unió a la marbox/rambox ubicada en la regió promotora de *marA.*
9. L'adquisició de la resistència a quinolones en *S*. Typhimurium s'associa a una disminució en la producció de biofilm degut a una repressió del gen *csgB*, que codifica per la fibra curli.
10. Existeix una associació entre expulsió activa, disminució en el creixement bacterià i en la producció de biofilm en *S*. Typhimurium.
11. L'adquisició de resistència antibiòtica es relaciona amb una pèrdua de la virulència en *S*. Typhi tal i com ho demostren la disminució en la capacitat invasiva i la supervivència dins la cèl·lula hoste així com una menor activació de la resposta immunitària innata.
12. La baixa prevalença d'aïllats de *S. enterica* altament resistents a ciprofloxacina en la clínica podria estar relacionada amb la disminució dels trets de virulència.
13. El nou gen *yedF* juga un paper potencial en la regulació de proteïnes implicades en el metabolisme anaeròbic en *S*. Typhimurium.
14. El nou gen *yedF* està potencialment implicat en la complexa regulació de la virulència en *S*. Typhimurium.
15. Als aïllats pertanyent al patotip ST19 els manca certs gens respecte el patotip ST313 entre els quals una fímbria funcional codificada pel gen *fimH*, implicada en l'adhesió a la cèl·lula hoste.
16. La soca pertanyent al patotip ST19 causant de gastroenteritis aïllada a Espanya presenta alteracions en gens implicats en virulència, relacionats amb el procés invasiu comparat amb els seus homòlegs provinents de Moçambic.
17. La identificació de noves proteïnes de membrana externa en *S*. Typhimurium SL1344 augmenta el coneixement actual sobre la composició de l'embolcall cel·lular en aquest patogen. A més, contribueix a la identificació d'antígens com a potencials dianes per al desenvolupament de vacunes.

ANNEX

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Chapter 5
Antimicrobial Resistance in *Yersinia enterocolitica*

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Chapter Outline

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INTRODUCTION

p0075 Bacteria within the genus *Yersinia* are Gram-negative, pleomorphic bacilli belonging to the family Enterobacteriaceae. There are currently 15 species within the genus *Yersinia*, three of which are pathogenic to humans. *Y. pestis* is the etiological agent of bubonic plague, while *Y. enterocolitica* and *Y. pseudotuberculosis* are known primarily as foodborne pathogens mainly acquired through ingestion of contaminated pork and related products, and water (Bottone, 1997). However, transmission from animal to human and from person to person can also occur (Fredriksson-Ahomaa et al., 2006). *Y. pseudotuberculosis* is not frequently detected as a cause of human infection and, therefore, in this chapter we will mainly focus on *Y. enterocolitica*.

p0080 Classification of *Y. enterocolitica* strains into biotypes or serotypes (or bioserotypes) is based on biochemical tests and the somatic O antigen (lipopolysaccharide or LPS), with six biotypes (1A, 1B, 2, 3, 4, and 5) and more than 57 serotypes (Wauters et al., 1987). Nonetheless, most of the strains belong to biotypes 2, 3, and 4 and to serotypes O:3, O:5,27, O:8, and O:9 (Fredriksson-Ahomaa et al., 2006). *Y. enterocolitica* is subdivided into the *Y. enterocolitica* subspecies *enterocolitica*, which includes mainly biotype 1B, and the *Y. enterocolitica* subspecies

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palearctica, which includes the remaining biotypes (Neubauer et al., 2000). Both pathogenic and non-pathogenic *Y. enterocolitica* strains have been described, and their virulence potential relies on the presence or absence of several chromosome- and plasmid-encoded virulence genes (see subsection on "Virulence Factors") (Fabrega and Vila, 2012). Pathogenic strains can be classified, in turn, into two groups: high-pathogenicity and low-pathogenicity. The first group is mainly composed of bioserotype 1B/O:8 strains, which are associated with more severe clinical manifestations since they harbor additional virulence traits. In contrast, the second group leads to milder infections and comprises biotypes 2 to 5, highlighting serotypes O:3 and O:9 as the most representative among clinical isolates (Fabrega and Vila, 2012; Lamps et al., 2006). In the case of strains belonging to biotype 1A, they have typically been considered as non-pathogenic since they usually do not carry virulence genes (Lee et al., 1977; Stephan et al., 2013). However, recent evidence suggests that some of these strains are virulent and able to cause gastroenteritis indistinguishable from that caused by other biotypes traditionally considered pathogenic (Burnens et al., 1996; Morris et al., 1991). Thus, biotype 1A strains likely deserve to be included in the low-pathogenicity group.

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Human Infections

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Human infection with *Y. enterocolitica* was first reported by Schleifsten and Coleman in 1939. The infections caused by this pathogen are named yersiniosis and occur worldwide, despite the incidence being higher in cooler climates. In several countries human yersiniosis has become a reportable disease since *Y. enterocolitica* is the third most commonly reported etiological agent after *Campylobacter* and *Salmonella* (Drummond et al., 2012). The populations at increased risk for infection include children under 4 years of age and immunocompromised patients (Greene et al., 1993; Hoogkamp-Korstanje and Stolk-Engelaar, 1995; Jouquan et al., 1984). The majority of cases are sporadic, however, several outbreaks have been occasionally described in different parts of the world and associated with the consumption of pork- and milk-related products (Black et al., 1978; Drummond et al., 2012; Grahek-Ogden et al., 2007; Jones, 2003; Tacket et al., 1984).

p0090

The onset of illness is usually within 24–48 h after ingestion of the contaminated product. The clinical manifestations usually last for 1–3 weeks or even longer. The symptoms reported for intestinal yersiniosis are fever, watery diarrhea (5–10 stools per day), and abdominal pain (Fabrega and Vila, 2012; Vantrappen et al., 1977). Mesenteric lymphadenitis and terminal ileitis are also frequent manifestations which mimic appendicitis, particularly in older children. Serious local complications include hemorrhage and necrosis of the small intestine, intestinal perforation, peritonitis, mesenteric vein thrombosis, or ileocecal intussusception (Abdel-Haq et al., 2000). Cases of sepsis, however, have also been reported and are associated with patients with iron overload or those receiving blood transfusion (Boelaert et al., 1987; Guinet et al., 2011). Among other less frequent clinical

manifestations, *Y. enterocolitica* can cause sporadic cases of endocarditis, pneumonia in immunocompromised patients and also community-acquired pneumonia in immunocompetent people (Greene et al., 1993; Lupi et al., 2013; Wong et al., 2013). Additionally, postinfection sequelae as a consequence of *Y. enterocolitica* infection have been observed, including reactive arthritis and erythema nodosum as the most frequently detected (Bottone, 1997).

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Diagnosis

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The diagnosis of enteritis caused by *Y. enterocolitica* is mainly carried out by culture on laboratory plates. Cefsulodin–irgasan–novobiocin (CIN) agar is the media most frequently used and is based on *Y. enterocolitica*'s resistance to the abovementioned antibiotics (Schiemann, 1979). Plates are incubated at 22–28°C for 48 h, preferentially, or at 30°C for 24 h. *Y. enterocolitica* colonies appear as a dark red "bull's eye" with a translucent edge allowing easy identification. Corroboration is carried out by the specific biochemical properties of *Y. enterocolitica* such as motility at 25°C but not at 37°C, production of urease, lack of oxidase activity and lactose fermentation, as well as absence of either gas or hydrogen sulfide on Kligler's iron agar. Alternatively, the API 20E system can also be used to identify the *Y. enterocolitica* (Bottone, 1997). However, recent technology such as MALDI-TOF mass spectrometry has been used as a rapid, accurate method to also identify the species of the genus *Yersinia* based on protein profiles (Ayyadurai et al., 2010). Additional methods to identify pathogenic *Y. enterocolitica* include both detection of the presence of virulence genes and tests to assess phenotypic characteristics associated with the production of plasmid-encoded virulence factors (Fredriksson-Ahomaa and Korkeala, 2003; Lambertz et al., 2008).

