3. CONCLUSIONES

3. CONCLUSIONES

Las principales conclusiones que se pueden extraer de los resultados obtenidos en el presente trabajo son las siguientes:

1. La virulencia de *S. typhimurium* no aumenta con el incremento de la tasa de mutagénesis espontánea producida por la inactivación del gen *mutS.*

2. La introducción en *S. typhimurium* de un plásmido portador de los genes *umuDC* de *E. coli,* responsables de la reparación tendente al error, no provoca un aumento de su virulencia.

3. El nivel de lesiones en el DNA que se produce a lo largo del proceso infectivo en las células de *S. typhimurium* no es suficiente para activar completamente el sistema de reparación de emergencia o SOS.

4. Los mutantes *fur* poseen una menor concentración intracelular de cAMP que origina un descenso en la expresión de los genes controlados por dicho nucleótido.

5. La baja concentración intracelular de cAMP de los mutantes *fur* es atribuible a la presencia en éstos de una mayor cantidad de Fe^{2+} libre que incrementa la actividad de la enzima 3',5'-fosfodiesterasa codificada por el gen *cpdA*.

6. El promotor *flhDC,* además de estar regulado por el complejo CRP-cAMP, se encuentra bajo el control directo de la proteína Fur.

7. Los mutantes Fur^- de *S. typhimurium* presentan un descenso de un orden de magnitud en su virulencia cuando son inoculados intraperitonealmente. Esta disminución es atribuible a la menor expresión del gen *fliZ* y por sus bajos niveles de cAMP.

8. La expresión del gen *fljB,* que codifica una de las dos flagelinas alternativas de *S. typhimurium,* no está regulada por el complejo CRP-cAMP ni por la proteína Fur.

9. La inactivación del gen *zur*, cuyo producto regula los sistemas de transporte de zinc, solo origina un descenso de un orden de magnitud en la virulencia de *S. typhimurium* cuando se inocula intraperitonealmente.

10. La virulencia de *S. typhimurium* sufre una disminución de aproximadamente tres ordenes de magnitud cuando se mutageniza el gen *znuC*, responsable del transporte de alta afinidad de zinc.

4.BIBLIOGRAFÍA

4. BIBLIOGRAFÍA

Aagaarad, A., y P. Brzezinski. 2001. Zinc ions inhibit oxidation of cytochrome *c* oxidase by oxygen. FEBS Lett.2001 62:173-182

Abdul-Tehrani, H., A. J. Hudson, Y. Chang, A. R. Timms, C. Hawkins, J. M. Williams, P. M. Harrison, J. R. Guest y S. C. Andrews.1999. Ferritin Mutants of *Escherichia coli* are iron deficient and growth impaired, and *fur* mutants are iron deficient. J. Bacteriol. 181:1415-1428

Achenbach, L. A., y W. Yang.1997. The *fur* gene from *Klebsiella pneumoniae*: characterization, genomic organization and philogenetic analysis. Gene 185: 201-207

Adilkashmi, T., P. D. Ayling y C. Ratledge.2000. Mutational analysis of a role for salicylic acid in iron metabolism of *Mycobacterium smegmatis*. J. Bacteriol.. 182:264-271

Aisen P.1998. Transferrin, the transferrin receptor and the uptake of iron by cells. Metal Ions Biol. Syst. 35:585-631

Andrewa, S. C., P. M. Harrison y J. R. Guest.1989.Cloning sequencing and maping the bacterioferritin gene (*bfr*) of *E. coli* K-12. J. Bacteriol.. 179:3939-3947

Andrews, S. C. 1998. Iron storage in bacteria. Adv. Microbiol. Physiol. 40:281-351

Andrews, S. C., J. M. A. Smith, C. Hawkins, J. M. Williams, P. M. Harrison y J. R. Guest.1993. Overproduction, purification and characterization of the bacterioferritin of *Escherichia coli* and a C-extended variant. Eur. J. Biochem. 213:329-338

Ankenbauer, R. G. y H. N. Quan.1994. FptA, the Fe(III)-pyochelin receptor of *Pseudomonas aeruginosa*: a phenolate siderophore receptor homologous to hydroxamate siderophore receptors. J. Bacteriol.. 176:307-319

Azaiwa, S. 2001. Bacterial flagella and type III secretion system. FEMS Microbiol. Lett. 10053:1-8

Bailey, S., R. W. Ewans, R. C. Garratt, B. Gorinsky, S. Hasnain, C. Horsburgh, H. Jhoti, P.F. Lindley, A. Mydin y R.Sarra. 1998. Molecular structure of serum transferrin at 3.3-Å resolution. Biochemistry 27:5804-58212

Baker E. N., B. F. Anderson, H.M. Baker, C. L. Day, M. Haridas, G.E. Norris, S. V. Rumball, C. A. Smith y D. H. Thomas.1994.Three-dimensional structure of lactoferrina in various functional states. Adv. Exp. Med. Biol. 357:1-12

Barghouthi, S., R. Young, M. O. Olson, J. E. Arceneaux, L. W. Clem y B. R. Byers. Amonabactin, a novel tryptophan- or phenylalanine-containing phenolate siderophore in *Aeromonas hydrophila*. J. Bacteriol. 171:1811-1816

Bäumler, A. J., T. L. Norris, T. Lasco, W. Voigt, R. Reissenbrodt, W. Rabsch y F. Heffron.1998. IroN, a novel outer membrane siderophore receptor characteristic of *Salmonella enterica*. J. Bacteriol. 180, No.6:1446-1453

Beard, S. J., M. N. Hughes y R. K. Poole. 1995. Inhibition of the cytochrome *bd*-terminated NADH oxidase system in *E. coli* K-12 by divalent cations. FEMS Microbiol. Lett. 131:205-210

Beard, S. J., R. Hashim, J. Membrillo-Hernández, M. N. Hughes y R. K. Poole. 1997. Zinc(II) tolerance in *Escherichia coli* K-12: evidence that the *zntA* gene (o732) encodes a cation transport ATPase. Mol. Microbiol. 25: 883-891

Beard, SJ,R. Hashim, G. Wu, M. R. Binet, M. N. Hughes y R. W. Poole.2000. Evidence for transport of zinc(II) ions via the *pit* inorganic phosphate transport system in *Escherichia coli*. FEMS Microbiol. Lett. 184 (2):231-235

Bearson, S., B. Bearson y J. W. Foster. 1997. Acid stress responses in enterobacteria. FEMS Microbiol. Lett. 147: 173-180

Berlett, B. S., P.B. Chock, M. B. Yim y E. R. Stadtman.1990. Manganese(II) catalyses the bicarbonate-dependent oxidation of amino acids by hydrogen peroxide and the amino acid-facilitated disminution of hydrogen peroxide. Proc. Natl. Acd. Sci. USA 87:389-393

Bijlsma, J.J.E., A. H. M. van Vliet, N. J. Hughes, S. Häg, S. Bereswill, D. J. Kelly. C. M. J. E. Vandenbrouke-Grauls, M, Kist y J. G. Kusters.2002. The Helicobacter pylori homologue of the ferric uptake regulator is involved in acid resistance. Infect. Immun. 70:606-611

Björkman, J., D. Hughes y D. I. Andersson.1998. Virulence of antibiotic-resistant *Salmonella typhimurium.* Proc. Natl. Acad. Sci. USA 95: 3949-3953

Bosch, M., R. Tarragó, M.E. Garrido, S. Campoy, A.R. Fernández de Henestrosa, A. M. Pérez de Rózas, I. Badiola y J. Barbé.2001.Expression of the *Pasteurella multocida ompH* gene is negatively regulated by Fur protein. FEMS Microbiol. Lett. 203: 35-40

Boyd, J. M. N. Oza y J. R. Murphy.1990. Molecular cloning and DNA sequence analysis of a diphtheria *tox* iron-dependent regulatory element (*dtxR*) from *Corynebacterium diphtheria*. Proc. Natl. Acad. Sci. USA 87:5968-5972

Brocklehurst, K. R. , J. L. Hobman, B. Lawley, L. Blank, S. J. Marshall, N. L. Brown y A. P. Morby. 1999. ZntR ia a Zn(II)-responsive Mer-like transcriptional regulator of znuA in E. coli. Mol. Microbiol. 31:893-902

Brown, M. R. W., H. Anwar y P. A. Lambert. 1984. Evidence that mucoid *Pseudomonas aeruginosa* in the cystic fibrosis in lung grown under iron-restricted conditions. FEMS Microbiol. Lett. 21:113-117

Bsat, N., A. Herbig, L. Casillas-Martínez, P. Setlow y J. D. Helmann.1998.*Bacillus subtilis* contains multiple Fur homologs: identification of the iron uptake (Fur) and peroxide regulon (PerR) repressors. Mol. Microbiol. 29:189-198

Buchmeier, N. A. y S. J. Libby. 1997. Dynamics of growth and death within a *Salmonella typhimurium* population during infection of macrophages. Can. J. Microbiol. 43: 29-34

Buchmeier, N. A., S. J. Libby, Y. Xu, P. C. Loewen, J. Switala, D. G. Guiney y F. C. Fang.1995. DNA repair is more important than catalase for *Salmonella* virulence in mice. American Society for Clinical Investigation, Inc. 95:1045-1053

Buettner, M. J., E, Spitz y H. V. Rickenberg.1973.Cyclic adenosine 3',5'-monophosphate in *Escherichia coli.* J. Bacteriol.. 14:1068-1073

Carrano, C. J., R. Bohnek y B. matzanke.1996.Ferritin from mycelia of *Absidia spinosa* is a bacterioferritin. FEBS Lett. 390:261-264

