Phylogenetic analysis of the genus *Aeromonas* based on two housekeeping genes

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SUMMARY

The phylogenetic relationships of all known species of the genus *Aeromonas*, and especially of the species *A. bestiarum* and *A. salmonicida*, were investigated using the sequence of *rpo*D, a gene that encodes the σ^{70} factor, which is one of the sigma factors that confers promoter-specific transcription initiation on RNA polymerase. This analysis was complemented with the sequence of *gyr*B, a gene that has already proved useful for determining the phylogenetic relationships of the genus. Nucleotide sequences of *rpo*D and *gyr*B were determined from 70 strains. Both genes had similar substitution rates (<2%) and a similar number of variable positions (34 % for *rpo*D versus 32 % for *gyr*B). Strain grouping by the analysis of *rpo*D, *gyr*B and the combination of both genes were consistent with the taxonomic organization of all *Aeromonas* species described to date. However the simultaneous analysis of both clocks improved the reliability and power to differentiate, in particular, closely related taxa. At inter-species level, *gyr*B showed a better resolution for differentiating *Aeromonas* sp. HG11 / *A. encheleia*, and *A. veronii* / *A. culicicola* / *A. allosaccharophila*, while *rpo*D more clearly differentiated *A. salmonicida* from *A. bestiarum*. The analysis of *rpo*D revealed initial evidence for a clear phylogenetic divergence between *A. salmonicida* and *A. bestiarum*.

INTRODUCTION

The taxonomy of the genus *Aeromonas* is complex. During the last two decades, the number of new species descriptions have dramatically increased, resulting in the following species being recognized: *A. hydrophila*, *A. bestiarum*, *A. salmonicida*, *A. caviae*, *A. media*, *A. eucrenophila*, *A. sobria*, *A. veronii* (biogroups sobria and veronii), *A. jandaei*, *A. schubertii*, *A. trota*, *A. allosaccharophila*, *A. encheleia*, and *A. popoffii*. The two DNA homology groups, *Aeromonas* sp. (HG11), *Aeromonas* sp. (HG13; formerly Enteric Group 501), remain without a species name. The new species *A. culicicola* (Pidiyar et al., 2002) was recently described. The species *A. ichthiosmia* (Schubert et al., 1990b) and *A. enteropelogenes* (Schubert et al., 1990a) are now considered synonyms of *A. veronii* and *A. trota*, respectively (Collins et al., 1993; Carnahan, 1993; Huys et al., 2001; Huys et al., 2002).

One of the controversial taxonomic issues is represented by the species *A. hydrophila*, type species of the genus, and related species *A. bestiarum*, *A. salmonicida* and *A. popoffii*, all of them included in the so-called “A. hydrophila” complex (Janda & Abbot, 1998). *A. salmonicida* includes four psycrophilic non-motile subspecies: *A. salmonicida* subsp. salmonicida (Griffin et al., 1953), *A. salmonicida* subsp. achromogenes (Smith, 1963), *A. salmonicida* subsp. masoucida (Kimura, 1969a, b) and *A. salmonicida* subsp. smithia (Austin et al., 1989); and the mesophilic subspecies *A. salmonicida* subsp. pectinolytica (Pavan et al., 2000). In addition, *A. salmonicida* includes motile mesophilic strains confusingly referred to as “A. hydrophila (HG3)” (Altwegg et al., 1990). Results obtained by Soler et al. (2003) indicated a lack of phenotypic and genotypic criteria to differentiate *A. bestiarum* from *A. salmonicida*, whether due to limitations of selected techniques or because we are dealing with a single, although heterogeneous, species. Moreover, a phylogenetic analysis using *gyr*B sequences confirmed a very close relationship between two clusters corresponding to

A. bestiarum and *A. salmonicida*, but definitive conclusions could not be drawn as the range of nucleotide substitutions was border-line to that considered characteristic for different *Aeromonas* species (Yañez et al., 2003).

The study of two or more housekeeping genes could be useful to improve the reliability of the phylogenies (Stackebrandt et al., 2002; Yamamoto & Harayama 1998). It has been reported that *gyr*B (encoding the B subunit of DNA gyrase, a type II DNA topoisomerase) and *rpo*D (encoding σ^{70} factor, which is one of the sigma factors that confers promoter-specific transcription initiation on RNA polymerase) could be suitable phylogenetic markers for bacterial systematics (Gruber & Bryant 1997; Kim et al., 1999; Huang 1996; Yamamoto et al., 2000; Watanabe et al., 2001). They seem good index genes for determining the course of genome evolution because they are indispensable single-copy genes on which horizontal gene transfer seldom occurs (Sawada et al., 1999). Recently, Yañez et al., (2003) demonstrated that the *gyr*B sequence is an excellent molecular chronometer for phylogenetic inference in the genus *Aeromonas*, but only 14 strains corresponding to species of the "A. hydrophila" complex were included. To our knowledge, no data is available on the phylogenetic usefulness of the *rpo*D sequence in the genus *Aeromonas*.