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Treatment

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Antimicrobial therapy is not usually recommended for treating enterocolitis in immunocompetent hosts since most of the gastrointestinal infections are self-limiting. However, immunocompromised patients with invasive infection, who are at increased risk for developing bacteremia or even septicemia, need special attention and antibiotic treatment since the mortality rate in these cases can be as high as 50% (Fabrega and Vila, 2012).

p0105

According to the common profile of susceptibility among *Y. enterocolitica* strains (see section on "Antimicrobial susceptibility"), the initial recommendations for antimicrobial chemotherapy from public institutions, such as the WHO, included tetracycline, chloramphenicol, gentamicin, and cotrimoxazole (Crowe et al., 1996). However, newer compounds such as ciprofloxacin, ceftriaxone, and cefotaxime are also considered since they have shown excellent *in vitro* activity and have been successfully used to treat complicated infections (liver abscess, endocarditis, and septicemia) (Abdel-Haq et al., 2006; Chiu et al., 2003; Crowe et al., 1996; Hoogkamp-Korstanje et al., 2000; Jimenez-Valera et al., 1998;

Lupi et al., 2013). Current protocols include ciprofloxacin 500 mg/12 h for 3–5 days to treat enterocolitis, whereas ciprofloxacin or third-generation cephalosporins in combination with gentamicin should be used for 3 weeks or more for treating complicated and invasive infections.

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Epidemiology

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Y. enterocolitica is widely distributed in nature and can be isolated in water, soil, and from many domestic and wild animals. Bioserotype 4/O:3 is the most prevalent isolated from animals, particularly pigs, and since indistinguishable genotypes among human and porcine strains have been reported, pigs are considered the major reservoir (Bonardi et al., 2013; Bonke et al., 2011; Fredriksson-Ahomaa et al., 2006; Tadesse et al., 2013). This predominant bioserotype, however, has also been recovered from domestic animals, such as dogs and cats, suggesting that pets may also represent a source of human infection, especially for young children (Fredriksson-Ahomaa et al., 2006). On the contrary, the prevalence of strains belonging to biotypes 2 and 3 and serotypes O:5,27, O:8, and O:9 is much lower, despite several studies reporting sporadic cases from the slaughter of pigs, cows, sheep, goats, monkeys, and wild rodents with potential transmission to humans (Bonke et al., 2011; Fearnley et al., 2005; Fukushima et al., 1993; Hayashidani et al., 1995). However, among all the bioserotypes isolated from animals, including the most and least frequently detected, only a few have been associated with human infections, 4/O:3, 2/O:5,27, 2/O:9, and 3/O:3 being the most prevalent (Fredriksson-Ahomaa et al., 2006).

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Even though bioserotype 4/O:3 is also the most prevalent type isolated from humans worldwide, a relatively significant prevalence of other types can also be detected in particular geographic areas as a cause of human yersiniosis. Bioserotype 1B/O:8 strains have been isolated in the United States and have also been sporadically recovered in Japan and Europe, where it has been increasingly reported in Poland (Bottone, 1997; Hayashidani et al., 1995; Rastawicki et al., 2009). In China, the dominant epidemic serotypes are O:5 and O:8, with biotype 1A being the dominant biotype accounting for 84.7% of isolates (Mu et al., 2013). Biotype 1A strains are also significantly recovered from Australia, India, and the United States (Bottone, 1997; Pham et al., 1991a; Sharma et al., 2004). Moreover, bioserotype 3/O:3 has also been responsible for human yersiniosis in Japan and China (Fukushima et al., 1997).

p0120

On analyzing the incidence of yersiniosis over time, statistically significant decreasing trends have been noted in six EU member states: Denmark, Germany, Lithuania, Slovenia, Spain, and Sweden; while on the contrary, increasing trends have been noted in Hungary, Romania, and Slovakia (EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control), 2013). In the United States, the incidence of yersiniosis decreased more than 50% comparing data obtained in 2009 with the period 1996–1998 (CDC (Centers for Disease Control and Prevention), 2010).

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Virulence Factors

p0125 The ability of *Y. enterocolitica* strains to cause disease is attributed to the presence of different virulence factors, either located in the chromosome or on a 70 kb virulence plasmid named pYV, which is only detected in virulent strains (Bottone, 1997; Cornelis et al., 1998; Fabrega and Vila, 2012).

p0130 Three proteins have been shown to take over the invasion process of the intestinal mucosa: Inv, the invasin detected in all isolates (Pepe and Miller, 1993), YadA, the plasmid-encoded adhesin (El Tahir and Skurnik, 2001) and Ail, involved in adhesion and invasion only found among pathogenic strains (Pierson and Falkow, 1993). Two type 3 secretion systems (T3SSs) have also been reported. The Ysc T3SS is encoded by the pYV plasmid and is important during systemic stages of infection (Gemski et al., 1980). The Ysa T3SS, only detected in highly pathogenic biotype 1B strains and involved in early stages of infection, is encoded in the chromosomal Ysa pathogenicity island (Haller et al., 2000; Venecia and Young, 2005). Additional virulence factors include the High-Pathogenicity Island, which is only present in the chromosome of biotype 1B strains and is involved in the production of the siderophore yersiniabactin (Carniel et al., 1996), the Yst enterotoxin, frequently detected in diarrheagenic biotype 1A strains (Singh and Virdi, 2004), and the *myf* operon, which encodes a fibrillar structure reportedly involved in adhesion (Iriarte et al., 1993). Lastly, flagella as well as LPS have also been reported to contribute to virulence (Bengoechea et al., 2004; Young et al., 2000).

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ANTIMICROBIAL SUSCEPTIBILTY

p0135 The resistance profiles to different antimicrobial agents have been examined among strains collected from animal and environmental reservoirs, meat products, as well as those recovered from the clinical setting. Heterogeneity of the antimicrobial resistance pattern is shown to be depending on the bioserotype and geographical distribution (Tables 5.1 and 5.2).

p0140 The levels of resistance to β-lactams, which are the major family of antibiotics currently used, have been extensively studied in strains of both animal and human origin. In general terms, high percentages of resistance to ampicillin are detected: values >85% resistance have been reported for non-clinical strains and >95% in the case of clinical isolates (Abdel-Haq et al., 2000; Baumgartner et al., 2007; Bhaduri et al., 2009; Bonardi et al., 2014; Bonke et al., 2011; Capilla et al., 2003, 2004; Fredriksson-Ahomaa et al., 2010, 2012; Gousia et al., 2011; Mayrhofer et al., 2004; Novoslavskij et al., 2013; Pham et al., 1991a; Prats et al., 2000; Preston et al., 1994; Rastawicki et al., 2000; Tadesse et al., 2013; Terentjeva and Berzins, 2010). Nonetheless, lower rates of resistance have been reported (13–57.1%) for strains obtained from the animal and, to a lesser extent from the human, setting. Strains were collected in the United States, Switzerland, and Canada and variability was shown to depend on