Cellier, M. F., I. Bergevin, E. Boyer y E. Richer. 2001. Polyphyletic origins of bacterial Nramp transporters. Trends Genet. 17:365-370

Cellier, M., A. Beiouchi, P. Gros.1996.Resistance to intracellular infections: comparative genomic analysis of NARMP1. Trends Genet. 12:201-4

Chasteen, N. D., y P. M. Harrison.1999.Mineralization of Ferritin: an efficient means of iron storage. J. Struct. Biol. 126:182-194

Chen, C., y S. A. Morse. 2001. Identification and characterization of a high-affinity zinc uptake system in *Neisseria gonorrhoeae*. FEMS Microb. Lett. 10054: 1-5

Chilcott, G. S. y K. T. Hughes.2000. Coupling of flagellar gene expression to flagellar assembly in *Salmonella enterica* serovar Thyphimurium and *Escherichia coli*. Microb. Mol. Biol. Rev. 64, No.4: 694-708

Conlin, C. A., K. Hakenson, A. Liljasy C. G. Miller.1994.Cloning and nucleotide sequence of the cyclic AMP receptor protein-regulated *Salmonella typhimurium pepE* gene and cristalization of its product, an α-aspartil dipeptidase.J. Bacteriol.. 176: 166-172

Cope, L.D., R. Yogev, C. A, Slaughter, U. Muller-Eberhard y E. J. Hansen. 1995. A gene cluster involved in the utilization of both free heme and heme-hemopexin by *Haemophilus influezae* type b. J. Bacteriol.. 177:2644-2653

Cornelissen, C. N., M. Kelley, M. M. Hobbs, J. E. Anderson y J. G. Cannon.1998.The transferrin receptor expressed by gonococcal strain FA1090 is required for the experimental infection of human male volunteers. Mol. Microbiol. 27:611-616

Courcol, R. J., D. trivier, M. C. Bissinger, G. R. Martin y M. R. Brown. 1997.Siderophore production by *Staphylococcus aureus* and identification of iron-regulated proteins. Infect. Immun. 65: 1944-1948

Curtiss, R., III y S.M. Kelly.1987.*Salmonella typhimurium* deletion mutants lacking adenylate cyclase and the cyclic AMP receptor protein are avirulent and immunogenic. Infect. Immun. 64: 663-673

Dalet, K., E. Gouin, Y. Cenatiempo, P. Cossart y Y. Hechard.1999. Characterization of a new operon encoding a Zur-like protein and associated ABC zinc permease in *Listeria monocytogenes*. FEMS Microbiol. Lett. 174:111-116

Davis, R. W., D. Botstein y J. R. Roth.1980.Advanced bacterial genetics, A manual for genetic engineering.Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.

de Lorenzo, V., M. Herrero, F. Giovannini y J.B. Neilands.1988. Fur (ferric uptake regulation) protein and CAP (catabolite-activator protein) modulate transcription of *fur* gene in *E. coli.*Eur. J. Biochem. 173: 537-546

De Voss, J. J., K. Rutter, B. G. Schroeder y C. E. BarryIII.1999. Iron adquisition and metabolism by Mycobacteria. J. Bacteriol. 181: 4443-4451

De Voss, J. J., K. Rutter, B. G. Schroeder, H. Su, Y. Zhu y C. E. Barry III.2000. The salicylate-derived mycobactin siderophores of *Mycobacterium tuberculosis* are essential for growth in macrophages. Prot. Natl. Acad. Sci. USA 97:1252-1257

Dintilhac, A., G. Alloing, C. Granadel y J. P. Clarverys.1997. Competence and virulence of *Streptococcus pneumoniae*: *adc* and PsaA mutants exhibit a requirement of Zn and Mn resulting from inactivation of a putative ABC metal permeases. Mol. Microbiol. 25: 727-739

Domenech, P., A. S. Pym, M. Cellier, C. E. Barry, S. T. Cole. 2002. Inactivation of the *Mycobacterium tuberculosis* Nramp orthologue (*mntH*) does not affect virulence in a mouse model of tuberculosis. FEMS Microbiol. Lett. 207(1):81-86

Drubrac, S., y D. Touati.2000. Fur positive regulation of iron superoxide dismutase in *Escherichia coli*: Functional análisis of the *sodB* promoter. J. Bacteriol. 182: 3802-3808

Eide, D., M. Borderius, J. Fett, y M. L. Guerinot. 1996. A novel iron-regulated metal transporter from plants identified by functional expresión in yeast. Proc. Natl. Acad. Sci. USA 93:5624-5628

Eide, D. J. 1998. The molecular biology of metal ion transport in *Saccharomyces cerevisiae*. Annu. Rev. Nutr. 18:441-469

Eisen, J.A. 1998. A phylogenomic study of the MutS family of proteins. Nucleic Acids Res. 26 : 4291-4300

Escolar, L., J. Pérez-Martín y V. de Lorenzo. 1999. Opening the iron box: Transcriptional metalloregulation by the Fur Protein. J. Bacteriol.181: 6223-6229

Ewing, W.H. 1986.Edwards and Ewing's identification of Enterobacteriaceae.4th edition.Elsevier Science Publishing Co., Inc., N.Y.

Fang, F. C., M. A. DeGroote, J. W. Foster, A. J. Bäumler, U. Ochsner, T. Teterman, S. Bearson, J. Giard, Y. Xu, G. Campbell y T. Laessig. 1999. Virulent *Salmonella typhimurium* has two periplasmic cu, Zn-superoxide dismutasas. Proc. Natl. Acad. Sci. USA 96: 7502-7507

Farrant, J. L., A. Sansone, J. R. Canvin, M. J. Pallen, P. R. Langford, T. S. Wallis, G. Dougan y J. S. Kroll.1997, Bacterial copper- and zinc-cofactored superoxide dismutase contributes to the pathogenesis of systemic salmonellosis. Mol. Microbiol. 25: 785-796

Foster, J. W., y H. K. Hall. 1992. Effect of *Salmonella typhimurium* ferric uptake regulator (*fur*) mutations on iron- and ph- regulated protein sintesis, J. Bacteriol. 174: 4317-4323

Foster, J.W., Y.K. Park, I. S. Bang, K. Karem, H. Betts, H. K. Hall y E. Shaw.1994. Regulatory circuits involved with pH-regulated gene expression in *Salmonella typhimurium*. Microbiology 140:341-352

Gaballa, A., y J. D. Helmann. 1998. Identification of Zinc-specific metalloregulatory protein, Zur, controlling zinc transport operons in *Bacillus subtilis*. J. Bacteriol. 180: 5815-5821

Gaither, LA., y DJ. Eide. 2001. Eucaryotic zinc transporters and their regulation. Biometals 14:251-70

Gilford, J. R 1998. Antigenic diversity and gene polymorphism in *Haemophilus influenzae*. Infect. Immun. 66:5035-5059

Gillen, K.L., y K. T. Hughes.1991.Negative regulatory loci coupling flagellin sintesis to flagellar assembly in *Salmonella typhimurium*. J. Bacteriol. 173:2301-2310

Goel, V. K., y A. Kapil. 2001. Monoclonal antibodies against the iron regulated outer membrane proteins of *Actinobacter baumannii* are bactericidal. BMC Microbiology 1:16

González de Peredo, A., C. Saint.Pierre, J. Latour, I. Michaud-Soret y E. Forest.2001. Conformational Changes of Ferric uptake Regulation Protein upon Metal activation and DNA Binding; First Evidence of Structural Homologies with the Diphteria Toxin Represor. J. Mol. Biol. 310: 83-91

Gort, A. S., D. M. Ferber y J. A. Imlay. 1999.The regulation and role of the periplasmic copper, zinc superoxide dismutase of *Escherichia coli*. Mol. Microbiol. 32: 179-191

Govoni, G., S. Vidal, S. Gauthier, E. Skamene, D. Malo y P. Gros. 1996. The Bcg/Ity/Lsh locus: genetic transfer of resistance to infections in C57/BL/6J mice transgenic for the Nramp Gly^{169} allele. Infect. Immun. 64: 2923-2929

Gralnick, J. y D. Downs.2001. Protection from superoxide damage associated with an increased level of the YggX protein in *Salmonella enterica*. Proc. Natl. Acad. Sci. USA 98: 8030-8035

Grass, G., B. Fan, B. P. Rosen, S. Franke, D. H. Nies y C. Rensing. 2001. ZitB (YbgR), a member of the cation diffusion facilitator family, is an additional zinc transporter in *Escherichia coli*. 2001. J. Bacteriol. 183: 4664-4667

Grass, G., M. D. Wong, B. P. Rosen, R. L. Smith y C. Rensing. 2002. ZupT is a Zn (II) uptake system in *Escherichia coli*. J. Bacteriol. 184: 864-866

Guyton.1994.Textbook of Medical Physiology 8th Ed. W. B. Saunders Co.

