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

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The phylogenetic relationships of all known species of the genus *Aeromonas*, and especially *Aeromonas bestiarum* and *Aeromonas salmonicida*, were investigated on 70 strains using the *rpoD* sequence, which encodes the σ^{70} factor. This analysis was complemented with the sequence of *gyrB*, which has already proven useful for determining the phylogenetic relationships in the genus. Nucleotide sequences of *rpoD* and *gyrB* showed that both genes had similar substitution rates (<2%) and a similar number of variable positions (34% for *rpoD* versus 32% for *gyrB*). Strain groupings by analysis of *rpoD*, *gyrB* and a 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 the power to differentiate, in particular, closely related taxa. At the inter-species level, *gyrB* showed a better resolution for differentiating *Aeromonas* sp. HG11/*Aeromonas encheleia* and *Aeromonas veronii*/*Aeromonas culicicola*/*Aeromonas allosaccharophila*, while *rpoD* more clearly differentiated *A. salmonicida* from *A. bestiarum*. The analysis of *rpoD* provided initial evidence for clear phylogenetic divergence between the latter two species.

INTRODUCTION

The taxonomy of the genus *Aeromonas* is complex. During the last two decades, the number of new species descriptions has progressively increased, resulting in the following recognized species: *Aeromonas hydrophila*, *Aeromonas bestiarum*, *Aeromonas salmonicida*, *Aeromonas caviae*, *Aeromonas media*, *Aeromonas eucrenophila*, *Aeromonas sobria*, *Aeromonas veronii* (biogroups *sobria* and *veronii*), *Aeromonas jandaei*,

Aeromonas schubertii, *Aeromonas trota*, *Aeromonas allosaccharophila*, *Aeromonas encheleia* and *Aeromonas popoffii*. The two DNA homology groups (HGs), *Aeromonas* sp. (HG11), *Aeromonas* sp. (HG13; formerly Enteric Group 501), remain without a species name. The new species *Aeromonas culicicola* was described recently (Pidiyar *et al.*, 2002). The species *Aeromonas enteropelogenes* (Schubert *et al.*, 1990a) and *Aeromonas ichthiosmia* (Schubert *et al.*, 1990b) are now considered to be synonyms of *A. trota* and *A. veronii*, respectively (Carnahan, 1993; Collins *et al.*, 1993; Huys *et al.*, 2001, 2002).

One of the controversial taxonomic issues is represented by the species *A. hydrophila*, type species of the genus, and related species *A. bestiarum* and *A. salmonicida*, all of them included in the so-called 'A. hydrophila' complex (Janda & Abbott, 1998). *A. salmonicida* includes four psychrophilic non-motile subspecies: *A. salmonicida* subsp. *salmonicida* (Griffin *et al.*, 1953), *A. salmonicida* subsp. *achromogenes* (Smith, 1963), *A. salmonicida* subsp. *masoucida* (Kimura,

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Abbreviation: HG, homology group.

The GenBank/EMBL/DDBJ accession numbers for the *gyrB* and *rpoD* sequences determined in this study are given in Fig. 1.

A matrix table showing nucleotide substitution rates in *gyrB* and *rpoD* sequences is available as supplementary material in IJSEM Online.

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). A lack of phenotypic and genotypic criteria to split *A. bestiarum* from *A. salmonicida* has been found (Martínez-Murcia and co-workers, unpublished data). Moreover, a phylogenetic analysis using *gyrB* sequences confirmed a very close relationship between the two clusters corresponding to *A. bestiarum* and *A. salmonicida*, but definitive conclusions could not be taken as the range of nucleotide substitutions was borderline of that observed for other *Aeromonas* species (Yáñez *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 *gyrB* (encoding the B-subunit of DNA gyrase, a type II DNA topoisomerase) and *rpoD* (encoding σ^{70} factor which is one of the sigma factors that confer promoter-specific transcription initiation on RNA polymerase) could be suitable phylogenetic markers for bacterial systematics (Gruber & Bryant, 1997; Huang, 1996; Kim *et al.*, 1999; Watanabe *et al.*, 2001; Yamamoto *et al.*, 2000). They seem to be good index genes for determining the course of genome evolution because they are essential single-copy genes on which horizontal genes transfer seldom occurs (Sawada *et al.*, 1999). Recently, Yáñez *et al.* (2003) demonstrated that the *gyrB* sequence is an excellent molecular chronometer for phylogenetic inference in the genus *Aeromonas*, but only 17 strains corresponding to species of the 'A. hydrophila' complex were included. To our knowledge, no data are available about the phylogenetic usefulness of the *rpoD* sequence in the genus *Aeromonas*.