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TABLE 5.1 Frequencies of Resistance to the Most Important Antimicrobial Compounds for *Y. enterocolitica* Clinical Isolates According to Bioserotype and Geographical Area

Area	Sample	Bioserotype	No of Strains	% of Resistance									References
				β-Lactams[a]				Aminoglycosides[b]			Quinolones[c]		
				AMP	AMC	CEF	CXM	GEN	KAN	STR	NAL	CIP	
Poland	Human stool	4/O:3	114	100	0	–	0	0	–	–	–	0	Rastawicki et al. (2000)
Switzerland	Human stool	4/O:3	52	100	1.9	98.1	0	0	–	9.6	–	0	Baumgartner et al. (2007)
		2/O:5	22	100	90.9	100	0	0	–	0	–	0	
		2/O:9	34	100	94.1	100	0	0	–	0	–	0	
Switzerland	Human stool	1A	51	100	92.2	100	0		5.9	0	2		Fredriksson-Ahomaa et al. (2012)
		2/O:5,27	4	100	75	100	0		0	0	0		
		2/O:9	22	100	81.8	100	0		0	0	0		
		3/O:3	2	100	100	100	0		0	0	0		
		4/O:3	47	100	0	100	0		0	14.9	4.3		
Spain	Human stool	4/O:3	46	100	–	–	–	0	–	97.8	–	0	Capilla et al. (2003)
Spain	Human stool	4/O:3	271	–	–	–	–	–	–	–	23	0	Capilla et al. (2004)
Spain	Human stool	4/O:3	20	100	–	100	–	0	–	90	5	0	Prats et al. (2000)

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Germany	Human stool	2/O:9	2	100	–	–	–	0	0	0	0	0	Bonke et al. (2011)
		4/O:3	23	100	–	–	–	0	0	21.7	0	0	
Sweden		4/O:3	45	95.5	–	–	–	0	0	2.2	0	0	
Croatia		4/O:3	7	100	–	–	–	0	0	28.6	0	0	
Lithuania	Human stool	4/O:3	19	100	–	–	–	–	–	5.2	–	0	Novoslavskij et al. (2013)
Australia	Human stool	4/O:3	64	100	0	–	–	0	–	–	–	0	Pham et al. (1991b)
		1A	24	100	100	–	–	0	–	–	–	0	
		3/O:5,27	12	100	100	–	–	0	–	–	–	0	
India	Human stool	1A	36	100	100	–	100	–	–	–	–	–	Sharma et al. (2004)
USA	Human stool	ND[d]	30	97.7	–	–	12	0	–	–	–	–	Abdel-Haq et al. (2006)
Canada	Human stool	O:3	945	99.9	26.3	99.9	–	–	–	–	–	–	Preston et al. (1994)
		O:5,27	58	96.6	98.3	98.3	–	–	–	–	–	–	
		O:8	54	13	1.8	96.3	–	–	–	–	–	–	

[a]*AMP, ampicillin; AMC, amoxicillin/clavulanic acid; CEF, cefalothin; CXM, cefuroxime.*
[b]*GEN, gentamycin; KAN, kanamycin; STR, streptomycin.*
[c]*NAL, nalidixic acid; CIP, ciprofloxacin.*
[d]*ND, Not determined.*

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TABLE 5.2 Frequencies of Resistance to the Most Important Antimicrobial Compounds for *Y. enterocolitica* Non-Clinical Isolates According to Bioserotype and Geographical Area

Area	Sample Source	Bioserotype	No of Strains	% of Resistance									References
				β-Lactams[a]				Aminoglycosides[b]			Quinolones[c]		
				AMP	AMC	CEF	CXM	GEN	KAN	STR	NAL	CIP	
Austria	Meat (chicken, beef, pork, turkey)	4/O:3; ND[d]	118	–	–	–	–	0	0	0	0	0	Mayrhofer et al. (2004)
Greece	Meat (pork)	ND	25	96	–	–	60	36	36	–		24	Gousia et al. (2011)
Italy	Pig tonsils	4/O:3	22	–	0	–	–	5	5	64	5	0	Bonardi et al. (2014)
Latvia	Pig tonsils	4/O:3	71	100	0	100	–	–	–	5.6	0	0	Terentjeva and Berzins (2010)
Lithuania	Pig feces, carcasses	4/O:3	41	100	–	–	–	–	–	7.3	–	0	Novoslavskij et al. (2013)
Switzerland	Pig feces	4/O:3	10	100	10	100	0	0	0	0	–	0	Baumgartner et al. (2007)
	Pig feces	2/O:5	25	100	100	100	0	0	0	8	–	0	
	Pork	2/O:5	26	100	100	100	0	0	0	0	–	0	
	Pig feces	2/O:9	8	100	100	100	0	0	0	0	–	0	

t0015

Switzerland	Pig feces, wild boar	2/O:5,27	9	33.3	–	–	–	0	0	0	0	0	Bonke et al. (2011)
	Pig feces	2/O:9	1	100	–	–	–	0	0	0	0	0	
	Pig feces	4/O:3	20	100	–	–	–	0	0	5	0	0	
Germany	Pig feces, pork	4/O:3	44	90.9	–	–	–	0	0	75	0	0	
Sweden	Pork	4/O:3	13	92.3	–	–	–	0	0	7.7	0	0	
Finland	Pig feces	4/O:3	7	85.7	–	–	–	14.3	14.3	28.6	0	0	
India	Pig, aquatic	1A	10	100	100	–	100	–	–	–	–	–	Sharma et al. (2004)
USA	Pig feces	4/O:3	80	100	0	85	–	0	0	0	0	0	Bhaduri et al. (2009)
	Pig feces	2/O:5	26	100	0	100	–	0	0	0	0	0	
USA	Pig feces	4/O:3	74	31	2.7	–	–	0	0	–	0	0	Tadesse et al. (2013)
	Pig feces	O:5	44	13.6	2.3	–	–	0	0	–	0	0	
	Pig feces	O:9	7	57.1	0	–	–	0	0	–	0	0	
	Pig feces	O:8	5	20	20	–	–	0	0	–	0	0	

[a]*AMP, ampicillin; AMC, amoxicillin/clavulanic acid; CEF, cefalothin; CXM, cefuroxime.*
[b]*GEN, gentamycin; KAN, kanamycin; STR, streptomycin.*
[c]*NAL, nalidixic acid; CIP, ciprofloxacin.*
[d]*ND, Not determined.*

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the bioserotype analyzed (4/O:3, O:8, O:9, 2/O:5,27, O:5) (Bonke et al., 2011; Tadesse et al., 2013; Preston et al., 1994).

