

Antonio J. Martínez-Murcia<sup>1\*</sup>  
Lara Soler<sup>2</sup>  
María José Saavedra<sup>1,3</sup>  
Matilde R. Chacón  
Josep Guarro<sup>2</sup>  
Erko Stackebrandt<sup>4</sup>  
María José Figueras<sup>2</sup>

## Phenotypic, genotypic, and phylogenetic discrepancies to differentiate *Aeromonas salmonicida* from *Aeromonas bestiarum*

<sup>1</sup>Molecular Diagnostics Center,  
and Univ. Miguel Hernández,  
Orihuela, Alicante, Spain

<sup>2</sup>Microbiology Unit,  
Dept. of Basic Medical Sciences,  
Univ. Rovira i Virgili, Reus, Spain

<sup>3</sup>Dept. of Veterinary Sciences,  
CECAV-Univ. of Trás-os-Montes  
e Alto Douro, Vila Real, Portugal

<sup>4</sup>DSMZ-Deutsche Sammlung von  
Mikroorganismen und Zellkulturen  
GmbH, Braunschweig, Germany

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\*Corresponding author:

A.J. Martínez-Murcia  
Molecular Diagnostics Center  
Aptdo. 169. 03300 Orihuela, Alicante, Spain  
Tel. +34-966754951. Fax +34-966735527  
E.mail: ammurcia@mdc-bt.com

**Summary.** The taxonomy of the “*Aeromonas hydrophila*” complex (comprising the species *A. hydrophila*, *A. bestiarum*, *A. salmonicida*, and *A. popoffii*) has been controversial, particularly the relationship between the two relevant fish pathogens *A. salmonicida* and *A. bestiarum*. In fact, none of the biochemical tests evaluated in the present study were able to separate these two species. One hundred and sixteen strains belonging to the four species of this complex were identified by 16S rDNA restriction fragment length polymorphism (RFLP). Sequencing of the 16S rDNA and cluster analysis of the 16S–23S intergenic spacer region (ISR)-RFLP in selected strains of *A. salmonicida* and *A. bestiarum* indicated that the two species may share extremely conserved ribosomal operons and demonstrated that, due to an extremely high degree of sequence conservation, 16S rDNA cannot be used to differentiate these two closely related species. Moreover, DNA–DNA hybridization similarity between the type strains of *A. salmonicida* subsp. *salmonicida* and *A. bestiarum* was 75.6 %, suggesting that they may represent a single taxon. However, a clear phylogenetic divergence between *A. salmonicida* and *A. bestiarum* was ascertained from an analysis based on *gyrB* and *rpoD* gene sequences, which provided evidence of a lack of congruence of the results obtained from 16S rDNA, 16S–23S ISR-RFLP, DNA–DNA pairing, and biochemical profiles. [Int Microbiol 2005; 8(4):259-269]

**Key words:** *Aeromonas* spp. · nucleic acids techniques · gene *gyrB* · gene *rpoD*

### Introduction

*Aeromonads* are autochthonous to aquatic environments worldwide and have been strongly implicated in the etiology of a variety of fish and human diseases. Particularly relevant to aquaculture is the species *A. salmonicida*, the causative agent of fish furunculosis. The taxonomy of the genus *Aeromonas* is complex and some of its species cannot be easily differentiated [17]. DNA–DNA reassociation studies carried out by Popoff in the early 1980s [44,45] identified three different hybridization groups (HGs) within the phenotypic “*A.*

*hydrophila*” complex: HG1, represented by *A. hydrophila*; HG2, initially represented by a group of unclassified strains; and HG3, *A. salmonicida*. The latter species now includes non-motile strains isolated from fish divided into four psychrophilic subspecies: *A. salmonicida* subsp. *salmonicida* [18], *A. salmonicida* subsp. *achromogenes* [47], *A. salmonicida* subsp. *masoucida* [32,33], *A. salmonicida* subsp. *smithia* [7], and the mesophilic subspecies *A. salmonicida* subsp. *pectinolytica* isolated from water [43]. In addition, *A. salmonicida* includes motile mesophilic strains isolated from diverse sources, confusingly referred to as “*A. hydrophila*” (HG3) and classically represented by the reference strain Popoff 316 [5].

The complexity of *A. salmonicida* taxonomy is reflected by the number of articles describing strains that cannot be included in any of the described subspecies due to the presence of atypical biochemical and genetic characteristics [6,7,22,35,46,50,52]. The unnamed HG2 was first called *A. salmonicida* subsp. *bestiarum* [10,30], but later, although it showed high (71%) DNA–DNA reassociation with “*A. hydrophila*” (HG3) and even higher (78%) with *A. hydrophila* (HG1), it was designated as a different species, *A. bestiarum* [3]. In addition, the type strain of *A. salmonicida* was not hybridized with *A. bestiarum* in the definition of the latter species. By contrast, the 16S rRNA gene sequence of the type strain of *A. bestiarum* is identical to that of *A. salmonicida* subsp. *achromogenes* and *A. salmonicida* subsp. *masoucida* and only shows two nucleotide differences from that of *A. salmonicida* subsp. *salmonicida* [36,38]. By using the RFLPs of the 16S rRNA gene, we obtained species-specific patterns for all of the type strains of the genus [9,17]. Recently, however, we have isolated numerous strains (referred to here as *A. bestiarum/A. salmonicida*) displaying a pattern that is a mixture of those shown by *A. bestiarum* and *A. salmonicida* strains. The present report attempts to determine whether there is genetic or phenotypic distinction between strains of these two species as well as among strains of other species that integrate the “*A. hydrophila*” complex, including the recently described species *A. popoffii*.