In the present study, nucleotide sequences of *gyrB* and *rpoD* genes were determined from a collection of 70 *Aeromonas* strains that includes all described species and DNA HGs. Derived phylogenetic analyses were inferred to investigate the *Aeromonas* inter-species relationships, in particular between *A. salmonicida* and *A. bestiarum*. Two genes and more strains were used to assess the congruence with previous *gyrB* phylogeny and results of 16S rRNA gene sequencing and DNA–DNA pairing studies.

METHODS

Bacterial strains, culture conditions and 16S rRNA 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 by 16S rRNA gene-RFLP (Figueroa *et al.*, 2000) and further sequencing (Martínez-Murcia *et al.*, 1999). Bacterial strains were grown on tryptic soy agar (Difco) at 28 °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 TE, vortexed at high speed for

1 min, and incubated at 96 °C 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 –20 °C.

PCR amplification and sequencing of *gyrB* and *rpoD*. A fragment of approximately 1100 bp of the *gyrB* gene was amplified by PCR and sequenced as described previously (Yáñez *et al.*, 2003). PCR amplification for the *rpoD* gene (fragment of approx. 820 bp) was performed in a Gene-AMP (PCR System Perkin Elmer 2400). A final PCR volume of 100 µl contained 1 µg genomic DNA, 20 mM Tris/HCl pH 8.4, 50 mM KCl, 3 mM MgCl₂, 0.3 mM dNTPs, 2.5 U *Taq* DNA polymerase (Invitrogen) and 1 µM each primer. The reaction mixture was subjected to a touch-down PCR regimen of: denaturation at 95 °C for 5 min, followed by 2 cycles at 94 °C for 1 min (denaturation); 63 °C for 1 min (annealing); 72 °C for 1 min (extension); 2 cycles at 94 °C, 1 min; 61 °C, 1 min; 72 °C, 1 min; 2 cycles at 94 °C, 1 min; 59 °C, 1 min; 72 °C, 1 min; and 30 cycles at 94 °C, 1 min; 58 °C, 1 min; 72 °C, 1 min. Amplified products were electrophoresed on 0.8% agarose/TBE gels, and purified using the GFX PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech). Nucleotide sequences were determined by using the ABI PRISM BigDye Terminators v2.0 Cycle Sequencing Kit 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 *gyrB* and *rpoD* are summarized in Table 2.

Phylogenetic data analysis. The nucleotide sequences of *gyrB* and *rpoD* 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 *gyrB*–*rpoD* phylogenetic tree was also constructed from the combination of these two genes and treating it as a single nucleotide sequence (Yamamoto *et al.*, 2000).

RESULTS AND DISCUSSION

In the present study, nucleotide sequences of *gyrB* and *rpoD* amplicons were determined from 26 and 68 *Aeromonas* strains, respectively (Table 1). The other *gyrB* sequences were published previously in a phylogenetic study of the genus *Aeromonas* (Yáñez *et al.*, 2003). Experiments were repeated at least twice from single colonies of original cultures to confirm readings and solve ambiguities. The derived *gyrB* sequences comprised between 960 and 1100 nucleotides covering more than 70% of the ATPase domain (amino acid residues 2–392 in *Escherichia coli*; Huang, 1996) and 191 nucleotides of the 3' flanking region. The *rpoD* sequences comprised between 813 and 825 nucleotides covering approximately 46% of the protein (amino acid residues 112–369 in *E. coli*; Barne *et al.*, 1997); however, the active domain is not included in this stretch. Identification of species by 16S rRNA gene sequencing is shown in Table 1.

Comparative analysis of *gyrB* and *rpoD* sequence alignments

The *gyrB* sequences from all aeromonads were aligned and percentages of nucleotide substitutions were calculated for a continuous stretch of 957 bases (positions 404–1364

Table 1. *Aeromonas* strains used in this study

DNA homology groups are given in parentheses. Culture collections: ATCC, American Type Culture Collection, Manassas, VA, USA; CCUG, Culture Collection of the University Göteborg, Göteborg, Sweden; CDC, Centers for Disease Control and Prevention, Atlanta, GA, USA; CECT, Colección Española de Cultivos Tipo, Universidad de Valencia, Valencia, Spain; CIP, Collection bactérienne de l'Institut Pasteur, Paris, France; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; LMG, Culture Collection of the Laboratorium voor Microbiologie Gent, Universiteit Gent, Ghent, Belgium; NCIMB, National Collection of Industrial and Marine Bacteria, Aberdeen, UK.