In the present study, nucleotide sequences of *gyr*B and *rpo*D genes were determined from a collection of 70 *Aeromonas* strains, which includes all described species and DNA Homology Groups (HGs). The derived phylogenetic analysis was assessed to investigate the *Aeromonas* inter-species relationships, in particular between the species *A. salmonicida* and *A. bestiarum*. Two genes and more strains were used to assess the congruence with previous *gyr*B phylogeny and results of 16S rDNA sequencing and DNA-DNA pairing studies.

METHODS

Bacterial strains, culture conditions, and 16S rDNA identification. A total of 70 strains comprising all known *Aeromonas* species, were examined in this study (Table 1). New isolates and reference strains from culture collections were identified genetically by the 16S rDNA RFLP (Figueras et al., 2000) and further sequencing (Martinez-Murcia et al., 1999). Bacterial strains were grown on tryptic soy agar (Difco, Barcelona, Spain) at 28^{o}C with the exception of those belonging to the psychrophilic species *A. salmonicida*, which were grown at room temperature.

DNA extraction and purification. A single colony from fresh cultures was resuspended in 50 μL of TE, vortexed at high speed for 1 min, and incubated at 96ºC for 10 min. The tube was again vortexed and centrifuged for 2 min at 12,000 g. The supernatant was transferred to a fresh tube and stored at –20ºC.

PCR amplification and sequencing of *gyr*B and *rpo*D. A fragment of *ca*.1100 bp of the *gyr*B gene was amplified by PCR and sequenced as previously described (Yañez et al., 2003). PCR amplification for the *rpo*D gene (fragment of *ca*. 820 bp) was performed in a Gene-AMP (PCR System Perkin Elmer 2400). A final PCR volume of 100 μl containing 1μg genomic DNA, 20 mM Tris-HCl pH8.4, 50 mM KCl, 3 mM $MgCl_2$, 0.3 mM dNTP´s, 2.5 U *Taq* DNA polymerase (Invitrogen, Barcelona, Spain) and 1 μM of each primer. The reaction mixture was subjected to a touchdown PCR regimen of: denaturation at 95°C for 5 min, followed by 2 cycles at 94^{o}C for 1 min (denaturation); 63^{o}C for 1 min (annealing); 72^{o}C for 1 min (extension); 2 cycles at 94^{o}C, 1 min; 61^{o}C, 1 min; 72^{o}C, 1 min; 2 cycles at 94^{o}C, 1 min; 59^{o}C, 1 min; 72^{o}C, 1 min; and 30 cycles at 94^{o}C, 1 min; 58^{o}C, 1 min; 72^{o}C, 1 min. Amplified products were electrophoresed on 0.8% agarose-TBE gels, and purified using the GFX^{TM} PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech, Barcelona, Spain). Nucleotide

sequences were determined by using the ABI PRISM® BigDye™ Terminators v2.0 Cycle Sequencing Kit (Applied Byosistems, Foster City, CA, USA) in the ABI PRISM® 310 Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions. Characteristics of primers used for PCR amplification and sequencing of *gyr*B and *rpo*D are summarized in Table 2.

Phylogenetic data analysis. The nucleotide sequences of *gyr*B and *rpo*D were independently aligned by the CLUSTAL X program, version 1.8 (Thompson et al., 1997). Genetic distances were obtained using Kimura's 2 parameter model (Kimura, 1980) and evolutionary trees were constructed by the neighbour-joining method (Saitou & Nei, 1987) with the MEGA program (Kumar et al., 2001). A *gyr*B-*rpo*D phylogenetic tree was also constructed from the combination of these two genes together and treating them as a single nucleotide sequence (Yamamoto et al., 2000).