p0145 In the case of amoxicillin/clavulanic acid, heterogeneous susceptibility profiles have been seen among *Y. enterocolitica* strains. High levels of resistance (100% for non-clinical strains and >75% for clinical isolates) have been shown to occur in strains belonging to biotypes 1A, 2, and 3 collected from around the world (Baumgartner et al., 2007; Fredriksson-Ahomaa et al., 2012; Pham et al., 1991a; Sharma et al., 2004), including serotype O:5,27 strains from Canada (presumably related to biotypes 2 or 3 according to the most prevalent biotype–serotype associations) (Preston et al., 1994). Tadesse et al., however, reported very low levels of resistance (<3%) for strains serotyped as O:5 and O:9 (putatively belonging to biotypes 2 or 3) obtained from animals in the United States (Tadesse et al., 2013). On the other hand, bioserotype 4/O:3 consistently shows the lowest resistance values, <10%, among all the isolates regardless of the country of isolation (Baumgartner et al., 2007; Bhaduri et al., 2009; Bonardi et al., 2014; Fredriksson-Ahomaa et al., 2012; Pham et al., 1991a; Rastawicki et al., 2000; Tadesse et al., 2013; Terentjeva and Berzins, 2010).

p0150 Similar to the resistance levels observed for ampicillin, most of the *Y. enterocolitica* strains are also resistant to cefalothin, a first-generation cephalosporin. Several studies have reported percentages of resistance >85% in strains of animal origin and >98% in strains isolated from humans (Baumgartner et al., 2007; Fredriksson-Ahomaa et al., 2010; Prats et al., 2000; Preston et al., 1994; Terentjeva and Berzins, 2010). Fortunately, most of the strains studied remain susceptible to more recent cephalosporins, including the second-generation compound cefuroxime and third-generation cephalosporins such as ceftriaxone, ceftazidime, and cefotaxime (Baumgartner et al., 2007; Bhaduri et al., 2009; Bonke et al., 2011; Capilla et al., 2003; Fredriksson-Ahomaa et al., 2010; Gousia et al., 2011; Prats et al., 2000; Preston et al., 1994; Rastawicki et al., 2000; Tadesse et al., 2013; Terentjeva and Berzins, 2010). Nonetheless, a few exceptions have been reported. In 2000, Abdel-Haq et al. reported low levels of resistance to cefuroxime (12%), ceftazidime (11%), and cefotaxime (1%) in strains isolated from patients at the Children's Hospital of Michigan (USA) (Abdel-Haq et al., 2000). Recently, in a study conducted in 2011 in Greece by Gousia et al., a high proportion of *Y. enterocolica* isolates from animals (60%) was resistant to cefuroxime, whereas lower values were observed for ceftriaxone (8%) (Gousia et al., 2011). Regrettably, in these two studies no information was provided regarding the bioserotype of the isolates. More importantly, Sharma et al. detected that all 36 clinical strains tested for antimicrobial susceptibility in India, only belonging to biotype 1A, were resistant to cefotaxime (Sharma et al., 2004).

p0155 To our knowledge little information is available on the susceptibility of *Y. enterocolitica* to cephamycins and carbapenems. Furthermore, data on cefoxitin susceptibility has only been reported in a few studies. First, in a study performed in Australia, the authors revealed that all the isolates belonging to

bioserotype 4/O:3 were susceptible to cefoxitin whereas those belonging to biotypes 1A (serotypes O:5 or O:8) and 3 (serotype O:5,27) were resistant (Pham et al., 1991a). Similarly, a more recent study conducted in Switzerland showed that all bioserotype 4/O:3 clinical isolates were susceptible, while higher resistance rates were observed for biotype 1A (45%) and bioserotypes 2/O:5,27, 2/O:9, and 3/O:3 (>80%) (Fredriksson-Ahomaa et al., 2012). Information regarding imipenem resistance is also scarce. To our knowledge, only one study from Greece has been conducted, showing 8% of the strains of animal origin to be resistant to this drug (Gousia et al., 2011). With respect to clinical isolates, no resistance to imipenem has been reported (Pham et al., 1991a; Rastawicki et al., 2000).

p0160 Other antimicrobials which deserve to be highlighted are those belonging to the group of aminoglycosides. Full susceptibility to kanamycin and gentamicin has been reported in almost all studies regardless of the source of isolation (Abdel-Haq et al., 2000; Baumgartner et al., 2007; Bhaduri et al., 2009; Bonke et al., 2011; Capilla et al., 2003; Mayrhofer et al., 2004; Pham et al., 1991a; Preston et al., 1994; Rastawicki et al., 2000; Tadesse et al., 2013; Terentjeva and Berzins, 2010). A few studies represent an exception since they report levels of resistance ranging from 5% to 36% in animal strains belonging to bioserotype 4/O:3, when information concerning bioserotyping is available, (Bonardi et al., 2014; Gousia et al., 2011; Tadesse et al., 2013); and in human strains belonging to biotype 1A, despite being reported in only one study (Fredriksson-Ahomaa et al., 2012). Higher heterogeneity has been observed for streptomycin among clinical and non-clinical strains. Several studies have revealed a lack of resistance, particularly concerning biotype 2 strains (Baumgartner et al., 2007; Bhaduri et al., 2009; Bonke et al., 2011; Mayrhofer et al., 2004), while others have reported levels of resistance ranging from 5.6% to 28.6% (Baumgartner et al., 2007; Bonke et al., 2011; Fredriksson-Ahomaa et al., 2010, 2012; Novoslavskij et al., 2013; Terentjeva and Berzins, 2010). The highest levels of resistance to streptomycin have been described for strains belonging to bioserotype 4/O:3. Among strains of animal origin, studies carried out in Italy and Germany showed percentages of resistance of 64% and 75%, respectively (Bonardi et al., 2014; Bonke et al., 2011). In the clinical setting, strains isolated in Spanish hospitals have reported the highest levels of resistance to streptomycin (≥90%) (Capilla et al., 2003; Prats et al., 2000); whereas the remaining studies, also performed in Europe, show a prevalence of resistance <30% (Baumgartner et al., 2007; Bonke et al., 2011; Novoslavskij et al., 2013). Cases in which high levels of resistance have been reported are attributed to horizontal transfer of plasmids carrying genes which confer resistance to streptomycin (see subsection on "Mechanisms of resistance to aminoglycoside") (Capilla et al., 2003).

p0165 A much more optimistic situation has been reported for quinolones. Almost all the *Y. enterocolitica* strains of any origin studied have shown a lack of resistance to nalidixic acid or a very low level of resistance (<5%) (Bhaduri et al.,

2009; Bonardi et al., 2014; Bonke et al., 2011; Fredriksson-Ahomaa et al., 2012; Mayrhofer et al., 2004; Prats et al., 2000; Tadesse et al., 2013; Terentjeva and Berzins, 2010). Nonetheless, one study from Spain revealed that up to 23% of the clinical isolates were resistant to this drug (Capilla et al., 2004). In most of the situations in which resistance is reported the strains belong to bioserotype 4/O:3. Moreover, full susceptibility to the fluoroquinolone ciprofloxacin has also been reported in the studies analyzed in this review (Baumgartner et al., 2007; Bhaduri et al., 2009; Bonardi et al., 2014; Bonke et al., 2011; Novoslavskij et al., 2013; Preston et al., 1994; Tadesse et al., 2013; Terentjeva and Berzins, 2010), except one study from Greece in which Gousia *et al*. reported that as many as 24% of strains they isolated from animal samples were ciprofloxacin-resistant (Gousia et al., 2011). Even though nalidixic acid was not evaluated in this study, it is very likely that ciprofloxacin-resistant strains might also be resistant to this drug, as acquisition of mutations in the target genes encoding resistance to quinolones (the most commonly found mechanism of resistance) confers resistance to nalidixic acid prior to ciprofloxacin (see subsection on "Mechanisms of resistance to quinolones").