Haag, H., H. P. Fiedler, J. Meiwers, H. Drechsel, G. Jung y H. Zahner.1994.Isolation and biological characterization of staphyloferrin B, a compound with siderophore activity from staphylococci. FEMS Microbiol. Lett. 115:125-130

Hall, H. K., y J. W. Foster.1996. The role of Fur in acid tolerance response of *Salmonella typhimurium* is physically and genetically separable from its role in iron acquisition. J. Bacteriol. 178: 5683-5691

Hammerschmidt, S., A. Müller, H. Sillman, R. Borroww, A. Fox, J. Putten, W. D. Zollinger, R. Gerardy-Schahn y M. Frosch. 1996. Capsule phase variation in *Neisseria meningitidis* serogroup B by slipped-strand mispairing in the polysialyltransferase gene (said):correlation with bacterial invasion and the outbreak of meningococcal disease.Mol. Microbiol. 20:1211-1220

Hamza, I., S. chauhan, R. Hasset y M. R. O'Brien.1998.The bacterial Irr protein is required for coordination of heme biosíntesis with iron availiability. J. Biol. Chem. 273:21669-21674

Hantke, K. 1987. Selection procedure for desregulated iron transport mutants (*fur*) in *Escherichia coli* K 12: *fur* not only affects iron metabolism. Mol. Gen. Genet. 210: 135-139

Hantke, Klaus.2001. Iron and Metal regulation in bacteria. Curr. Opin. Microbiol. 4:172-177

Harrison, P.M., y P- Arosio.1996.Ferritins: molecular properties, iron storage function and cellular regulation. Biochim. Biophys. Acta 1275:161-203

Hassett, D. J., P. A. Sokol, M. L. Howell, J. Ma, H. T. Schweizer, U. Ochsner y M. L. Vasil.1996. Ferric uptake regulator (Fur) mutants of *Pseudomonas aeruginosa* demonstrate defective siderophore-mediated iron uptake, altered aerobic growth, and decreased superoxide dismutase and catalase activities. J. Bacteriol. 178:3996-4003

Headley, V., M. Hong, M. Galko y S. Payne.1997. Expression of aerobactina genes by *Shigella flexneri* during extracellular and intracellular growth. Infect. Immun. 65: 818-823

Henderson, D. P., y S. M. Payne.1994. *Vibrio cholerae* iron transport systems: roles of heme and siderophore iron transport in virulence and identification of a gene associated with multiple iron transport systems. Infect. Immun. 62: 5120-5125

Henle, E. S., y S. Lin.1997. Formation, Prevention, and Repair of DNA damage by Iron/hidrogen peroxide. J. Biol. Chem. 272: 19095-19098

Hill, P.J., A. Cockayne, P. Landers, J. A. Morrissey, C. M. Sims y P. Williams.1998. SirR, a novel iron-dependent repressor in *Staphylococcus epidermidis*. Infect. Immun. 66: 4123-4129

Hoffmann, T., A. Schültz, M. Brosius, A. Völker, U. Völker y E. Bremer.2002. High-salinity-induced iron limitation in *Bacillus subtilis*. J. Bacteriol. 184:718-727

Horsburgh, M. J., E. Ingham y S. J. Foster. 2001. In *Staphylococcus aureus*, Fur is an interactive regulator with PerR contributes to virulence, and is necessary for oxidative

stress resistance through positive regulation of catalase and iron homeostasis. J. Bacteriol. 183: 468-475

Horsburgh, M. J., M. O. Clements, H. Crossley, E. Ingham and S. J. Foster. 2001.PerR controls oxidative stress resistance and iron storage proteins and is required for virulence in *Staphylococcus aureus*. Infect. Immun.69: 3744-3754

Horst, J., T. Wu y M. G. Marinus. 1999. *Escherichia coli* mutator genes. Trends in Microbiol. 7: 29-36

Howard, S.P.,C. Herrmann, C. W. Stratilo y V. Braun. 2001. In vivo synthesis of the perimplasmic domain of TonB inhibits transport through FecA and FhuA iron siderophore transporters of *Escherichia coli*. J. Bacteriol. 183: 5885-5895

Hudson, A.J., S. C. Andrews, C. Hawkins, J. M. Williams, M. Izuhara, F. C. Meldrum, S. Mann, P. M. Harrison y J. R. Guest.1993.Overexpression, purification and characterization of *Escherichia coli* ferritin. Eur. J. Biochem. 218:985-995

Ikeda, J. S. , C. K. Schmitt, S. C. Darnell, P. R. Watson, J. Bispham, T. S. Walls, D. L. Weistein, E. S. Metcalf, P. Adams, C. D. O'Connor y A. D. O'Brien. 2001. Flagellar phase variation of *Salmonella enterica* serovar Typhimurium contributes to virulence in murine typhoid infection model but does not influence *Salmonella*-induced enteropathogenesis. Infect. Immun. 69: 3021-3030

Ikeda, J. S., C. K. Schmitt, S. C. Darnell, P.R. Watson, J. Bispham, T. S. Wallis, D. L. Weinstein, E. S. Metcalf, P. Adams, C. D. O'Connor y A. D. O'Brien.2001.Flagellar phase variation of *Salmonella enterica* Serovar Typhimurium contributes to virulence in the Murine Typhoid infection model but does not influence Salmonella-induced enteropathogenesis. Infect. Immun. 69:3021-3030

Imamura, R., K. Yamanaka, T. Ogura, S. Hiraga, N. Fukita, A. Ishihama y H. Niki.1996. Identification of the *cpdA* gene encoding cyclic 3',5'-adenosine monophosphate phosphodiesterase in *E. coli*. J. Biol. Chem. 271: 25423-25429

Immamura, R., K. Yamanaka, T. Ogura, S. Hiraga, N. Fujita, A. Ishihama y H. Niki. 1996. Identification of the *cpdA* gene encoding cyclic3',5'-adenosine monophosphate phodphodiesterase in *E. coli*. J.Biol. Chem. 271:25423-25429

Iyoda, S., T. Kamidoi, K. Hirose, K. Kutsukake y H. Watanabe. 2001. A flagellar gene *fliZ* regulates the expresion of invasion genes and virulence phenotype in *Salmonella enterica* serovar Typhimurium. Microbial Pathog. 30:81-90

Jacques, M., M. Bélanger, M.s. Diarra, M. Dargis y F. Malouin. 1994. Modulation of *Pasteurella multocida* capsular polysaccharide during growth under iron-restricted conditions and *in vivo*. Microbiology 140:263-270

Jakubovics, N. S., A. W. Smith y H. F. Jenkinson. 2000. Expression of the virulence related Sca (Mn^{2+}) permease in *Streptococcus gordonii* is regulated by a diphtheria toxin metallorepressor-like protein ScaR. Mol Microbiol. 38:140-153

Janakiraman, A. y J. M. Slauch.2000.The putative iron transport system SitABCD encoded on SPI1 is required for full virulence of *Salmonella typhimurium*. Mol. Microbiol. 35: 1146-1155

Kadner, R. J.1990.Vitamin B_{12} transport in *E. coli*: energy coupling between membranes. Mol. Microbiol 4: 2927-2033

Kajalainen, T. K., D.G. Evans, D. J. Evans Jr, D. Y. Graham y C. Lee. 1991. Iron represses the expression of CFA/I fimbriae of enteroroxigenic *E. coli*. Microbial. Pathog. 11:317-323

Kcoh, W. H., G. Kopsidas, B. Meffle, A. S. Levine y R. Woodgate.1996. Analysis of chimeric UmuC proteins: identification of regions in *Salmonella typhimurium* UmuC important for mutagenic activity. Mol. Gen. Genet. 23: 121-129

Kehres, D. G., A. Janakiraman, J.M. Slauch y M. E. Maguire. 2002. Regulation of *Salmonella enterica* serovar Typhimurium *mntH* transcription by H_2O_2, Fe^{2+} and Mn^{2+}. J. Bacteriol. 184:3151-3158

Kehres, D. G., M. L: Zaharik, B. B. Finlay y M. E. Maguire.2000. The Nramp proteins of *Salmonella typhimurium* and *Escherichia coli* are selective manganese transporters involved in the response to reactive oxygen. Mol. Microbiol. 36: 1085-1100

Kehres, D.G., A, Janakiraman, J.M. Slauch y M. E. Maguire. 2002. SitABCD is the alkaline Mn^{2+} transporter of *Salmonella enterica* serovar Typhimurium. J. Bacteriol. 184:3159-3166

Kingsley, R., W. Rabsch, P. Stephens, M. Roberts, R. Reissbrodt, P. H. Williams.1995. Iron supplying systems of *Salmonella* in diagnostics, epidemiology and infection. FEMS Imm. Medical Microbiol. 11: 257-264

Ko, M., y C. Park.2000.H-NS-dependent regulation of flagellar síntesis is mediated by a LysR Family protein. J. Bacteriol.. 182:4670-4672

Kolb, A., S. Busby, Y. Ohya y T. Iino.1993. Transcriptional regulation by cAMP and its receptor protein. Annu. Rev. Biochem. 62:749-795

Komoriya, K., N. Shibano, T. Higano, N. Azuma, S. Yamaguchi y S. Aizawa. 1999. Flagellar proteins and type-III exported virulence factors are the predominant proteins secreted into the culture media of *Salmonella typhimurium*. Mol. Microbiol. 34:764-779

Konetschny-Rapp, S., G. Jung, J. Meiwers y H. Zahner.1990. Staphyloferrin A: a structurally new siderophore from staphylococci. Eur. J. Biochem. 191: 65-74

Kutsukake, K., Y. Ohya y T. Iino.1990. Transcriptional analysis of the flagellar regulon of *Salmonella typhimurium*. J. Bacteriol.. 172: 741-747

Kutsukake, K.1997. Autogenous and global control of the flagellar master operon, *flhD*, in *Salmonella typhimurium.* Mol. Gen. Genet. 254:440-448

Lai, E., K. L. Clarck, S. K. Burkley y J. E. Darnell. 1993. Hepatocyte nuclear factor 3/fork head or “winged helix” proteins: a family of transcription factors of diverse biologic function. Proc. Natl. Acad. Sci. USA 90:10421-10423

Larsen, R. A., M. G. Thomas y K. Postle.1999.Proton motive force, ExbB and ligand-bound FepA drive conformational changes in TonB. Mol. Microbiol. 31:1809-1824

Le Cam, E., D. Fréchon, M. Barray, A. Fourcade y E. Delain.1994.Observation of bonding and polymerization of Fur represor onto operator-containing DNA with electron and atomic force microscopes. Proc. Natl. Acad. Sci. USA 91:11816-11820

Le Clerc, J. E., B. li, W. L. Payne y T. A. Cebula. 1996. High mutation frequencies among *Escherichia coli* and *Salmonella* pathogens. Science 274 : 1208-1211

Lee, Y. H., R.K. Deka, N. V. Norgand, J. D. Radolf y C. A. Hasemann.1999. *Treponema pallidum* TroA is a periplasmic binding protein with a helical backbone. Nat. Struct. Biol. 6:628-633

Lehninger, A.L., D. L. Nelson y M.M. Cox.1993.Principles of Biochemistry 2on Ed. Worth Publishers, Inc.