Species	Strain no.	Other designation(s)	16S rRNA
<i>A. hydrophila</i> (HG1)	CECT 839 ^T	ATCC 7966 ^T , DSM 30187 ^T , Popoff 543 ^T	<i>A. hydrophila</i>
<i>A. bestiarum</i> (HG2)	CECT 4227 ^T	ATCC 51108 ^T ; CDC 9533-76 ^T , LMG 13444 ^T , Popoff 218 ^T	<i>A. salmonicida</i> / <i>A. bestiarum</i>
	LMG 13662	A295	<i>A. salmonicida</i> / <i>A. bestiarum</i>
	LMG 13448	A169	<i>A. salmonicida</i> / <i>A. bestiarum</i>
<i>A. salmonicida</i> (HG3)	CECT 894 ^T	ATCC 33658 ^T , CIP 103209 ^T , LMG 3780 ^T	<i>A. salmonicida</i> / <i>A. bestiarum</i>
	CECT 5173	Popoff 316	<i>A. salmonicida</i> / <i>A. bestiarum</i>
	CECT 4237	JCM 8199, NCIMB 1103	<i>A. salmonicida</i> / <i>A. bestiarum</i>
subsp. <i>masoucida</i>	CECT 896 ^T	ATCC 27013 ^T , CIP 103210 ^T , LMG 3782 ^T	<i>A. salmonicida</i> / <i>A. bestiarum</i>
subsp. <i>achromogenes</i>	CECT 895 ^T	ATCC 33659 ^T , LMG 14900 ^T , NCIMB 1110 ^T	<i>A. salmonicida</i> / <i>A. bestiarum</i>
subsp. <i>pectinolytica</i>	DSM 12609 ^T	34mel ^T	<i>A. salmonicida</i> / <i>A. bestiarum</i>
<i>A. caviae</i> (HG4)	CECT 838 ^T	ATCC 15468 ^T , LMG 3775 ^T , Popoff 545 ^T	<i>A. caviae</i>
	CECT 4221	ATCC 15467, CIP 7615, LMG 3755, Popoff 544	<i>A. caviae</i>
<i>A. media</i> (HG5)	CECT 4232 ^T	ATCC 33907 ^T , LMG 9073 ^T , NCIMB 2237 ^T	<i>A. media</i>
	CECT 4234	ATCC 35950	<i>A. media</i>
<i>A. eucrenophila</i> (HG6)	CECT 4224 ^T	ATCC 23309 ^T , LMG 3774 ^T , NCIMB 74 ^T , Popoff 546 ^T	<i>A. eucrenophila</i>
	CECT 4827	LMG 13057, CDC 9179, A914, CCUG 30341	<i>A. eucrenophila</i>
<i>A. sobria</i> (HG7)	CECT 4245 ^T	ATCC 43979 ^T , CIP 74.33 ^T , LMG 3783 ^T , Popoff 208 ^T	<i>A. sobria</i>
<i>A. veronii</i> bg. <i>sobria</i> (HG8)	CECT 4246	ATCC 9071, NCIMB 37, LMG 3785	<i>A. veronii</i>
<i>A. jandaei</i> (HG9)	CECT 4228 ^T	A1642 ^T , ATCC 49568 ^T , LMG 12221 ^T	<i>A. jandaei</i>
<i>A. veronii</i> bg. <i>veronii</i> (HG10)	CECT 4257 ^T	A901 ^T , ATCC 35624 ^T , LMG 9075 ^T	<i>A. veronii</i>
	CECT 4260	ATCC 35625, LMG 16334	<i>A. veronii</i>
<i>Aeromonas</i> sp. (HG11)	CECT 4253	A902, ATCC 35941, NCIMB 13014	<i>Aeromonas</i> sp. (HG11)
	CECT 4856	A926, LMG 13076, CCUG 30365	<i>Aeromonas</i> sp. (HG11)
<i>A. schubertii</i> (HG12)	CECT 4240 ^T	ATCC 43700 ^T , CDC 2446-81 ^T , LMG 9074 ^T	<i>A. schubertii</i>
<i>Aeromonas</i> sp. (HG13)	CECT 4254	ATCC 43946, CDC 2478-85	<i>Aeromonas</i> sp.
<i>A. trota</i> (HG14)	CECT 4255 ^T	A1646 ^T , ATCC 49657 ^T , LMG 12223 ^T	<i>A. trota</i>
	CECT 4935	LMG 13080, ATCC 49659, AS 370	<i>A. trota</i>
<i>A. allosaccharophila</i> (HG15)	CECT 4199 ^T	ATCC 51208 ^T , CCUG 31218 ^T , S289 ^T , LMG 14059 ^T	<i>A. allosaccharophila</i>
	CECT 4220	ATCC 35942, CDC 0715-84, LMG 14021	<i>A. allosaccharophila</i>
	CECT 4200	S290, LMG 14058	<i>A. allosaccharophila</i>
<i>A. encheleia</i> (HG16)	CECT 4342 ^T	ATCC 51929 ^T , S181 ^T , NCIMB 13442 ^T	<i>A. encheleia</i>
	CECT 4341	ATCC 51930, S176, LMG 16329	<i>A. encheleia</i>
<i>A. popoffii</i> (HG17)	CECT 5176 ^T	LMG 17541 ^T , IK-O-a-10-3 ^T	<i>A. popoffii</i>
	LMG 17543–17546		<i>A. popoffii</i>
<i>A. culicicola</i>	MTCC 3249 ^T		<i>A. culicicola</i>
<i>Aeromonas</i> sp.	Isolates 140P, 830, 874		<i>A. hydrophila</i>
	Isolate 63c	CECT 5216	<i>A. hydrophila</i>
	Isolate A7	LMG 13452	<i>A. salmonicida</i> / <i>A. bestiarum</i>
	Isolate 101F	CECT 5219	<i>A. salmonicida</i> / <i>A. bestiarum</i>
	Isolate 220c	LMG 18998	<i>A. salmonicida</i> / <i>A. bestiarum</i>