p0170 In addition to these three main families of antibacterial drugs, tetracyclines, chloramphenicol, and cotrimoxazol can also represent potential treatments for human yersiniosis. Low rates of tetracycline-resistant strains of both animal and human origin have been documented among studies conducted in Europe and Canada, ranging from full susceptibility, particularly observed in biotype 2 strains collected from animals and all biotypes of human isolates, to 12% of resistance for bioserotype 4/O:3 (Baumgartner et al., 2007; Bonke et al., 2011; Fredriksson-Ahomaa et al., 2010; Gousia et al., 2011; Mayrhofer et al., 2004; Novoslavskij et al., 2013; Pham et al., 1991a; Preston et al., 1994; Rastawicki et al., 2000; Terentjeva and Berzins, 2010). Nonetheless, higher rates of tetracycline resistance have been reported in two studies from the United States assessing animal settings: Bhaduri et al. showed that up to 69.2% of serotype O:5 strains were resistant versus 13.8% among serotype O:3 (Bhaduri et al., 2009), whereas Tadesse et al. revealed that intermediate percentages (28.6% and 20%) were observed for serotypes O:9 and O:8, respectively, despite the recovery of a reduced number of isolates (Tadesse et al., 2013).

p0175 Regarding chloramphenicol, most studies report very low levels of resistance, from 0% to 4% of the isolates (Baumgartner et al., 2007; Bhaduri et al., 2009; Bonke et al., 2011; Fredriksson-Ahomaa et al., 2010; Mayrhofer et al., 2004; Pham et al., 1991a; Preston et al., 1994; Tadesse et al., 2013; Terentjeva and Berzins, 2010). Nonetheless, a few exceptions have been reported, although all concerned bioserotype 4/O:3 strains. In the animal setting Bonardi et al. showed 55% of resistance to this drug in a report from Italy (Bonardi et al., 2014), whereas clinical strains analyzed from Spanish hospitals have shown higher levels of resistance: Prats et al. detected an increase in resistance from 20% to 60% comparing strains collected during the period 1985–1987 versus 1995–1998 (Prats et al., 2000), whereas Capilla et al. showed 100% of resistance

and suggested the clonal expansion of a particular isolate in their study (Capilla et al., 2003).

p0180 Similarly, the levels of resistance reported for trimethoprim and cotrimoxazole indicate that most strains remain susceptible (Abdel-Haq et al., 2000; Baumgartner et al., 2007; Fredriksson-Ahomaa et al., 2010; Mayrhofer et al., 2004; Pham et al., 1991a; Preston et al., 1994; Rastawicki et al., 2000; Tadesse et al., 2013; Terentjeva and Berzins, 2010). Only a single study performed in Spain by Capilla et al. reported high levels of resistance to cotrimoxazol of approximately 91% among clinical isolates which belonged to bioserotype 4/O:3 (Capilla et al., 2003).

p0185 Lastly, all the studies reviewed agree that erythromycin is the only antibiotic to which 100% of the *Y. enterocolitica* strains recovered either from animal reservoirs and human feces are resistant, likely associated with the constitutive expression of the efflux pump AcrAB-TolC, the main efflux pump characterized to confer the multidrug resistance (MDR) phenotype among Enterobacteriaceae (Fredriksson-Ahomaa et al., 2010; Novoslavskij et al., 2013; Preston et al., 1994; Terentjeva and Berzins, 2010; Vila et al., 2011).

p0190 Few reports have analyzed the rates of antimicrobial resistance in *Y. enterocolitica* over time in order to assess whether these values have increased in more recent study periods. Preston et al. compared the antimicrobial profile for strains collected during the period 1972–1976 and in 1980, 1985 and 1990. Their results showed that, in general terms, no major change was observed. The most important difference was observed for tetracycline resistance which increased from 0.4% in 1985 to 2% in 1990 (Preston et al., 1994). More recently, Prats et al. evaluated antimicrobial resistance in *Y. enterocolitca* isolated over two periods of time, 1985–1987 and 1995–1998. Their results showed an increase in resistance regarding streptomycin (from 72% to 90%) and chloramphenicol (from 20% to 60%), and, to a lesser extent, nalidixic acid (from 0% to 5%) (Prats et al., 2000).

p0195 Concerning the prevalence of MDR isolates, the results published in the literature have been analyzed in this chapter according to the definition of MDR proposed by Magiorakos et al. in which resistance to three or more antimicrobial classes is required for an organism to be considered MDR (Magiorakos et al., 2012). Several studies, conducted in different countries such as Switzerland, Italy, and the United States, have reported low levels of MDR (<2%) among clinical and non-clinical strains, with most of them belonging to bioserotype 4/O:3 (Baumgartner et al., 2007; Bonardi et al., 2013; Fredriksson-Ahomaa et al., 2012; Tadesse et al., 2013). On the contrary, other reports have shown increased percentages of MDR reaching levels as high as 50–60% (Bonardi et al., 2014; Prats et al., 2000; Sihvonen et al., 2011). This situation may, at least in part, be due to the fact that strains of bioserotype 4/O:3 were the only or predominant group collected and tested in these studies, and/or that a great proportion of these MDR strains may be clonally related and disseminated as an outbreak. This latter consideration was assessed in the study performed by

Sihvonen et al. (2011) which revealed different percentages, 59.1% and 18.3%, for outbreak-related strains and for sporadic cases, respectively (Sihvonen et al., 2011). The antibiotics most frequently found in the MDR patterns include streptomycin, sulfonamides, and chloramphenicol, and to a lesser extent cotrimoxazol, tetracycline, and nalidixic acid. The first association, resistance to streptomycin, sulfonamides, and chloramphenicol, has been shown to be carried by a conjugative plasmid (Sihvonen et al., 2011). Moreover, the association of sulfonamide-streptomycin resistance has also been suggested to be part of a mobile genetic element such as an integron (Prats et al., 2000).

p0200 According to this information and on comparing the percentage of resistance regarding all bioserotypes, most of the resistant strains reported from both the animal and clinical settings belong to bioserotype 4/O:3. Moreover, resistant isolates reported in human settings are likely to originate in the animal environment in which several antimicrobial compounds or related drugs are used or have been used in the veterinary field (e.g., streptomycin, apramycin, tetracycline, chloramphenicol, thiamphenicol, florphenicol, quinolones). Nonetheless, the low percentages of resistance to second- and third-generation cephalosporins, as well as cephamycins, are detected among clinical isolates, suggesting that resistance might emerge directly in the clinical setting as a consequence of human antimicrobial therapies.

p0205 Although the use of ciprofloxacin, third-generation cephalosporins, and gentamicin is the current treatment of choice for yersiniosis, a modest trend toward increased antimicrobial resistance has been observed in *Y. entercolitica*, hence underscoring the need for surveillance of resistance as well as for efforts focused on encouraging a rational use of the antimicrobial compounds.

s0045 MECHANISMS OF ANTIMICROBIAL RESISTANCE

p0210 The success of antibiotics in inhibiting bacterial growth relies on the ability of the drug to recognize its target and achieve the appropriate concentration of the antibiotic for efficient inhibition. Mutations leading to antimicrobial resistance are usually located in the chromosome and their spread depends on the dissemination of a particular clone. Acquisition of antibiotic resistance genes can also be spread by clonal expansion, although since they are located in transferable elements (i.e., transposons and plasmids) they can be horizontally transferred between bacteria (Martinez and Baquero, 2014).