Létoffé, S., J. M. Ghigo y C. Wandersman.1994.Iron acquissition from heme and hemoglobin by a *Serratia marcescens* extracellular protein. Proc. Natl. Acad. Sci. USA 91:9876-9880

Lewis, D. A., J. Klesney-Tait, S. R. Lumbley, C. K. Ward, J. L. Latimer, C. A. Ison y E. J. Hansen. 1999. Identification of the znuA-encoded periplasmic zinc transport protein of *Haemophilus ducreyi*. Infect. Immun. 67: 5060-5068

Li, C., C. J. Louise, W. Shi y J. Adler.1993. Adverse conditions which cause lack of flagella in *Escherichia coli*. J. Bacteriol.1575:2229-2235

Lindsay, J. A., y S. J. Foster.2001. *zur*: a $Zn^{(2+)}$- responsive regulatory element of *Staphylococcus aureus*. Microbiology 147: 1259-1266

Lissner, C.R., D. L. Weinstein y A. D. O'Brien.1985. Mouse chromosome 1 Ity locus regulates microbicidal activity of isolated peritoneal macrophages against a diverse group of intracellular and extracellular bacteria. J. Immun. 135:544-547

Litwin, M., S.A, y S. B. Calderwood.1996.Role of iron in regulation of virulence genes. Clin. Microbiol. Rev. 6:137-149

Liu, H., S. R. Hewit y J. B. Hays. 2000. Antagonism of ultraviolet-light mutagenesis by the methyl-directed mismatch-repair system of *Escherichia coli*. Genetics 154: 503-512

Lockman, H. y R. Curtiss III.1990.*Salmonella typhimurium* mutants lacking flagella or motility remain virulent in BALB/c mice. Infect. Immun. 58:137-143

Lockman, H.A., y R. Curtis.1992.Virulence of non-type 1-fimbriated and non-frimbiated nonflagellated *Salmonella typhimurium* mutants in murine typhoid fever. Infect. Immun. 60:491-496

Lu, D., B. Boyd y C. A. Lingwood. 1997. Identification of the key protein for Zinc Uptake in *Haemophilus influenzae*. J. Biolog. Chem. 272: 29033-29038

Luke, N. R., R. J. Karalus y A. A. Campagnari.2002. Inactivation of the *Moraxella catarrhalis* superoxide dismutasa SodA induces constitutive expression of Iron-repressible outer membrane proteins. 2002. Infect. Immun. 70:1889-1895

Makui, H., E. Roig, S. T. cole, J. D. Helmann, P. Gros y M. F. M. Cellier.2000. Identification of the *Escherichia coli* K-12 Nramp orthologe (MntH) as a selective divalent metal ion transporter. Mol. Microb. 35: 1065-1078

Manabe, Y. C., B. J. Saviola, L. Sun, J. R. Murphy y W. R. Bishai.1997. Attenuation of virulence in *Mycobacterium tuberculosis* expressing a constitutively active iron repressor. Proc. Natl. Acad. Sci. USA 96:12844-12848

Massé, E. y S. Gottesman.2002.A small RNA regulates the expression of genes involved in iron metabolism in *Escherichia coli*. Prot. Natl. Acad. Sci. USA 99:4620-4625

McCarter, L., y M. Silverman. 1989. Iron regulation of swarmer cell differentiation of *Vibrio parahaemolyticus*. J. Bacteriol. 171: 731-736

Meyer, J. M. , D. Hohandel, A. Khan y P. Corneleis. 1990. Pyoverdin facilitated iron uptake in *Pseudomonas aeruginosa*: A immunological characterization of pyoverdin receptor. Mol. Microbiol. 4:1401-1405

Meyer, J. M., D. Hohnadel y F. Halle. 1989. Cepabactin from *Pseudomonas cepacia*, a new type of siderophore. J. Gen. Microbiol. 135: 1479-1487

Miller, R. A., y B. E. Britigan.1997. Role of oxidants in microbial pathophysiology. Clin. Microbiol. Rev. 10:1-8

Minamino, T., R. Chu, S. Yamaguchi y R. M. Macnab.2000. Role of flagellar protein export in *Salmonella*. J. Bacteriol.. 182:4207-4215

Mizrahi, V y S. J. Andersen. 1998. DNA repair in *Mycobacterium tuberculosis*. What have we learnt from the genome sequence? Mol. Microbiol. 29:1331-1339

Mizushima, T., A. Tomura, T. Shinpuku,T. Miki y K. Sekimizu.1994. Loss of flagellation in *dnaA* mutants of *Escherichia coli.* J. Bacteriol.. 176:5544-5546

Moeck, G. S., y J. W. Coulton. TonB-dependent iron acquisition; mechanism of siderophore-mediated active transport. Mol. Microbiol. 28: 675-681

Moeck, G. S., y L. Letellier.2001. Characterization of in vitro interactions between a truncated TonB protein from *Escherichia coli* and outer membrane receptors FhuA and FepA. J. Bacteriol. 183: 2755-2764

Möllmann, U., A. Ghosh, E. K. Dolence, J. A. Dolence, M. J. Miller et al.1998.Selective growth promotion and growth inhibition of Gram-negative and Gram-positive bacteria by synthetic siderophore-β-lactam conjugates. Biometals 11:1-12

Morrissey, J. A., A. Cockayne, P. J. Hill y P. Williams. 2000. Molecular cloning and analysis of putative siderophore ABC transporter from *Staphylococcus aureus.* Infect Immun. 68: 6281-6288

Mukarami, Y., S. Kato, M. Nakajima, M. Matsuoka, H. Kawai, K. Shin-Ya y H. Seto. Formobactin, a novel free radical scavenging and neuronal cell protecting substance from *Nocardia sp*. J. Antibiot. 49:839-845

Nanassy, O. Z., y K. T. Hughes.1998. *In vivo* identification of intermediate stages of the DNA Inversion reaction catalysed by the *Salmonella* Hin recombinase. Genetics 149:1649-1663

Neilands, J. B. 1995. Siderophores: Structure and fuction of microbial iron transport compounds. J. Biol. Chem. 270:26723-26726

Nielsen, L. D., D. Monard y H. V. Rickenberg.1973.Cyclic 3',5'-adenosine monophosphate phosphodiesterase of *E. coli*. J. Bacteriol. 116:857-866

Ochsner, U. A., y M. L. Vasil.1996, Gene repression by the ferric uptake regulator in *Pseudomonas aeruginosa*: Cycle selection of iron-regulated genes, Proc. Natl. Acad. Sci. USA 93: 4409-4414

Ohnishi, K., K. Kutsukake, H. Suzuki, y T. Iino.1990.Gene *fliA* encodes an alternative sigma factor specific for flagellar operon in *Salmonella typhimurium.* Mol. Gen. Genet. 221: 139-149

Okujo, N., M. Saito, S. Yamamoto, T. Yoshida, S. Miyoshi y S. Shinoda.1994. Structure of vulnibactin, a new polyamine-containing siderophore from *Vibrio vulnificus*. Biometals 7:109-116

Panina, E. M., A. A. Mirinov y M. S: Gelfand. 2001. Comparative analysis of FUR regulons in gamma-proteobacteria. Nucleic Acid Res. 29: 5195-5206

Paradis, S., D. Dubreuil y M. Jacques. 1996. Examination of surface polysaccharides of *Actinobacillus pleuropneumoniae* serotype 1 grown under iron-restricted conditions. FEMS Microb. Lett. 137:201-206

Patzer, S. I. y K. Hantke. 2000. The zinc-responsive regulator Zur and its control of the *znu* gene cluster encoding the ZnuABC zinc uptake system in *Escherichia coli*. J. Biol. Chem. 275: 2432-24332

Patzer, S. I., y K. Hantke. The ZnuABC high-affinity zinc uptake system and its regulator Zur in *Escherichia coli*. Mol. Microbiol. 28: 1199-1210

Patzer, S. I., y K. Hantke.2001. Dual Represión by Fe^{2+} -Fur and Mn^{2+}-MntR of *mntH* gene, encoding an NRAMP-like Mn^{2+} transporter in *Escherichia coli*. J. Bacteriol. 183: 4806-4813

Perry, R. D., P. B. Balbo, H. A. Jones, J. D. Fetherston y E. DeMoll.1999. Yersiniabactin from *Yersinia pestis*: biochemical characterization of the siderophore and its role in iron transport and regulation. Microbiology 145: 1181-1190