Table 1. cont.

Species	Strain no.	Other designation(s)	16S rRNA
	Isolates 37, 119P, 120P, 156, A99, 440, 130, 361c, 108F, 22, 107F, 26, PIW-98, 40, 117P, 116P		<i>A. salmonicida</i> / <i>A. bestiarum</i>
	Isolate 665c		<i>A. caviae</i>
	Isolate 239, 480, 280, 610, 57		<i>Aeromonas</i> sp.
	Isolate 741		<i>A. media</i>
	Isolate 350		<i>A. sobria</i>
	Isolate 344		<i>A. jandaei</i>

according to *E. coli* numbering). Ranges of inter-species nucleotide substitution rates were determined (available as supplementary material in IJSEM Online) by taking the maximum and minimum divergence values obtained when comparing each single species (including all isolates) with all strains of all other *Aeromonas* species. Sequence similarity between all *Aeromonas* strains was 86.3–100 %, corresponding to 0–131 nucleotide differences. Number of total variable positions was 306 (approx. 32 % of the determined fragment) in addition to insertion/deletion of a single triplet. At the intra-species level, the rates of nucleotide substitutions were 0–2.3 %, often showing values under 2 %, with an overall value of approximately 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 percentage nucleotide substitutions (Table A in IJSEM Online) were calculated for a continuous stretch of 813 bases (ranging from positions 332–1108 according to

E. coli numbering). Sequence similarity between all *Aeromonas* strains was 81.7–100 %, corresponding to 1–148 nucleotide differences. The alignment exhibited a total of 281 variable positions (approx. 34 % of the fragment sequenced) and number of insertions/deletions was 12 bp, corresponding to 4 codons. At the intra-species level, the rates of nucleotide substitutions were 0–2.6 %, being under 2 % for most *Aeromonas* species, and overall value of approximately 1.6 %. Inter-species nucleotide substitution was over 3 % except for the following pairs of species: *A. encheleia*/*Aeromonas* sp. HG11 (1.4–1.7 %) and *A. veronii*/*A. culicicola* (1.6–1.7 %).

The comparison of *gyrB* and *rpoD* data indicated that both genes show similar substitution rates. The number of variable positions is not very different (32 % for *gyrB* versus 34 % for *rpoD*) and the ranges of nucleotide mutations between all strains are also almost the same (0–131 and 0–148, respectively). It has to be considered that 70 % of determined *gyrB* fragment corresponded to the ATPase domain while the *rpoD* fragment did not contain the protein active-site (Lonetto *et al.*, 1992).