p0215 Most of the transferable genes are embedded in structures called gene cassettes, which usually contain a single coding sequence (most commonly an antibiotic resistance gene) and an integrase-specific recombination site (*attC*). Integrons are not mobile genetic elements, but are characterized by their ability to capture and accumulate gene cassettes by site-specific recombination. Most of the clinically relevant antimicrobial-resistance-encoding integrons belong to class 1, whereas integrons belonging to class 2 and class 3 similarly carry cassettes predominantly composed of antibiotic resistance genes and are also of

clinical concern (Hall, 2012). However, integrons can only be mobilized, and are responsible for transmission of resistance genes, when incorporated into transposons. Transposons are segments of DNA flanked by terminal inverted repeats carrying a transposition region. In some cases this transposition region includes the transposase gene (*tnpA*), which allows self-replication and mobilization to a new position in the chromosome or plasmid (Griffiths et al., 1999). Furthermore, integrons, transposons or standalone resistance genes are often harbored in plasmids, the major source for dissemination of antimicrobial resistance genes. In particular, conjugative plasmids can be transferred among different pathogens at a high frequency, thereby favoring their rapid and broad dissemination.

s0050

Mechanisms of Resistance to β-lactams

p0220

As mentioned previously, *Y. enterocolitica* is intrinsically resistant to certain β-lactam antibiotics (e.g., ampicillin and the first-generation cephalosporin cefalothin). It is well established that this phenotype is due to the presence and expression of two chromosomally encoded β-lactamase genes, namely *blaA* and *lab* (Cornelis and Abraham, 1975). The *blaA* gene is constitutively expressed and encodes the broad-spectrum class A penicillinase (Cornelis and Abraham, 1975). The presence of clavulanic acid (20 μM) completely inhibits the activity of this enzyme, whereas no change is detected in the presence of aztreonam (Pham et al., 1991a). Unlike BlaA, the BlaB enzyme is a class C cephalosporinase (AmpC-type β-lactamase), which can be inhibited by aztreonam and cloxacillin but not by clavulanic acid (Cornelis and Abraham, 1975; Pham et al., 1991a). Moreover, imipenem has been shown to induce BlaB activity (Pham and Bell, 1992).

p0225

In order to study the individual contribution of these two enzymes, Seoane et al. reported the antimicrobial susceptibility profile to different penicillins and cephalosporins by cloning the *blaA* and *blaB* genes from strains belonging to bioserotypes 4/O:3 and 2/O:5b, respectively, and expressing them individually in an *E. coli* recipient strain. The results showed an increase in resistance to all of the antibiotics tested for the two enzymes, with only one exception; there was no increase in the MIC of cefoxitin in the case of BlaA. The most representative results for BlaA were detected for ampicillin and carbenicillin, whereas for BlaB the highest increments were observed for ampicillin, cefalothin, and cefotaxime. BlaB conferred higher levels of resistance to almost all the cephalosporins tested (Table 5.3) (Seoane and Garcia-Lobo, 1991).

p0230

Among clinical and non-clinical *Y. enterocolitica* strains, the presence and differential expression of these two enzymes have been characterized and associated with different levels and spectra of resistance. These strain variations have been reported to largely depend on the bioserotype and the geographic origin of the isolate (Table 5.4). Analyses on the presence or absence of the *blaA* and *blaB* genes by PCR amplification among clinical and non-clinical strains

t0020 **TABLE 5.3 Effect of BlaA and BlaB Expression on the MICs of Several β-lactams**

Strain	Enzyme Produced	Antimicrobial Susceptilities (MICs, μg/mL)							
		Penicillins[a]			Cephalosporins[b] (Generation)				
		AMP	BPC	CAR	CFL (1st)	CEF (1st)	MAN (2nd)	FOX (2nd)	CTX (3rd)
E. coli HB101		2	ND[c]	4	2	2	<1	2	<0.03
E. coli HB101	BlaA	1024	ND	>1024	16	128	32	2	0.12
E. coli HB101	BlaB	512	ND	16	128	1024	16	64	4

[a]*MP, ampicillin; BPC, benzylpenicillin; CAR, carbenicillin.*
[b]*CFL, cephaloridine; CEF, cefalothin; MAN, cefamandole; FOX, cefoxitin; CTX, cefotaxime.*
[c]*ND, Not determined.*

collected from all over the world and belonging to various biotypes (1A, 1B and 2–5) and serotypes (O:3, O:9 and O:5,27) indicate that almost all the strains harbor both genes (Bonke et al., 2011; Sharma et al., 2006; Stock et al., 1999, 2000b). While Stock et al. initially observed variable results in strains belonging to biotypes 1A, 1B, and 3 (Stock et al., 2000b), this situation was later suggested to be a problem due to lack of amplification in the previous protocol (Sharma et al., 2006). Only one bioserotype 3/O:3 strain isolated from a chinchilla in Germany lacked both genes (Bonke et al., 2011).

p0235 The results obtained from such qualitative studies are complemented by phenotypic analysis. Both the β-lactam hydrolyzing activity from crude enzyme extracts and the MIC of several β-lactams are tested for inhibition with clavulanic acid, aztreonam or cloxacillin or induction with imipenem (0.5 mg/L) (Bonke et al., 2011; Pham et al., 1991a; Stock et al., 1999). Inhibition by clavulanic acid is an indicator of BlaA activity, whereas inhibition by aztreonam or cloxacillin indicates BlaB production, which may or may not be induced by imipenem. If a complete inhibition was observed for a given compound, the corresponding enzyme was assumed to be the only enzyme expressed (Stock et al., 1999).

Concerning expression of the class A β-lactamase, BlaA, a repeated pattern can be concluded from several studies including isolates from different geographic regions and different sources (Table 5.4). Most of the strains (93–100%) of clinical and non-clinical origin belonging to biotypes 1A, 1B, 2 (only serotype O:9), and 4 expressed the BlaA enzyme (Bonke et al., 2011; Pham et al., 1991a, 1995; Sharma et al., 2006; Stock et al., 1999, 2000b). Only

t0025

TABLE 5.4 Presence, Production, and Induction of *blaA*/BlaA and *blaB*/BlaB in *Y. enterocolitica* Clinical and Non-Clinical Isolates According to Bioserotype and Geographic Area

Sample	Geographic Area	Bioserotype	No of Strains	% of Presence, Production, and Induction					References
				blaA	BlaA	*blaB*	BlaB	BlaB Induction	
Clinical origin	Australia	1A	24	–[a]	100	–	100	100	Pham et al. (1991a), Pham and Bell (1992)
		3	12	–	0	–	100	100	
		4/O:3	64	–	100	–	0	0	
	Europe, Asia, Brazil, South Africa	4/O:3	31	–	100	–	100	100	Pham et al. (1995)
	Australia, New Zealand	4/O:3	10	–	100	–	0	0	
	Canada	4/O:3	3	–	100	–	0	0	
	Canada	4/O:3	4	–	100	–	100	100	
	Germany	2/O:9	2	100	100	100	100	–	Bonke et al. (2011)
	Germany	4/O:3	23	100	100	100	91.3	–	
	Sweden	4/O:3	45	100	100	100	91.1	–	
	Croatia	4/O:3	7	100	100	100	100	–	
	India	1A	35	100	100	100	94.3	94.3	Sharma et al. (2006)
	Europe	1A	15	100	100	100	100	100	
	USA	1A	1	100	100	100	100	100	