Persmark, M., T. Frejd y B. Mattiasson.1991.Purification, characterization, and structure of pseudobactin 589A, a siderophore from a plant growth promoting *Pseudomonas*. Biochemistry 30:2010-2018

Petterson, A., B. Kuipers, M. Pelzer, E. Verhagen, R. H. Tiesjema, J. Tommassen y J. T. Polman. 1990. Monoclonal antibodies against the 70 kilodalton iron regulated outer membrane proteins of *Neisseria meningitidis* are bactericidal and strain specific. Infect. Immun. 58:3036-3041

Posey, J. E., J. M. Hardham, S. J. Norris y F. C. Gherardini.1999. Characterization of manganese-dependent regulatory protein, TroR, from *Treponema pallidum*. Proc. Natl. Acad. Sci. USA 96: 10887-10892

Prince,R. W., C. D: Cox y M. L. Vasil.1993.Coordinate regulation of siderophore and endotoxin A production: molecular cloning and sequencing of the *Pseudomonas aeruginosa fur* gene. J. Bacteriol. 175:2589-2598

Prüβ, B. M., X. Liu, W. Hendrickson, P. Matsumura.2001.FlhD/FlhC-regulated promoters analysed by gene array and *lacZ* gene fusions. FEMS Microbiol. Lett. 9854:1-7

Que, Q., y J. D. Helmann.2000. Manganese homeostasis in *Bacillus subtilis* is regulated by MntR, a bifunctional regulator related to the diphtheria toxin repressor family of proteins. Mol. Microbiol. 35: 1454-1468

Ratledge, C., y L. G. Dover. 2000. Iron Metabolism in pathogenic bacteria. Ann. Rev. of Microb. 54: 881-941

Reichard, P. 1994.J. Biol. Chem. 268:8383-8386

Rensing, C., B. Mitra y B. P. Rosen. 1997. The *zntA* gene of *Escherichia coli* encodes a Zn(II)- translocating P-type ATPase. Proc. Natl. Acad. Sci. USA 94: 14326-14331

Rutz, J. M., T. Abdullah, S. P. Singh, V. I. Kalve y P. E. Klebba. 1991. Evolution of ferric enterobactin receptor in Gram- Negative bacteria. J. Bacteriol. 173:5964-5974

Ruyen-Janecky, L. J., M. Hong y S. M. Payne.1999. The virulence plasmid-encoded *impCAB* operon enhances survival and induced mutagénesis in *Shigella flexneri* after exposure to UV radiation. Infect. Immun. 67:1415-1423

Saken, E., A. Rakin y J. Hessemann. 2000. Molecular characterization of a novel siderophore-independent iron transport system in *Yersinia*. Int. J. Med. Microbiol. 290:51-60

Schmiedeskamp, M., y R. E. Klevit.1997.Paramagnetic cobalt as a probe of the orientation of an accessory DNA binding by a zinc-finger domain from the yeast ADR1 zinc-finger protein. Biochemistry 36:14003-14011

Schmitt, C. K., S. C. Darnell y A. O'Brien.1996.The attenuated phenotype of a *Salmonella typhimurium flgM* mutant is related to expression of FliC flagellin.J. Bacteriol.. 178:2911-2915

Schmitt, M. P., M. Predich, L. Doukhan, I. Smith y R. K. Holmes.1995.Characterization of an iron-dependent regulatory protein (IdeR) of *Mycobacterum tuberculosis* as a functional homolog of the diphtheria toxin repressor (DtxR) from *Corynebacterium diphtheriae.* Infect. Immun. 63:4284-4289

Schoffler, H., y V. Braun.1989.Transport across the outer membrane of *Escherichia coli* K12 via the FhuA receptor is regulated by the TonB protein of the cytoplasmatic membrane. Mol. Gen. Genet. 217:378-383

Schwyn, B. y J.B. Neilands.1987.Universal chemical assay for the detection and determination of siderophores. Analytical Biochem. 160:47-56

Serkin, C. D. y H. S. Seifert. 2000. Iron avaiability regulates DNA recombination in *Neisseria gonorrhoeae*. Mol. Microbiol. 37(5): 1075-1086

Shi, W., C. Li, C.J. Louise y J. Adler.1993.Mechanism of adverse condition causing lack of flagella in *E. coli.* J. Bacteriol. 175:2236-2240

Smith, H. What happens to bacterial pathogens in vivo?. 1998. Trends in Microbiol.6: 239-243

Smith, N. H., y R. K. Selander.1991.Molecular genetic basis for complex flagellar antigen expresión in a triphasic serovar of *Salmonella*. Proc. Natl. Acad. Sci. USA 88:956-960

Smith, R. L., M. T. Kaczamarek, L. M. Kucharski y M. E. Maguire.1998. Magnesium transport in *Salmonella typhimurium*: regulation of *mgtA* y *mgtCB* during invasion of epithelial and macrophage cells. Microbiology 144:1835-1843

Sokol, P. A., y D. Woods.1983. Demonstration of an iron siderophore binding protein in the outer membran of *Pseudomonas aeruginosa*. Infect. Immun. 40:665-669

Sokurenko, E. V., D. L. Hasty y D. E. Dykhuizen. 1999. Pathoadaptative mutations: gene loss and variation in bacterial phatogens. Trends in Microbiol. 7: 191-195

Soutourina, O., A. Kolb, E. Krin, C. Laurent-Winter, S.Rimsky, A. Danchin y P. Bertin.1999. Multiple control of flagellum biosynthesis in *E. coli*: Role of H-NS protein and the cyclic AMP-Catabolite activator protein complex in the transcription of the *flhDC* master operon. J. Bacteriol.. 181:7500-7508

Stojiljkovic, I. , A. J. Bäumler y K. Hantke.1994. Fur regulon in Gram-negative bacteria identification and characterization of a New Iron J. Mol. Biol. 236: 531-545

Stojiljkovic, I., y K. Hantke. 1995. Functional domains of the *Escherichia coli* ferric uptake regulator protein (Fur). Mol. Gen. Genet. 247: 199-205

Theil, E. C. 1987.Ferritin: structure, gene regulation, and cellular function in animals, plants, and microorganisms. Annu. Rev. Biochem. 56:289-315

Thewell, C., N. J. Robinson y J. S. Turner-Cavet.1998. An SmtB-like repressor from *Synechocystis* PCC6803 regulates a zinc exporter. Proc. Natl. Acad. Sci. USA 95:10728-10733

Thompson, J. M., H. A. Jones y R. D. Perry.1999.Molecular characterization of the hemin uptake locus (*hmu* from *Yersinia pestis* and analysis of *hmu* mutants for hemin and hemoprotein utilization. Infect. Immun. 67: 3879-3892

Torres, A. G., P. Redford, R. A. Welch y S. M. Payne.2001. TonB-dependent systems of uropathogenic *Escherichia coli*: Aerobactin and Heme transport ant TonB are required for virulence in mouse. Infect. Immun. 69:6179-6185

Touati, D. 2000. Iron and oxidative stress in bacteria. Arch. Biochem Biophys. 373:1-6

Touati, D., M. Jacques, B. Tardat, L. Bouchard y S. Despied. 1995. Lethal oxidative damage and mutagénesis are generated by iron in Δ*fur* mutants of *Escherichia coli*: Protective role of superoxide dismutase. J. Bacteriol. 177: 2305-2314

Tsolis, R. M. T., A. J. Bäumler y F. Heffron. 1995. Role of *Salmonella typhimurium* Mn-Superoxide Dismutasa (SodA) in protection against early killing by J774 Macrophages. Infect. Immun, 63: 1739-1744

Tsolis, R. M., A. J. Bäumler, I. Stojiljkovic y F. Heffron.1995. Fur Regulon of *Salmonella typhimurium*: Identification of New Iron-Regulated Genes. J. Bacteriol. 177:4628-4637

Tsui, H. T., G. Feng y M. E. Winkler. 1997. Negative regulation of *mut*S and *mut*H repair gene expresión by the Hfq and RpoS Global regulators of *Escherichia coli* K-12. J.Bacteriol. 179: 7476-7487

Turner, J. S., A. P. Morby, B. A. Whitton, A, Gupta y N. J. Robinson.1993.Construction of Zn^{2+}/Cd^{2+}hypersensitive cyanobacterial mutants lacking a functional metallothinein locus. J. Biol. Chem. 268:4494-4498

Usher, K. C., E. Özkan, K. H. Gardner y J. Deisenhofer.2001.The plug domain of FepA, a TonB- dependent transport protein from *Escherichia coli*, binds its siderophore in absence of the transmembrane barrel domain. Prot. Natl. Acad. Sci. USA 98:10676-10681

Vasil, M.L., y U. A. Ochsner.1999. The response of *Pseudomonas aeruginosa* to iron: genetics, biochemistry and virulence. Mol. Microbiol. 34:399-413

Vassinova, N., y D. Kozyrev.2000.A meted for direct cloning of Fur-regulated genes: identification of seven new Fur-regulated loci in *Escherichia coli*. Microbiolgy 146: 3171-3182

Wang, J., A. Mushegian, S. Lory y S. Jin. 1996. Large-scale isolation of candidate virulence genes of *Pseudomonas aeruginosa* by in vivo selection. Proc. Natl. Acad. Sci. USA 93:10434-10439

Wardrop, S. L., y D. R. Richardson.1999.The effect of intracellular iron concentration and nitrogen monoxide on Nramp2 expression and non-transferrin-bound iron uptake. Eur. J. Biochem. 263:41-49