Table 2. Primers used for PCR amplification and sequencing of *gyrB* and *rpoD* genes

Primer	Position*	Sequence (5'→3')†	Reference
<i>gyrB</i>			
<i>gyrB</i> 3F	334–354	TCCGGCGGTCTGCACGGCGT	Yáñez <i>et al.</i> (2003)
<i>gyrB</i> 9Rs	980–960	CCTTGACCGAAATGACCGCC	Yáñez <i>et al.</i> (2003)
<i>gyrB</i> 7F	792–812	GGGGTCTACTGCTTCACCAA	Yáñez <i>et al.</i> (2003)
<i>gyrB</i> 9R	979–959	ACCTTGACGGAGATAACGGC	Yáñez <i>et al.</i> (2003)
<i>gyrB</i> 14R	1464–1444	TTGTCCGGGTTGTACTCGTC	Yáñez <i>et al.</i> (2003)
<i>rpoD</i>			
<i>rpoD</i> 70F	280–323	ACGACTGACCCGGTACGCATGTAYATGMNGARATGGGNACNGT	Yamamoto <i>et al.</i> (2000)
<i>rpoD</i> 70Fs	280–302	ACGACTGACCCGGTACGCATGTA	Yamamoto <i>et al.</i> (2000)
<i>rpoD</i> 70Fs1	740–757	GTCAATTCCGCCTGATGC	This study
<i>rpoD</i> 70R	1139–1096	ATAGAAATAACCAGACGTAAGTTNGCYTCNACCATYTCYTTYTT	Yamamoto <i>et al.</i> (2000)
<i>rpoD</i> 70Rs	1139–1117	ATAGAAATAACCAGACGTAAGTT	Yamamoto <i>et al.</i> (2000)
<i>rpoD</i> 70Rs1	800–782	ATCATCTCGCGCATGTTGT	This study

*Positions according to *E. coli* numbering.

†Symbols: N=any nucleotide; R=A or G; S=C or G; Y=C or T; M=A or C.

At the intra-species level, substitution rates are very similar: 0–2.3 % for *gyrB*, 0–2.6 % for *rpoD* and around 1.6 % in both gene sequences. Identical *rpoD* and *gyrB* sequences were found for *A. media* strains CECT 4234 and CECT 4232^T and also for *A. allosaccharophila* strains CECT 4200 and CECT 4199^T. However, while identical *rpoD* sequences were found in *A. encheleia* strains CECT 4341 and CECT 4342^T, and also for *A. eucrenophila* CECT 4827 and CECT 4224^T, the corresponding *gyrB* sequences determined in a previous work (Yáñez *et al.*, 2003) showed 2 and 8 nucleotide differences, respectively. Therefore, we repeated the sequencing of both genes for the above strains, starting from fresh cultures, and concluded that these pairs of strains contain identical *rpoD* and *gyrB* sequences. The finding of perfect matches in two different markers in four cases suggests that these pairs may be strain duplicates. Identical genetic profiles obtained for the respective pairs of strains by using ERIC and REP-PCR typing methods reinforced this possibility (data not shown).

At the inter-species level, however, the two phylogenetic markers behaved differently and depending on the species. Between *Aeromonas* species, the ranges of nucleotide substitution rates for *rpoD* were notably higher (very often almost double) than those of *gyrB* (see Table A in IJSEM Online). But, two pairs of species, *A. encheleia*/*Aeromonas* sp. HG11 (2.1–2.2 %, *gyrB*; 1.4–1.7 %, *rpoD*) and *A. veronii*/*A. culicicola* (3.5–3.7 %, *gyrB*; 1.6–1.7 %, *rpoD*), were exceptions. An example of this higher inter-species mutation rate exhibited by *rpoD* is *A. salmonicida*/*A. bestiarum* with a range of 1.8–4.3 % for *gyrB*, but 6.8–8.7 % for the *rpoD* sequence (see Table A in IJSEM Online).

In conclusion, in *Aeromonas* species, *rpoD* showed a better inter-species resolution to split *A. salmonicida* from *A. bestiarum*; however, *gyrB* was better for differentiating *Aeromonas* sp. HG11/*A. encheleia* and *A. veronii*/*A. culicicola*/*A. allosaccharophila*.