RESULTS AND DISCUSSION

In the present study, nucleotide sequences of *gyr*B and *rpo*D amplicons were determined from 26 and 68 *Aeromonas* strains, respectively (Table 1). The other *gyr*B sequences were previously published in a phylogenetic study of the genus *Aeromonas* (Yañez et al., 2003). Experiments were repeated at least twice from single colonies of original cultures to confirm readings and solve ambiguities. The derived *gyr*B sequences comprised between 961 and 964 nucleotides covering more than 70% of the ATPase domain (amino acid residues 2-392 in *E.coli*; Huang 1996) and 191 nucleotides of the 3' flanking region. The *rpo*D sequences comprised between 813 and 825 nucleotides covering approximately 46% of the protein (amino acid residues 112 to 369 in *E.coli*; Barne et al., 1997), although the active domain is not included in this stretch. Identification of species by 16S rDNA RFLP and/or sequencing is shown in

Table1.

Comparative analysis of *gyr*B and *rpo*D sequence alignments. The *gyr*B sequences from all aeromonads were aligned and percentages of nucleotide substitutions were calculated for a continuous stretch of *ca.* 961 bp (ranging from positions 404 to 1364 according to *E. coli* numbering). Ranges of inter-species nucleotide substitution rates were calculated (Table 3) by taking the maximum and minimum divergence values obtained when comparing each single species (including all isolates) with all the other strains of *Aeromonas* spp. Sequence similarity between all *Aeromonas* strains ranged from 86.3 to 100%, corresponding to 0-131 nucleotide differences. The number of total variable positions was 306 (*ca*. 32% of the determined fragment) in addition to an insertion/deletion of a single triplete. At the intra-species level, the rates of nucleotide substitutions ranged from 0 to 2.3 %, often showing values under 2 %, with an overall value of ca. 1.6%. However, inter-species nucleotide substitutions were usually over 3 %, except for the following two pairs of species: *A. salmonicida* and *A. bestiarum* (1.8-4.3 %), and *A. encheleia* and *Aeromonas* sp. HG11 (2.1-2.2 %).

The *rpoD* sequences from all aeromonad strains were aligned and percentages of nucleotide substitutions (Table 3) were calculated for a continuous stretch of *ca.* 820 bp (ranging from positions 332 to 1108 according to *E. coli* numbering). Sequence similarity between all *Aeromonas* strains ranged from 81.7 to 100 %, corresponding to 1-148 nucleotide differences. The alignment exhibited a total of 281 variable positions (ca. 34% of the fragment sequenced) and the number of insertions/deletions was 12 bp, corresponding to 4 codons. At the intra-species level, the rates of nucleotide substitutions ranged from 0 to 2.6 %, being under 2 % for most *Aeromonas* species, with an overall value of ca. 1.6 %. Inter-specie nucleotide substitution was over 3%

except for the following pairs of species: *A. encheleia* / *Aeromonas* sp. HG11 (1.4-1.7 %), *A. veronii* / *A. culicicola* (1.6-1.7 %).

The comparison of these data, between *gyr*B and *rpo*D, indicated that both genes show similar substitution rates. The number of variable positions was not very different (32 % for *gyr*B versus 34 % for *rpo*D) and the ranges of nucleotide mutations between all strains were also almost the same (0-131 and 0-148, respectively), considering that 70% of determined *gyr*B-fragment corresponded to the ATPase domain while the *rpoD*-fragment did not contain a protein active-site. However, depending on the taxonomic level subjected to comparison, these two molecular clocks may behave differently. At the intra-species level, substitution rates were very similar: the range of values for *gyr*B was 0-2.3 %, for *rpo*D was 0-2.6 %, and most likely around 1.6 % in both gene sequences. But, while identical *rpo*D sequences were determined for the pair of strains CECT4341 and CECT4342^{T} of *A. encheleia*, and CECT4827 and CECT4224^{T} of *A. eucrenophila*, the corresponding *gyr*B sequences showed 2 and 8 differences, respectively. As a consequence of this analysis, *gyr*B seems more useful to differentiate closely related strains. Also, in agreement with *gyr*B, identical *rpo*D sequences were found for strain CECT4234 and type strain of *A. media*, for strain CECT4200 and type strain of *A. allosaccharophila*, but they were considered strain duplicates, which has been confirmed by present results. At the inter-species level, the nucleotide substitution rate of *rpo*D is notably higher than (very often almost double that of) *gyr*B (Table 3), except for two pairs of species: *A. encheleia* / *Aeromonas* sp. HG11 (2.1-2.2 %, *gyr*B; 1.4-1.7 %, *rpo*D), and *A. veronii* / *A. culicicola* (3.5-3.7 %, *gyr*B; 1.6-1.7 %, *rpo*D). An illustrative example of this higher inter-species mutation rate exhibited by *rpo*D is represented by the case *A. salmonicida* / *A. bestiarum* showing a range of 1.8-4.3 % on *gyr*B, but 6.8-8.7 % on the *rpo*D sequence.