Watnick, P., T. Eto, H. Takahashi y S. B. Calderwood.1997. Purification of *Vibrio cholerae* Fur and estimation of its intracellular abundance by antibody sandwich enzym-linked immunosorbent assay. J. Bacteriol.. 179:243-247

Weiser, J.N. y N. Pan.1998.Adaptation of *Haemophilus influenzae* to acquired and innate hummoral immunity based on phase variation of lipopolisaccharide. Mol. Microbiol 30:765-775

Wilmes-Riesenberg, M. R., B. Bearson, J. W. Foster y R. Curtiss III. 1996. Role of the acid tolerance response in virulence of *Salmonella typhimurium*. Infect. Immun. 64:1085-1092

Wyckoff, E.E., J. A. Stoebner, K. E. Reed y S. M. Payne.1997. Cloning a *Vibrio cholerae* Vibriobactin gene cluster: Identification of genes required for early steps in siderophore biosynthesis. J. Bacteriol. 179: 7055-7062

Xiong, A., V. K. Singh, G. Cabrera y R. Jayaswal. 2000. Molecular characterization of the ferric-uptake regulator, Fur, from *Staphylocoocus aureus*. Microbiology 146: 659-668

Xu, X., S. C. Holt y D. Kolodrubetz. 2001. Cloning and expression of two novel hemin binding protein genes in *Treponema senticola*. Infect. Immun. 69:4465-4472

Yamamoto, S., N. Okujo, Y. Fujita, T. Yoshida y S. Shinoda. 1993. Structures of two polyamine-containing catecholate siderophores from *Vibrio fluvialis*. Biochemistry 113:538-544

Yang, A. H. W., R T. A. McGillivray, J. Chen, Y. Luo, Y. Wang, G. D. Brayer, A. B. Manson, R. C. Woodworth y M. E. P. Murphy. 2000. Crystal structures of two mutants (K206Q, H207E) of the N-lobe of mutant transferrin with increased affinity for iron. Prot. Science 9: 49-52

Yoshioka, K., S. Aizawa y S. Yamaguchi. 1995. Flagellar filament structure and cell motility of *Salmonella typhimurium* mutants lacking part of the outer domain of flagellin. J. Bacteriol.. 177:1090-1093

Zahrt, T. C., N. Buchmeier y S. Maloy. 1999. Effect of *mut*S mutations on *Salmonella* virulence. Infect. Immun, 67: 6168-6172

Zhao, H., y D. Eide. 1996. The yeast ZRT1 gene encodes the zinc transporter protein of a high-affinity uptake system induced by zinc limitation. Proc. Natl. Acad. Sci USA 93:2454-2458

Zheng, M., B. Doan, T. D. Schneider y G. Storz. 1999. OxyR and SoxRS regulation of *fur*. J. Bacteriol. 181, No.15: 4639-4643

5. ANEXOS

ANEXO I

FEMS Microbiology Letters 187 (2000) 145–150

www.fems-microbiology.org

Virulence and mutation rates of *Salmonella typhimurium* strains with increased mutagenic strength in a mouse model

Susana Campoy [a], Ana M. Pérez de Rozas [b], Jordi Barbé [a,*], Ignacio Badiola [b]

[a] *Department of Genetics and Microbiology, Faculty of Sciences, Universitat Autònoma de Barcelona, 08193 Barcelona, Spain*
[b] *Unitat de Sanitat Animal, Institut de Recerca i Tecnologia Agroalimentària, Barcelona, Spain*

Received 16 March 2000; received in revised form 26 April 2000; accepted 26 April 2000

Abstract

Two strains of *Salmonella typhimurium* presenting increased mutation rates, either spontaneous or mediated by DNA damage, have been constructed. One of the strains carries a null *mutS* mutation, while the other harbors plasmid pRW30, which contains the *Escherichia coli umuDC* operon. The virulence of these strains has been determined by inoculating BALB/c or Swiss mice. The 50% lethal dose of both strains is identical to that obtained for the wild-type. Likewise, the two strains and the wild-type contribute equally to animal death in mixed infections. The frequency of Nal^R mutants recovered from animals inoculated with either wild-type or $MutS^-$ cells was not affected by the presence of pRW30. These results indicate that the DNA damage which *S. typhimurium* cells can suffer during the infectious process by host cell metabolites does not cause induction of the SOS response at levels able to trigger the error-prone DNA repair pathway. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: *Salmonella typhimurium*; *mutS* gene; Virulence; SOS mutagenesis

1. Introduction

Pathogenic bacteria rely on genetic variability to adapt to the changing environmental conditions found during infection, as well as to facilitate the evasion of host defenses. One source of genetic variation is mutation, either spontaneous or induced [1], and the importance of this factor has been shown in *Neisseria meningitidis* and *Haemophilus influenzae* [2]. In other pathogenic bacteria, data about the importance of mutagenic strength during infection remain inconclusive and based on indirect evidence. Thus, it has been reported that in isolates of commensal and pathogenic *Escherichia coli* and *Salmonella enterica* strains there is a higher percentage of mutator mutants than that found randomly in laboratory strain cultures [3,4]. These mutator strains present a higher spontaneous mutation rate because they are defective in either the *mutS* or the *mutL* genes belonging to the mismatch repair system which is responsible for removing misincorporations produced during DNA replication [5]. A role in the adaptational ability to environmental conditions and, as a consequence, a higher fitness in comparison with wild-type cells has been proposed for these mutator clones [1,3–5].

Another way to increase bacterial mutation rates is to activate the SOS repair system. When chromosomal replication is inhibited in bacterial cells as a consequence of DNA damage, a coordinated set of functions (known as the SOS response) is displayed to repair lesions and to increase cell survival. This system is positively regulated by the RecA protease and negatively controlled by the LexA repressor [6]. Among the genes belonging to the SOS network is the *umuDC* operon encoding the DNA polymerase V which, together with RecA and a modified form of the DNA polymerase III holoenzyme, is required for translesion synthesis to give rise to the SOS-dependent mutagenesis [7].

The presence of different *umuDC*-like genes in virulence plasmids has been reported in several pathogenic bacteria [8]. In some cases (*Shigella flexneri*) the DNA damage-mediated mutagenesis is due to these plasmid-encoded genes [8], whereas in others (*S. typhimurium*) it is *umuDC*-dependent [9]. Nevertheless, and unlike what happens in *E. coli, S. typhimurium* presents a very low level of both spontaneous and DNA damage-mediated mutagenesis because of several substitutions present in residues 26–

* Corresponding author. Tel.: +34 (93) 5811837; Fax: +34 (93) 5812387; E-mail: jbarbe@einstein.uab.es

0378-1097/00/$20.00 © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.
PII: S0378-1097(00)00192-0

59 of its UmuC protein [10]. However, a selective advantage for cells presenting a higher DNA damage-mediated mutagenesis as a consequence of the presence of either very active *umuDC* or related genes has been suggested [8].

Furthermore, little is known about the dynamics of spontaneous mutant appearance during the bacterial infectious process in vivo as well as the role which DNA repair genes play in this process. In this respect, *S. typhimurium* is an appropriate organism in which to study this aspect because of its decreased mutagenic ability, which may be easily increased by introducing *E. coli umuDC* genes.

For these reasons, we have studied the virulence and competitiveness of *S. typhimurium* in the murine model under two situations that increase mutagenic rates: inactivation of mismatch repair and introduction of *E. coli umuDC* genes. Likewise, the frequency of mutant recovery from infected animals in these conditions has also been determined.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The bacterial strains, plasmids, phages and primers used in this study are listed in Table 1. *E. coli* and *S. typhimurium* strains were grown in LB broth [11]. Antibiotics were added to the culture media at the concentrations previously reported [11].

2.2. Genetic and biochemical methods, and DNA techniques

Triparental matings using pRK2013 as the mobilizing plasmid were as previously reported [11]. *S. typhimurium* chromosome exchange markers, P22 HT-mediated transductions and plasmid electroporation were performed as described [11]. In all cases, absence of the P22 HT prophage in the transductants obtained was determined by streaking them in green plates. UV-mediated mutagenesis experiments were carried out as previously described [12]. All DNA methodology used was as published [11].

2.3. Animal studies

To determine the 50% lethal dose (LD_{50}), groups of four Swiss or BALB/c 6–8-week-old female mice were inoculated intraperitoneally with serial dilutions of the *S. typhimurium* desired strain. Mortality was recorded at 28 days post infection, and the LD_{50} value was calculated by the method of Reed and Muench [13] without statistically significant differences in obtained values for each strain. For the competitive experiments, animals were inoculated with either 2×10^3 or 2×10^4 cfu depending on whether they were BALB/c or Swiss, respectively.