Phylogenetic relationships of *Aeromonas* spp.

Unrooted phylogenetic trees for *gyrB* (Fig. 1a), *rpoD* (Fig. 1b) and the combined *gyrB*–*rpoD* gene sequences (1770 bp; Fig. 1c) were constructed from the derived genetic matrices. All obtained phylogenies showed considerable divergence (branch lengths) between all *Aeromonas* species under study. Strain grouping was consistent in all three trees in agreement with a recently published phylogenetic study based on *gyrB* (Yáñez *et al.*, 2003). Some differences were observed in the topology at the deepest branching points of single *gyrB* and *rpoD* trees (Fig. 1a, b). This reflected the differences in the inter-species nucleotide substitution rates between *gyrB* and *rpoD* sequences and, as a consequence, lower bootstrap values appeared at the deepest branching points of the combined *gyrB*–*rpoD* tree (Fig. 1c). Because *rpoD* yielded a better resolution than *gyrB* 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 *gyrB*–*rpoD* tree, as it comprised the combined capacities of both molecular clocks. The study of several housekeeping gene sequences has already been recommended for improving the reliability of phylogenetic inference (Stackebrandt *et al.*, 2002).

Addition of *rpoD* analysis to the systematics of the genus *Aeromonas* has increased the advantages found previously on the basis of *gyrB* sequences (Yáñez *et al.*, 2003), compared to the reference 16S rRNA gene phylogeny (Martínez-Murcia *et al.*, 1992; Martínez-Murcia, 1999). Examples of that were 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 rRNA gene sequences, but were clearly separated by *gyrB* sequences (7.3, 6.5 and 6.3 %, respectively) and even more by *rpoD* (9.8, 9.7 and 7.2 %). Agreements between *gyrB* and 16S rRNA (Yáñez *et al.*, 2003) were here confirmed, but also improved by the results obtained in the *gyrB*–*rpoD* 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) represented two heterogeneous phenotypes of a single species. The phylogenetic distinctiveness of *A. allosaccharophila* and *A. veronii* was greater for *gyrB* sequences (3.3–4.0 %); however, although very closely related by *rpoD*, the range of nucleotide substitution rates (2.6–3.2 %) was greater than the observed overall value (under 2 %) for considering them to be the same species. However, *A. culicicola* and *A. veronii* showed a range of *rpoD* mutation rates of 1.6–1.7 %, which is borderline to the more likely intra-species value observed in the present analysis (approx. 1.6 %). 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; Yáñez *et al.*, 2003). Divergence determined by *gyrB* was over 2 %, but *rpoD* differences between these groups of strains were 1.4–1.7 %. This result could be considered critical as the overall analysis of the genus *Aeromonas* indicated that these values fall within the limits for considering these strains to be a single species. Nevertheless, only two strains for each group (HG11 and *A. encheleia*) have been subjected to *rpoD* sequencing. We recommend the analysis of several housekeeping genes to investigate if any considerable phylogenetic divergence can be detected between these micro-organisms.

In the study of Yáñez *et al.* (2003), a group of isolates (531c, 610 and 520) showed a considerable *gyrB* relationship to the cluster corresponding to *A. media*, but exhibited substitution rates of 2.7–3.1 %. As reported, these strains showed 2–9 nucleotide differences in their 16S rRNA gene sequences compared to that of the *A. media* type strain; but all these mutations were located at a hypervariable stem-loop (positions 457–476), a region considered to be unreliable for making phylogenetic conclusions. This nucleotide diversity may be detected by 16S rRNA gene-RFLP as

they yielded profiles differing from these corresponding to type strains (Figueras *et al.*, 2000). In the present study, more of those isolates previously selected by 16S rRNA gene-RFLP analysis were included (280, 480, 57, 239 and 741), and their 16S rRNA, *gyrB* and *rpoD* gene sequences were determined. In the *gyrB* tree (Fig. 1a), the isolates 741 and 239 clustered with the *A. media* type strain, but the rest (isolates 57, 480, 610 and 280), although closely related to *A. media*, formed an 'independent cluster', indicating that this may represent a distinct phylogenetic line of *Aeromonas*. Differences in mutations rates were discerned for some of

these isolates when comparing *gyrB* with *rpoD* sequences, which is why they branched slightly differently in the corresponding trees. An example was isolate 239 which showed a *gyrB* divergence range of 2.9–3.9 % to strains of the above indicated 'independent cluster', but only 1.2–1.6 % for *rpoD*. This was again a case where different mutational behaviour in two housekeeping genes was found for closely related strains. The possibility of horizontal gene transfer, although not very likely, must be taken into account. Any hypothesis based on these results needs to be supported by a more comprehensive set of gene data.