In a conclusion, in *Aeromonas* spp., *rpo*D showed a better inter-species

resolution for differentiating *A. salmonicida* from *A. bestiarum*; however, this gene is superseded by *gyr*B for differentiating *Aeromonas* sp. HG11 / *A. encheleia*, and *A. veronii* / *A. culicicola* / *A. allosaccharophila*.

Phylogenetic relationships of *Aeromonas* spp. Unrooted phylogenetic trees for *gyr*B (Fig. 1A), *rpo*D (Fig. 1B), and the combined *gyr*B-*rpo*D gene sequences (1770 bp.; Fig. 1C), were constructed from the derived genetic matrices. All obtained phylogenies show considerable divergence (branch lengths) between all *Aeromonas* species under study. Strain grouping is consistent in all three trees, also in agreement with a preliminary phylogenetic study based on *gyr*B, recently published (Yañez et al., 2003). Some differences in topology at the deepest branching points of single *gyr*B and *rpo*D trees are observed (Figs. 1 and 2). This may be due to the differences in the inter-species nucleotide substitution rates found between *gyr*B and *rpo*D sequences and, as a consequence, lower bootstrap values appear at the deepest branching points of the combined *gyr*B-*rpo*D tree (Fig. 1C). Because *rpo*D yielded a better resolution than *gyr*B, for splitting *A. salmonicida* from *A. bestiarum*, and the opposite was found for other closely related species (viz.: *A. encheleia* / *Aeromonas* sp. HG11; or *A. veronii* / *A. culicicola*), the phylogeny of the genus *Aeromonas* was expected to be improved by constructing the *gyr*B-*rpo*D tree, as it comprises the combined capacities of both molecular clocks. The study of several housekeeping gene sequences has already been recommended to improve the reliability of phylogenetic inferences (Stackebrandt et al., 2002).

Addition of *rpo*D analysis to the systematics of the genus *Aeromonas* has increased the advantages previously found on the basis of *gyr*B sequences (Yañez et al., 2003), compared to the referenced 16S rDNA phylogeny (Martínez-Murcia et al., 1992; Martínez-Murcia 1999). Examples of that are the pairs of species *A. trota* / *A.*

caviae, *A. hydrophila* / *A. media*, and *A. culicicola* / *A. jandaei*, which show only 1, 3, and 1 nucleotide differences, respectively, in their 16S rDNAs, are clearly separated by *gyr*B sequences (7.3 %, 6.5 %, and 6.3% respectively) and even more by *rpo*D (9.8 %, 9.7%, and 7.2%). Agreements between *gyr*B and 16S rDNA (Yañez et al., 2003) are here confirmed, but also improved by the results obtained in the *gyr*B-*rpo*D inference. For instance, *A. schubertii* clustered at the deepest branch of the genus, and *A. veronii* biogroup sobria (HG8) and *A. veronii* biogroup veronii (HG10) represent two heterogeneous phenotypes of a single species. The phylogenetic distinctiveness of *A. allosaccharophila* and *A. veronii* is greater in the *gyr*B sequences (3.3-4.0%); but, although very closely related by *rpo*D, the range of nucleotide substitution rates (2.6 to 3.2 %) were still over the observed overall value (ca. 1.6%) to consider them the same species. However, *A. culicicola* and *A. veronii* showed a range of *rpo*D mutation rates of 1.6-1.7 %, which is border-line to the more likely intra-species value observed in the present analysis (under 2%). One of the most controversial taxonomic issues is currently represented by the species *A. encheleia* and *Aeromonas* sp. HG11 (Huys et al., 1997; Martínez-Murcia 1999; Yañez et al., 2003). Divergence determined by *gyr*B was over 2 %, but *rpo*D differences between these groups of strains ranged from 1.4 to 1.7 %. This result is critical as, following the overall analysis of the genus *Aeromonas*, these values fall within the limits to consider these strains as belonging to a single species. Nevertheless, only two strains for each group (HG11 and *A. encheleia*) have been subjected to *rpo*D sequencing. We recommend the analysis of several housekeeping genes to investigate if any considerable phylogenetic divergence can be detected between these microorganisms.