(*Continued*)

Clinical and non-clinical origin	Germany	1A	14	0	92.9	100	100	100	Stock et al. (2000b)
		1B	12	16.7	100	100	100	91.7	
		3	22	40.9	45.5	100	100	45.5	
	Germany	2	12	100	41.7	100	100	41.7	Stock et al. (1999)
		4	13	100	100	100	100	100	
		5	10	100	80	100	100	60	
Non-clinical origin	India	1A	30	100	100	100	100	100	Sharma et al. (2006)
	Switzerland	2/O:5,27	9	100	0	100	100	–	Bonke et al. (2011)
	Switzerland	2/O:9	6	100	100	100	100	–	
	Germany	3/O:3	2	50	50	50	50	–	
	Germany, Sweden, Switzerland, Finland, Croatia	4/O:3	92	100	97.8	100	96.7	–	

[a]*Not determined in this study.*

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three strains were categorized as non-producers: one belonging to biotype 1A collected in Germany and two belonging to biotype 4 collected from pigs in Germany and Finland (Bonke et al., 2011; Stock et al., 2000a). In contrast, none of the strains belonging to biotype 2 (serovar O:5,27 strains isolated from animals in Switzerland) and biotype 3 (collected from humans in Australia) expressed BlaA. The former group harbored the gene whereas in the latter group the presence of *blaA* was not determined (Bonke et al., 2011; Pham et al., 1991a). Approximately 50% of the strains belonging to biotypes 2 (undetermined serotype) and 3 isolated in Germany expressed *blaA*, however, the reasons were different: all the biotype 2 strains harbored the gene but only some strains expressed it, whereas all biotype 3 strains demonstrating BlaA activity were also positive for the presence of the gene (Bonke et al., 2011; Stock et al., 1999, 2000b). Lastly, 80% of the biotype 5 strains, predominantly of animal origin, expressed BlaA.

p0245 If expression of the BlaB enzyme is considered, a great percentage (91–100%) of the clinical and non-clinical strains belonging to biotypes 1A, 1B, 2 (both serotypes O:9 and O:5,27), 3, and 5 produced this β-lactamase (Bonke et al., 2011; Pham et al., 1991a, 1995; Sharma et al., 2006; Stock et al., 1999, 2000b). Two clinical 1A strains isolated in India, which were positive for the presence of the gene, and a single biotype 3 strain collected from a chinchilla in Germany, which did not harbor *blaB*, did not express the BlaB enzyme (Bonke et al., 2011). The remaining strains belonging to the most commonly isolated bioserotype 4/O:3 showed different results according to the geographical region from which they were isolated. Strains collected in Australia, New Zealand, and Canada did not express the BlaB cephalosporinase (the presence or absence of *lab* was not determined), whereas most of the strains isolated in Europe, Asia, Brazil, South Africa, and Canada did express the enzyme (Bonke et al., 2011; Pham et al., 1991a, 1995; Stock et al., 1999). Six clinical isolates did not express the protein; two isolated in Germany and four in Sweden (Bonke et al., 2011) (Table 5.4).

p0250 The results concerning induction of BlaB activity in the presence of imipenem are also shown in Table 5.4. The results have revealed that, when BlaB is produced it is almost always induced in the presence of imipenem regardless of the biotype. All strains belonging to biotypes 1A and 4, which are positive for BlaB production, show imipenem induction (Pham and Bell, 1993; Sharma et al., 2006; Stock et al., 2000b). More than 91% of the biotype 1B strains also produce BlaB which can be further induced (Stock et al., 2000b). Different results have been observed regarding strains belonging to biotypes 2 and 3. Stock et al. detected an association between BlaB inducibility and BlaA production: less than 50% of the strains only producing BlaB showed an inducible phenotype, suggesting that this enzyme could not be further induced because maximal levels were constitutively produced to compensate for the lack of BlaA (Stock et al., 1999, 2000b). Nonetheless, no clear results have been observed for biotype 5 strains and in other studies. Stock et al. showed that 60% of biotype

5 isolates showed BlaB induction regardless of the predominant expression of the enzymes (Stock et al., 1999), whereas Pham et al. reported induction of BlaB for all biotype 3 strains even in those producing only BlaB (Pham and Bell, 1993).

p0255 In view of these results, the high percentage of resistance to ampicillin (>85%) can be attributed to the fact that *Y. enterocolitica* strains always produce at least one of the two chromosomal β-lactamases, and both contribute to ampicillin resistance (Seoane and Garcia-Lobo, 1991). However, a few strains have been characterized to express both proteins and were susceptible or intermediate susceptible based on the MIC of ampicillin (Bonke et al., 2011). Cefalothin resistance (>85%) can be easily attributed to BlaB production, a trait generally detected in most of the strains (>91%). Nonetheless, there is no information regarding the MIC of cefalothin for the subset of 4/O:3 strains which do not express the cephalosporinase BlaB (Pham et al., 1991a, 1995). Although these strains may be considered cefalothin-susceptible, there are no data available to confirm this.

p0260 Conversely, there is no easy explanation for *Y. enterocolitica* susceptibility to amoxicillin-clavulanic acid. On one hand, Pham et al. have reported that all bioserotype 4/O:3 strains that only express BlaA, collected from Australia, New Zealand, and Canada, are susceptible to this combination since BlaA can be inhibited by clavulanic acid; whereas those expressing both β-lactamases are resistant and show increased MICs of cefoxitin (Pham et al., 1991a, 1995; Pham and Bell, 1993). On the other hand, other reports have shown that bioserotype 4/O:3 strains expressing both BlaA and BlaB are susceptible to amoxicillin-clavulanic acid, with no explanation being suggested (Bonardi et al., 2014; Fredriksson-Ahomaa et al., 2012; Rastawicki et al., 2000; Terentjeva and Berzins, 2010).

p0265 Thus, even though the phenotype of β-lactam resistance in most strains can be explained by the ability to express one or both of the chromosomal β-lactamases, there are particular situations in which this association does not provide a reasonable explanation. The levels of expression of these two enzymes, attributed to different regulatory effects which may depend on the intrinsic properties of the strain or a particular bioserotype, might account for such differences (Bonke et al., 2011; Pham et al., 1991b; Stock et al., 1999). Moreover, it is worth mentioning that, to the best of our knowledge, to date no isolate has been reported to carry an extended-spectrum β-lactamase (ESBL) which may contribute to resistance to such compounds.

s0055

Mechanisms of Resistance to Aminoglycosides

p0270 Aminoglycosides require an energy-dependent uptake system to reach their target site, a highly conserved motif of the 16S rRNA, which is part of the 30S ribosomal small subunit and essential for correct protein synthesis. There are two major mechanisms of resistance to aminoglycosides characterized so

far: (i) reduction in the intracellular concentration of the antimicrobial via either decreased aminoglycoside uptake or increased active efflux; and (ii) inactivation of the antimicrobial by enzymatic modification preventing binding between the drug and the ribosomal RNA (Galimand et al., 2003; Magnet et al., 2001; Taber et al., 1987).