Fig. 1. Unrooted phylogenetic trees based on *gyrB* (a), *rpoD* (b) and the combined stretch of *gyrB-rpoD* (c) gene sequences, showing relationships in the genus *Aeromonas*. Type strains are in bold. Numbers shown at nodes indicate bootstrap values (percentage of 1000 replicates). Sequence accession numbers are in parentheses.

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

Our previous *gyrB* study (Yáñez *et al.*, 2003) showed the inter-relationship between *A. salmonicida* (HG3) and *A. bestiarum* (HG2) as a difficult case to be solved, because a

relatively low range of substitutions (2.2–3.3 %) was detected. Nevertheless, a limited number of strains was included in the mentioned work. This was consistent with 16S rRNA gene studies as, although type strains of these species differ in two single nucleotides (Martínez-Murcia, 1999), many strains simultaneously contained copies of both sequences of 16S molecules (present study and unpublished data). Therefore, the presence of one or another sequence of 16S rRNA, or both at the same time, seems absolutely random and, as a consequence, splitting *A. salmonicida* from *A. bestiarum* was not possible by using this genetic marker (Table A in IJSEM Online). All these difficulties were also found in the separation of HG2 from HG3 strains by DNA–DNA hybridization (Ali *et al.*, 1996; Hänninen, 1994). A recent molecular and phenotypic investigation into the taxonomy of the ‘*A. hydrophila*’ complex indicated a lack of criteria for splitting *A. salmonicida* from *A. bestiarum* (unpublished data).

In the present study, the number of strains of these species was greatly increased in an attempt to clarify their phylogenetic relationships. The *gyrB* inter-species ranges found were 2.2–4.3 % (between *A. salmonicida* and *A. bestiarum*), 2.7–4.3 % (*A. bestiarum* and *A. popoffii*) and 3.1–5.2 % (*A. salmonicida* and *A. popoffii*), very low values but still above the overall intra-species ranges observed for *Aeromonas*. However, the *rpoD* analysis yielded ranges of 6.8–8.7, 4.0–5.6 and 8.1–9.3 %, respectively, indicating that the resolving power to split *A. salmonicida* from *A. bestiarum* was twice that obtained by *gyrB* sequences. Consequently, we report in this study a phylogenetic marker that was able to separate these two species, not clearly distinguishable by previously used sequences. This is also the advantage of using several housekeeping genes for phylogeny-based systematics (Stackebrandt *et al.*, 2002). Apart from the genetic transfer hypothesis, the study of different genes (obviously not totally synchronized from an evolutionary point of view) may help us to differentiate species more effectively. Whether or not *rpoD* has been transferred during the evolution of these species should be investigated by sequencing other housekeeping genes.

At the time of submission of this report, a phylogenetic analysis based on *gyrB* sequences of *Aeromonas* species appeared (Pidíyar *et al.*, 2003). This study included only type strains and the obtained phylogenetic tree showed notable differences from that published by Yáñez *et al.* (2003) and from the trees from the present study. We compared the sequence with GenBank/EMBL/DDBJ accession no. AF417635 (Pidíyar *et al.*, 2003) to that with accession no. AY101799 (Yáñez *et al.*, 2003), both obtained from the type strain of *A. encheleia*, and 58 mismatches were found. Moreover, Pidíyar *et al.* (2003) consider *A. veronii* biogroup *sobria* and *A. veronii* biogroup *veronii* to be different species, which contradicts previous phylogenetic results based on 16S rRNA (Martínez-Murcia *et al.*, 1992), *gyrB* (Yáñez *et al.*, 2003) and the present work. Our phylogenetic analysis included sequences, checked several

times, from several strains for each species and the same conclusions were obtained by using two genetic markers, *gyrB* and *rpoD*.

In conclusion, *gyrB* and *rpoD* gene sequences, independently, have proved to be excellent molecular makers for assessing phylogeny in the genus *Aeromonas*. Moreover, the simultaneous analysis of both clocks improved the reliability and the 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* has been shown in this work. The sequences of more genes are obviously needed and may contribute to understanding the evolution of some *Aeromonas* groups.

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