Table 1
Bacterial strains, plasmids and oligonucleotide primers used in this work

	Relevant features		Source or reference
Organism			
E. coli			
DH5α	*supE4*, *ΔlacU169* (φ80 *lacZ*ΔM15), *hasdR17, recA1, endA1, gyrA 96, thi-1, relA1*		This laboratory
MC1061 (λ*pir*)	*lacY1 hsdR*, lysogenized with λ*pir* bacteriophage		This laboratory
S. typhimurium			
ATCC 14028	Wild-type strain		SLSA[a]
UA1770	As ATCC 14028, but Rif^R		This work
UA1771	As UA1770, but *mutS*::Gm		This work
UA1772	As ATCC 14028, but carrying the pRW30 plasmid		This work
UA1773	As ATCC 140128, but *mutS*::Gm		This work
UA1774	As UA1773, but carrying the pRW30 plasmid		This work
Plasmid			
pRK2013	Tra^+ of RK2, ColE1 replicon, Km^R		D.R. Helinski
pGEM-T	Amp^R		Stratagene
pGP704	Amp^R. Ori R6K Mob RP4		[21]
pRW30	pBR322 derivative containing the *E. coli umuDC* genes. Amp^R		R. Woodgate
pUA873	As pGem-T, but carrying the *S. typhimurium* ATC 14028 *mutS* gene. Amp^R		This work
pUA874	As pUA873, but containing a 3.2-kb Gm-resistant cassette inserted into the internal *Sac*II point of the *S. typhimurium* ATC 14028 *mutS* gene. Amp^R Gm^R		This work
pUA875	As pGP704, but carrying the *mutS*::Gm construction		This work
Primer	**Sequence**	**Position[b]**	
MutS1	5′-*GGATCC*CTGTTACAGGAGCGTCAGG-3′	+374	Upper primer used to obtain the internal fragment of the *mutS* gene
MutS2	5′-*GGATCC*GGAATAATGTAGCGTTCGGC-3′	+1509	Lower primer used to obtain the internal fragment of the *mutS* gene

[a]SLSA, Servei de Laboratoris de Sanitat Agrària de la Generalitat de Catalunya.
[b]Position of the 5′-end of the oligonucleotide with respect to the translational starting point of the *S. typhimurium mutS* gene. Added restriction sites are shown in italics.

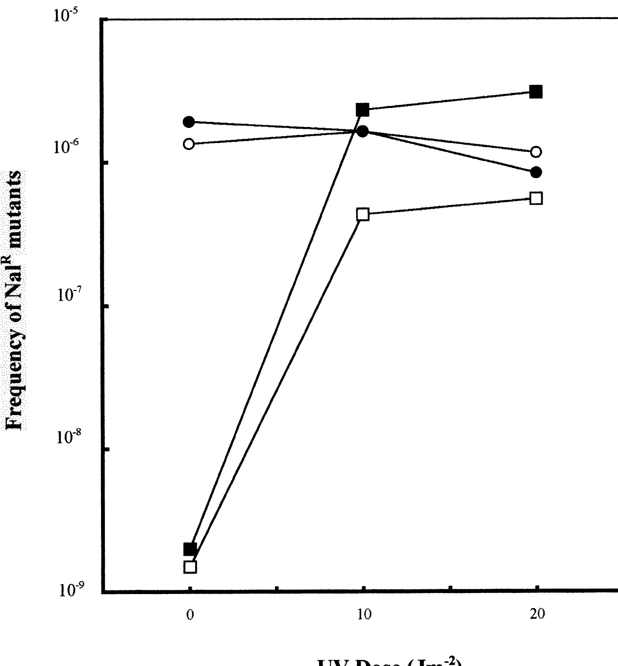

Fig. 1. UV-induced mutagenesis of *S. typhimurium* ATCC 14028 (□), *S. typhimurium* ATCC 14028 (pRW30) (■), *S. typhimurium* ATCC 14028 MutS$^-$ (○) and *S. typhimurium* ATCC 14028 MutS$^-$ (pRW30) (●) strains. For each dose of UV radiation the frequency of Nal^R mutants is the number of Nal^R clones divided by the number of survivors obtained. The values given are the mean of three independent experiments and they were reproducible to within an error of ±10%. Three plates were used for each point.

Segregational stability of plasmids was monitored by replica plating of the cells recovered from each dead animal. For mixed infections, each strain was grown separately and mixed in an approximately 1:1 ratio before injection. The concentration of both strains was checked by plating serial dilutions of the bacterial suspensions onto LB medium before mixing. Samples of blood were recovered by heart puncture immediately after the death of the mouse (generally 7 days after inoculation), and the concentration of bacterial cells was determined by plating a dilution series in the appropriate medium. The number of Nal^R mutants in infected animals was determined by directly plating the blood sample recovered in LB medium supplemented with nalidixic acid (50 μg ml^{-1}). For each strain, the frequency of mutagenesis was calculated by dividing the concentration of Nal^R clones by that of total cells recovered from each blood sample. In strains containing antibiotic resistance because of the presence of either the *mutS* mutation or the pRW30 plasmid, or both, the concentration of Nal^R and total cells was determined by plating in LB medium supplemented with the appropriate antibiotics. Mutagenesis experiments were only carried out in Swiss animals because of the low concentration of bacterial cells recovered from BALB/c [14], which is lower than the value of the mutation rate obtained for *S. typhimurium* ATCC 14028 wild-type strain under laboratory conditions.

3. Results and discussion

3.1. Construction of S. typhimurium ATCC 14028 derivatives with increased mutagenic rates

To construct a *mutS* null mutant of the ATCC 14028 virulent strain of *S. typhimurium*, an internal fragment of its *mutS* gene was amplified using oligonucleotide primers MutS1 and MutS2 (Table 1) corresponding to nucleotides 374–393 and 1509–1489 (with respect to its translational starting point) of the reported sequence of the *S. typhimurium* LT2 *mutS* gene. The 1.1-kb PCR fragment obtained with these primers was cloned in the pGEM-T vector, and to confirm that no mutation was introduced into the amplification reaction, its sequence was determined. Afterwards, a 3.2-kb gentamicin-resistant cassette was inserted into the internal *Sac*II of the *S. typhimurium* ATCC 14028 *mutS* gene. This 4.3-kb fragment containing the *mutS*::Gm construction was then cloned in the pGP704 suicide vector and introduced by triparental mating in a Rif^R derivative of the *S. typhimurium* ATCC 14028 wild-type strain. Gentamicin-resistant transconjugants were screened for loss of vector-mediated ampicillin resistance to detect putative mutants which had exchanged their wild-type gene for the inactivated *mutS* as a consequence of a double cross-over event. For one of these strains (UA1771), this was unequivocally confirmed by PCR amplification of chromosomal DNA using MutS1 and MutS2 primers (data not shown).

It has been described that most rifampicin-resistant mu-

Table 2
LD_{50} of *S. typhimurium* ATCC 14028, *S. typhimurium* ATCC 14028 (pRW30), *S. typhimurium* MutS$^-$ and *S. typhimurium* MutS$^-$ (pRW30) cells in BALB/c and Swiss mice[a]

Mouse strain	*S. typhimurium* strain			
	ATCC 14028	ATCC 14028 (pRW30)	MutS$^-$	MutS$^-$ (pRW30)
BALB/c	9	13	21	16
Swiss	2×10^3	2.1×10^3	3.5×10^3	3.3×10^3

[a]The LD_{50} was calculated as reported [12].

Table 3
Competition of *S. typhimurium* ATCC 14028 with either *S. typhimurium* ATCC 14028 (pRW30), *S. typhimurium* MutS$^-$ or *S. typhimurium* MutS$^-$ (pRW30) cells in BALB/c mice[a]

Strain inoculated	Percentage of strains recovered				
	ATCC 14028	ATCC 14028 (pRW30)	MutS$^-$	MutS$^-$ (pRW30)	
ATCC 14028+ATCC 14028 (pRW30)	53	47	–	–	0.88
ATCC 14028+MutS$^-$	54	–	46	–	0.85
ATCC 14028+MutS$^-$ (pRW30)	55	–	–	45	0.82

[a]Mice were inoculated intraperitoneally with a mixture of the indicated strains (1:1 proportion) and blood samples were taken as described in Section 2.
[b]Ratio of test strain to wild-type strain corrected for deviation observed in the corresponding inoculum ratio from a value of 1:1. Each competition experiment was performed on three separate occasions without statistically significant differences. Similar results were obtained when Swiss mice were used.

tants of *S. typhimurium* are avirulent in mice [15]. To prevent any putative interference of the Rif^R mutation in the behavior of our MutS$^-$ mutant during the infectious process, the *mutS*::Gm region from the UA1771 strain was transferred by P22-mediated transduction to wild-type (Rif^S) cells of *S. typhimurium* ATCC 14028. The PCR profile of the chromosome of 10 Gm^R transductants, when amplified with MutS1 and MutS2 primers, revealed that all of them contained the desired *mutS*::Gm mutation. One of these transductants (UA1773) was kept out for further work.

In agreement with the PCR profiles, Fig. 1 shows how the *S. typhimurium* ATCC 14028 MutS$^-$ mutant presents about a 1000-fold increase in the frequency of spontaneous Nal^R mutants over the wild-type strain. These values are in concordance with those obtained with transposon-induced MutS$^-$ mutants of *S. typhimurium* [16].

After the *S. typhimurium* MutS$^-$ mutant had been constructed, it was decided to further increase its mutagenic ability by transformation with plasmid pRW30, which encodes the *E. coli* wild-type *umuDC* operon. The presence of this plasmid does not increase the spontaneous mutation frequency in wild-type or in MutS$^-$ cells (Fig. 1). As expected, the frequency of UV-mediated mutagenesis was about 10-fold higher in wild-type ATCC 14028 cells harboring pRW30. UV irradiation did not increase the mutation rates of MutS$^-$ cells regardless of the presence of pRW30 (Fig. 1). This absence of UV mutagenesis in a MutS$^-$ strain has recently been also reported for *E. coli* [17], but it is a novel observation in *S. typhimurium*. The reasons for this behavior are so far unknown.