p0275 These structural modifications represent the most common mechanism of aminoglycoside resistance and are the result of particular plasmid-borne genes encoding enzymes belonging to three different classes: aminoglycoside nucleotidyltransferases (ANTs), aminoglycoside phosphotransferases (APHs), and aminoglycoside acetyltransferases (AACs). Each particular group includes enzymes with different regional specificities for aminoglycoside modifications: there are four nucleotidyltransferases (ANT(6), ANT(4″), ANT(3″), and ANT(2″)), seven phosphotransferases (APH(3′), APH(2″), APH(3″), APH(6), APH(9), APH(4), and APH(7″)), and four acetyltransferases (AAC(2′), AAC(6′), AAC(1), and AAC(3)). There also exists a bifunctional enzyme, AAC(6′)–APH(2″), that can sequentially acetylate and phosphorylate its aminoglycoside substrates (Kotra et al., 2000). The occurrence of the genes *ant(3″)-Ia* and *ant(3″)-Ib*, which confer resistance to streptomycin-spectinomycin, have been described as the most commonly found in clinical strains (Capilla et al., 2003; Levesque et al., 1995). These genes are integrated in mobile genetic elements as part of gene cassettes, integrons, and transposons and, thereby, can be disseminated among bacteria (Kotra et al., 2000). Moreover, in addition to their potential spread, cross-resistance has also been reported to be a problem that could limit the effective use of aminoglycoside compounds. For example, the use of apramycin in pig production has been correlated to the increased occurrence of cross-resistance with gentamicin and other aminoglycosides in *E. coli* strains collected from pigs carrying the *aac(3)-IV* gene in conjugative plasmids (Jensen et al., 2006). This phenomenon should be taken into account when determining the impact of antimicrobial use in animals on the dissemination of resistance into the clinical setting.

s0060 ## Mechanisms of Resistance to Quinolones

p0280 Overall, resistance to quinolones can be mediated by both chromosome- and plasmid-related mechanisms. Chromosomal mutations are the most prevalent mechanism contributing to quinolone resistance. These mutations can affect the genes encoding the type-II topoisomerase protein targets (*gyrA* and *gyrB* for the DNA gyrase, and *parC* and *parE* for the topoisomerase IV) and hence decrease the binding affinity towards the drug. In addition, chromosomal mutations resulting in decreased expression of porins, increased expression of efflux pumps, or the interplay of both, can be acquired leading to decreased accumulation of quinolones inside the bacteria. In terms of plasmid-mediated quinolone resistance, the following mechanisms have been found: (i) the expression of an aminoglycoside-modifying enzyme (AAC(6′)Ib-cr) which has the ability

to acetylate an amino group in the piperazine ring of the quinolone structure; this enzyme affects all quinolones with the exception of those which have the amino group blocked, such as levofloxacin; (ii) the *qnr* gene family, which encode a peptide able to protect the complex between the DNA and the DNA gyrase or topoisomerase IV, thereby preventing quinolone binding; and (iii) two efflux pumps, OqxAB and QepA. All of these proteins lead to relatively small increases in the MICs of quinolones, but these changes are sufficient to facilitate the selection of mutants, mainly in the *gyrA* gene, with higher levels of resistance (Fabrega et al., 2009).

p0285 Since wild-type strains of *Y. enterocolitica* are usually susceptible to quinolones, few reports have focused on the study and characterization of the mechanisms involved. In a study conducted in Spain, 23% of the *Y. enterocolitca* strains were resistant to nalidixic acid, all of which had a mutation in the *gyrA* gene and, in some cases, overexpression of an efflux pump was also detected (Capilla et al., 2004). Similar results have recently been published by Drummond et al. in a study in which three nalidixic acid-resistant strains were isolated from human samples. All three strains carried a *gyrA* mutation in addition to increased efflux (Drummond et al., 2013). Although the authors in these two studies did not identify the efflux pump involved, AcrAB/TolC would be the most likely candidate according to its relevance in Enterobacteriaceae and taking into account that quinolones can be found among its substrates (Okusu et al., 1996; Vila et al., 2011). Moreover, four mutations, one in each of the *gyrA* and *gyrB* genes and two in *parC*, were found in addition to the overexpression of AcrAB-TolC in a quinolone-resistant *Y. enterocolitica* mutant obtained *in vitro*, showing a ciprofloxacin MIC of 64 µg/mL as well as resistance to other unrelated antibiotics (tetracycline, erythromycin, chloramphenicol, and cefoxitin). This combination of mechanisms explained the high level of resistance to ciprofloxacin and the MDR pattern in this mutant. The overexpression of AcrAB-TolC was found to be related to the increased expression of *marAYe*, an ortholog of the global regulator *marA* shown to activate expression of the *acrAB* and *tolC* genes (Fabrega et al., 2009, 2010). As far as we know, no plasmid-mediated quinolone resistance has been reported so far in *Y. enterocolitica.*

s0065

CONCLUDING REMARKS

p0290 Yersiniosis is a foodborne disease acquired via the ingestion of food or water mainly contaminated with *Y. enterocolitica*. Despite not being among the most frequent food-related pathogens, it is of great concern in terms of food safety as it can replicate at refrigeration temperature. The clinical manifestations and severity of the disease are diverse but, fortunately, the pathogen generally colonizes the gastrointestinal tract and causes local infection, which resolves naturally without antibiotic treatment. However, in some cases, mainly among inmunocompromised patients and children under 4 years of age, complications and/or systemic infection may occur, leading to the need for antimicrobial therapy.

p0295 Bioserotype 4/O:3 is widely distributed worldwide in both the clinical and non-clinical settings. Although resistance to most antimicrobial compounds still remains low in *Y. enterocolitica* clinical isolates, strains belonging to this bioserotype are more likely to be resistant to antimicrobials than are strains of other biotypes. Thus, in life-threatening situations the recommended antibacterial therapy includes either fluoroquinolones or third-generation cephalosporins (the antibiotics showing the highest levels of susceptibility) in combination with gentamicin for which the pathogen is still broadly susceptible. In this way, possible treatment failure as well as infrequent resistance to certain compounds should be overcome. Nevertheless, since pigs are the main reservoir of *Y. enterocolitica* and cross-resistance with antibiotics used in the veterinary field occurs, continuous surveillance of the antimicrobial susceptibility profile of *Y. enterocolitica* is needed in the swine industry. In addition, clonal dissemination of strains carrying multiple-resistance genes in the hospital environment also deserves special attention.

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NON-PRINT ITEM

Abstract

Yersinia enterocolitica are zoonotic bacteria, capable of being transmitted from animals to humans via ingestion of contaminated products. This pathogen causes mainly enterocolitis characterized by fever, watery diarrhea, and abdominal pain. *Y. enterocolitica* strains can be classified into biotypes or serotypes based on biochemical tests and the somatic O antigen, with six biotypes (1A, 1B and 2–5) and more than 57 serotypes. In both humans and animals, bioserotype 4/O:3 is the most prevalent type isolated worldwide. Currently, most *Y. enterocolitica* isolates are susceptible to aminoglycosides, chloramphenicol, tetracycline, trimethoprim-sulfamethoxazole, ciprofloxacin, and third-generation cephalosporins *in vitro*. The last two antibiotics, in combination with gentamicin, represent the treatment of choice for enteritis and systemic infections, respectively. However, resistance to several of these compounds has been reported and the mechanisms to the most representative families (β-lactams, aminoglycosides, and quinolones) are described in this chapter.

Keywords

Yersinia enterocolitica, bioserotype, antimicrobial susceptibility, β-lactamase, foodborne pathogen, animal reservoir, third-generation cephalosporin, ciprofloxacin

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