In the study of Yañez et al. (2003), a group of isolates (531c, 610, 520) have shown a considerable *gyr*B relationship to the cluster corresponding to *A. media*, but exhibited a range of substitution rates of 2.7-3.1 %. As reported, these strains showed

from 2 to 9 nucleotide differences in their 16S rDNA compared to the *A. media* type strain, but all these mutations were located at the hypervariable stem-loop (positions 457-476), a region considered unreliable for making phylogenetic conclusions. This nucleotide diversity may be detected by 16S rDNA-RFLP as they yielded profiles differing from these corresponding to type strains. In the present study, more isolates selected by 16S rDNA analysis were included (280, 480, 57, 239, 741) and their phylogenetic location can be seen on the *gyr*B tree (Fig. 1A). Two strains (741 and 239) clustered together with the type strain of *A. media*, but the rest (isolates 57, 480, 610, and 280) formed an independent cluster, although closely related to *A. media*, indicating that this may represent a distinct phylogenetic line of *Aeromonas*. Differences in mutation rates were discerned for some of these isolates when comparing *gyr*B with *rpo*D sequencing results. An example is the isolate 239 that showed a *gyr*B divergence range of 2.9-3.9 % to strains of the so-called "independent cluster", but only 1.2-1.6 % by *rpo*D. This is again a case where different mutation behaviour on two housekeeping genes is found for closely related strains. The possibility of horizontal transfer of these genes, although not very likely, must be considered. Any hypothesis about these kind of results needs to be supported by a more comprehensive set of gene data.

The species *A. salmonicida* and *A. bestiarum*.

Our previous *gyr*B study (Yañez et al., 2003) showed that the inter-relationship between *A. salmonicida* (HG3) and *A. bestiarum* (HG2) is a difficult case to solve because a relatively low range of substitution (2.2-3.3 %) was detected. Nevertheless, a limited number of strains was included in the mentioned work. This was consistent with 16S rDNA studies as, although type strains of these species differ in two single nucleotides (Martínez-Murcia 1999), many strains simultaneously contained copies of

both types of 16S molecules (Soler et al., 2003; present study and unpublished results). Therefore, the presence of one or other type of 16S rDNA, or both at the same time, seems absolutely random and, as a consequence, splitting of *A. salmonicida* from *A. bestiarum* is not possible by using this genetic marker (Table 3). All these difficulties were also found in the separation of HG2 from HG3 strains by DNA-DNA hybridization (Hänninen, 1994; Ali et al., 1996). In a recent molecular and phenotypic approach to the taxonomy of the "Aeromonas hydrophila" complex, Soler et al. (2003) indicated a lack of criteria for splitting *A. salmonicida* from *A. bestiarum*.

In the present study, the number of strains of these species have been notably increased in an attempt to clarify their phylogenetic relationship. The *gyr*B inter-species ranges found were 2.2-4.3 % (between *A. salmonicida* and *A. bestiarum*), 2.7-4.3 % (*A. bestiarum* versus *A. popoffii*), and 3.1-5.2 % (*A.salmonicida* and *A. popoffii*), still very low values, but above the overall intra-species ranges observed for *Aeromonas*. The *rpo*D analysis has yielded, however, ranges of 6.8-8.7 %, 4.0-5.6 %, and 8.1-9.3 %, respectively, where it is worth nothing that resolution power to split *A. salmonicida* from *A. bestiarum* is twice that obtained by *gyr*B sequences. Consequently, we report in this paper a phylogentic marker that is able to separate these two species, indistinguishable by other sequences previously used. This is also the advantage of using several housekeeping genes for systematics based on phylogeny (Stackebrandt et al., 2002). Apart from the genetic transfer hypothesis, different genes (obviously not totally synchronized from a evolutionary point of view) may show a clear ability to split, depending on the taxa subjected to analysis. Whether or not *rpo*D has been transferred during the evolution of these species must be investigated by sequencing a large number of housekeeping genes.

In conclusion, *gyr*B and *rpo*D gene sequences, independently, have proved to

be excellent molecular markers for assessing phylogeny in the genus *Aeromonas*. Moreover, the simultaneous analysis of both clocks improve the reliability and power to differentiate, in particular, closely related taxa. This advantage derives from the differences in mutation rates that distinct proteins (different functional compromises) contain. Although controversial taxonomic issues remain unsolved, initial evidence for a clear phylogenetic divergence between *A. salmonicida* and *A. bestiarum* is reported in this work. The sequence of more genes is obviously needed and they may contribute to an understanding of the evolutionary meaning of some *Aeromonas* groups.

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Figure legends.

Fig. 1. Unrooted phylogenetic trees based on *gyr*B (A), *rpo*D (B), and the combined stretch of *gyr*B-*rpo*D gene sequences (C), showing the relationships of the genus *Aeromonas*. Type strain for each species is in bold. The numbers shown next to each node indicate bootstrap values (percentage of 1000 replicates).