3.2. Behavior of constructed *S. typhimurium ATCC 14028* derivatives during animal infection

Table 2 presents the virulence of *S. typhimurium* wild-type, *S. typhimurium* (pRW30) and *S. typhimurium* MutS$^-$ strains. Their LD_{50} values are virtually identical, indicating that neither an increase of the spontaneous mutagenesis rate nor the DNA damage-mediated mutagenic ability significantly affects *S. typhimurium* virulence. It must be pointed out that pRW30 is stable in both MutS$^-$ and wild-type cells during the infectious process, since more than 99% of colony-forming units recovered from dead animals were ampicillin-resistant. In agreement with these results, it has also been demonstrated that the virulence of *S. typhimurium* cells is not affected by the presence of a *mutL* mutation [18]. Likewise, it is known that *S. typhimurium mutS recD* double mutants show a general downshift in the purine metabolism interfering

Table 4
Mutation rate of *S. typhimurium* ATCC 14028, *S. typhimurium* ATCC 14028 (pRW30), *S. typhimurium* MutS$^-$, and *S. typhimurium* MutS$^-$ (pRW30) strains in Swiss mice[a]

Strain inoculated	Frequency of Nal^R clones	Percentage of Nal^R recovered, being			
		Wild-type	Wild-type (pRW30)	MutS$^-$	MutS$^-$ (pRW30)
ATCC 14028	1.4×10^{-9}	100	–	–	–
ATCC 14028 (pRW30)	1.2×10^{-9}	–	100	–	–
MutS$^-$	5.3×10^{-6}	–	–	100	–
MutS$^-$ (pRW30)	3×10^{-6}	–	–	–	100
ATCC 14028+ATCC 14028 (pRW30)	1.1×10^{-9}	51	49	–	–
ATCC 14028+MutS$^-$	4.6×10^{-6}	0.03	–	99.97	–
ATCC 14028+MutS$^-$ (pRW30)	2.6×10^{-6}	0.05	–	–	99.95

[a]Single or mixed infections and evaluation of the frequency of Nal^R mutants were carried out as described in Section 2. The values given are the mean of three independent experiments. For each experiment the average of the results obtained with four animals was calculated. In all cases, data obtained were reproducible to within an error of ±10%. Three plates were always used to determine the number of Nal^R clones present in each blood sample.

with its infective process but without varying its LD_{50} value [19].

To further analyze the behavior of these strains, competition assays upon mixed infection were carried out. Neither the *mutS* mutation nor the presence of the pRW30 plasmid affected the competitiveness of *S. typhimurium* cells (Table 3).

To investigate the impact of increased mutagenic activity upon the mutation rate beyond the infectious process, the frequencies of Nal^R mutants recovered from animals inoculated with either wild-type or $MutS^-$ cells carrying or not the pRW30 plasmid in single or mixed infections were calculated. Data show that in all cases the obtained values in both single or mixed infections (Table 4) were practically the same as those found in the in vitro experiments (Fig. 1). So, from wild-type-inoculated animals, Nal^R clones were isolated at a frequency of about 10^{-9}, regardless of the presence of the pRW30 plasmid. Likewise, the frequency of Nal^R mutants recovered from animals inoculated with either $MutS^-$ or $MutS^-$ (pRW30) cells was about 10^{-6}. From these results, two points indicating that the putative damage produced in the bacterial DNA under these conditions is not enough to largely induce the *S. typhimurium* SOS response (and, consequently the error-prone DNA repair pathway) can be noted. The first point was the observation of similar rates of *S. typhimurium* wild-type Nal^R clones isolated from animals (Table 4) and in the in vitro experiments (Fig. 1). The second one, in contrast to the results obtained in the in vitro experiments (Fig. 1), was that the presence of the pRW30 plasmid in *S. typhimurium* cells did not increase the rate of their Nal^R derivatives recovered from animals (Table 4).

It has been proposed that intracellular pathogens like *S. typhimurium* are exposed to several putative mutagenic factors such as iron, hydrogen peroxide and alkylating agents, among others, during the infection process of an animal which directly or indirectly, through the SOS response, should be an important source of genetic variability in these environments [20]. Our data clearly indicate that other factors must be more determinant in the induction of this variability.

Furthermore, results reported in this work point out that cells of *S. typhimurium* with greater mutagenic strength are able to develop an infectious process normally and that they are neither excluded by nor dominant over the wild-type cells. Moreover, the non-exclusion of cells with a high mutagenic rate by the wild-type ones would enable the mixed population to attain a rapid adaptation to any possible new stimulus which may appear during animal infection.

Acknowledgements

This work was funded by Grants PB97-0194 and BIO99-0779 of the Ministerio de Educación y Ciencia de España and 1999SGR-106 from the Comissionat per Universitats i Recerca de la Generalitat de Catalunya. S.C. was the recipient of a predoctoral fellowship from the Direcció General d'Universitats de la Generalitat de Catalunya. We thank Dr. Roger Woodgate for his generous gift of the pRW30 plasmid. We are deeply indebted to Joan Ruiz and M. Mar López for their excellent technical assistance.

References

[1] Arber, W. (2000) Genetic variation: molecular mechanisms and impact on microbial evolution. FEMS Microbiol. Rev. 24, 1–7.

[2] Gilsdorf, J.R. (1998) Antigenic diversity and gene polymorphism in *Haemophilus influenzae*. Infect. Immun. 66, 5053–5059.

[3] LeClerc, j.E., Li, B., Payne, W.L. and Cebula, T.A. (1996) High mutation frequencies among *Escherichia coli* and *Salmonella* pathogens. Science 274, 1208–1211.

[4] Matic, I., Radman, M., Taddei, F., Picard, B., Doit, C., Bingen, E., Denamur, E. and Elion, J. (1997) Highly variable mutation rates in commensal and pathogenic *Escherichia coli*. Science 277, 1833–1834.

[5] Horst, J.P., Wu, T. and Marinus, M.G. (1999) *Escherichia coli* mutator genes. Trends Microbiol. 7, 29–36.

[6] Walker, G.C. (1984) Mutagenesis and inducible responses to deoxyribonucleic acid damage in *Escherichia coli*. Microbiol. Rev. 48, 60–93.

[7] Tang, M., Shen, X., Frank, E.G., O'Donnell, M., Woodgate, R. and Goodman, M.F. (1999) $UmuD'_2C$ is an error-prone DNA polymerase, *Escherichia coli* pol V. Proc. Natl. Acad. Sci. USA 96, 8919–8924.

[8] Runyen-Janecky, L.J., Hong, M. and Payne, S.M. (1999) The virulence plasmid-encoded *impCAB* operon enhances survival and induced mutagenesis in *Shigella flexneri* after exposure to UV radiation. Infect. Immun. 67, 1415–1423.

[9] Koch, H.W., Cebula, T.A., Foster, P.L. and Eisenstadt, E. (1992) UV mutagenesis in *Salmonella typhimurium* is *umuDC* dependent despite the presence of *samAB*. J. Bacteriol. 174, 2809–2815.

[10] Koch, W.H., Kopsidas, G., Meffle, B., Levine, A.S. and Woodgate, R. (1996) Analysis of chimeric UmuC proteins: identification of regions in *Salmonella typhimurium* UmuC important for mutagenic activity. Mol. Gen. Genet. 23, 121–129.

[11] Jordan, A., Aragall, E., Gibert, I. and Barbé, J. (1996) Promoter identification and expression analysis of *Salmonella typhimurium* and *Escherichia coli nrdEF* operons encoding one of two class I ribonucleotide reductases present in both bacteria. Mol. Microbiol. 19, 777–790.

[12] Clerch, B., Bravo, J.M. and Llagostera, M. (1996) Efficiency of MucAB and *Escherichia coli* UmuDC proteins in quinolone and UV mutagenesis *in Salmonella typhimurium*: effect of MucA and UmuD processing. Mutat. Res. 349, 201–208.

[13] Reed, L.J. and Muench, H. (1938) A simple method for estimating fifty percent endpoints. Am. J. Hyg. 27, 493–497.

[14] Kahn, S., Everest, P., Servos, S., Foxwell, N., Zähringer, U., Brade, H., Rietschel, E.T., Dougan, G., Charles, I.G. and Maskell, D.J. (1998) A lethal role for lipid A in *Salmonella* infections. Mol. Microbiol. 29, 571–579.

[15] Björkman, J., Hughes, D. and Andersson, D.I. (1998) Virulence of antibiotic-resistant *Salmonella typhimurium*. Proc. Natl. Acad. Sci. USA 95, 3949–3953.

[16] Shanabruch, W.G., Behlau, I. and Walker, G.C. (1981) Spontaneous mutators of *Salmonella typhimurium* LT2 generated by insertion of transposable elements. J. Bacteriol. 147, 827–835.

[17] Liu, H., Hewitt, S.R. and Hays, J.B. (2000) Antagonism of ultraviolet-light mutagenesis by the methyl-directed mismatch-repair system of *Escherichia coli*. Genetics 154, 503–512.

[18] García del Portillo, F., Pucciarelli, M.G. and Casadesús, J. (1999) DNA adenine methylase mutants of *Salmonella typhimurium* show defects in protein secretion, cell invasion, and M cell cytotoxicity. Proc. Natl. Acad. Sci. USA 96, 11578–11583.

[19] Zahrt, T.C., Buchmeier, N. and Maloy, S. (1999) Effect of *mutS* and *recD* mutations on *Salmonella* virulence. Infect. Immun. 67, 6168–6172.

[20] Mizrahi, V. and Andersen, S.J. (1998) DNA repair in *Mycobacterium tuberculosis*. What have we learnt from the genome sequence? Mol. Microbiol. 29, 1331–1339.

[21] Herrero, M., de Lorenzo, V. and Timmis, K.N. (1990) Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. J. Bacteriol. 172, 6557–6567.