## Materials and methods

**Bacterial strains and culture conditions.** A total of 116 strains identified by the 16S rDNA-RFLP technique [9,17] were included in the study. The isolates were grown on TSA (Trypticase Soy Agar, Difco; Barcelona, Spain) at 30°C, with the exception of those belonging to *A. salmonicida* subsp. *salmonicida*, *A. salmonicida* subsp. *masoucida*, *A. salmonicida* subsp. *achromogenes*, *A. salmonicida* subsp. *smithia* and “atypical” *A. salmonicida* isolates, all of which were grown at room temperature (20–22). Ninety-three of isolates (18 *A. hydrophila*, 7 *A. bestiarum*, 26 *A. bestiarum/A. salmonicida*, 18 *A. salmonicida* and 24 *A. popoffii*) were predominantly environmental isolates from our collection, 22 were type and reference strains, and five were “atypical” *A. salmonicida* strains supplied by B. Austin (Heriot-Watt University, Edinburgh, Scotland) (Table 1).

**Biochemical studies.** The following biochemical tests were done in all strains according to conventional protocols: esculin hydrolysis, lysine decarboxylase, citrate utilization, motility, indole production, *N*-acetyl-D-glucosamine utilization, gluconate oxidation and acid production from rhamnose, sorbitol, lactose, D-sucrose, and salicin. DL-lactate utilization was tested according to the method of Janda et al. [29], urocanic acid according to the method of Hänninen [19] and elastase according to the method of Hasan et al. [20]. Test samples were incubated under the same conditions as used for bacterial growth. Readings were made every day for 3 days, and up to 5 days for the elastase test. All tests were carried out in duplicate, and appropriate positive and negative controls were included. Growth temperature (4°C, 20–22°C, 30°C, 37°C, and 44.5°C) and brown diffusible pigment production were evaluated on TSA [6]. Readings were taken every 24 h for up to 5 days for growth and up to 7 days for pigment production.

**16S rDNA-RFLP identification.** Briefly, the PCR-amplified 16S rRNA gene (1502 bp) was digested using two endonucleases (*AluI* and *MboI*) simultaneously, thus enabling the identification of all the species of the genus, with the exception of *A. bestiarum*, *A. salmonicida*, *A. popoffii*, and *A. encheleia*. To differentiate the latter two, further digestion with *NarI*, followed by digestion with *HaeIII* and *AlwNI*, was necessary, while *A. salmonicida* and *A. bestiarum* were distinguished by digestion with *NarI* followed by *PstI* [17].

**16S rRNA gene sequencing.** A fragment of ca. 560 bp of the 16S rRNA gene of the *A. bestiarum* and *A. salmonicida* type strains and of a subgroup of 14 isolates characterized as *A. bestiarum/A. salmonicida* by 16S rDNA-RFLP was sequenced. This fragment contains the characteristic variable positions (1011 and 1018 bp) for *A. bestiarum* and *A. salmonicida* [38]. Genomic DNA extraction and PCR amplification were performed as previously described [9,38]. PCR products were purified using Concert Rapid PCR Purification System (Life Technologies; Barcelona, Spain) following the manufacturer’s instructions. Sequencing primers were those described in Martínez-Murcia et al. [37] and sequences were determined using the ThermoSequenase fluorescently labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech; Barcelona, Spain) and the ALFexpressII sequencer (Amersham) according to the manufacturer’s indications.

**DNA–DNA hybridization.** DNA was isolated by chromatography on hydroxyapatite [11] and further submitted to DNA–DNA hybridization at 66°C according to the method of De Ley et al. [13], with some modifications [14,23]. Samples were read using a 2600 spectrophotometer equipped with a 2527-R thermoprogrammer and plotter (Gilford, Oberlin, Ohio, USA). Renaturation rates were computed with the TRANSFER.BAS program [27].

**16S–23S ISR-RFLP.** Seventy-six of the strains, including a representative number of each species, were analyzed by the 16S–23S intergenic spacer region–restriction fragment length polymorphism (ISR-RFLP) technique. Genomic DNA extraction and PCR amplification were performed as previously described [39,48]. Two double digestions of the 16S–23S ISR were carried out using the *HinfI*–*CfoI* and *HinfI*–*TaqI* (Gibco BRL, Barcelona, Spain). Digestion products were electrophoresed on a 4% metaphore agarose gel (FMCS Bioproducts Europe, Denmark). Gel images were saved as TIFF files and further analyzed by BioNumerics software, version 1.5 (Applied Maths, Kortrijk, Belgium). Levels of similarity between the profiles were calculated using the band-matching Dice coefficient ( $S_D$ ), and the cluster analysis of similarity matrices was calculated with the unweighted pairgroup method with arithmetic means (UPGMA).

**PCR amplification and sequencing of *gyrB* and *rpoD*.** Fragments of ca. 1100 bp and 820 bp of the *gyrB* and *rpoD* genes, respectively, were amplified by PCR and sequenced as previously described [49,54].

**Phylogenetic data analysis.** The nucleotide sequences of *gyrB* and *rpoD* were aligned, treated as a single nucleotide sequence [53], and the evolutionary tree was constructed as previously described [49].

## Results

**Biochemical studies.** Table 2 shows the biochemical responses obtained with the type and reference strains of *Aeromonas* included in the study. All strains of *A. salmonicida* subsp. *pectinolytica* and “*A. hydrophila*” LMG 13451 (Popoff 316) were able to grow from 4 to 37°C. The remaining *A. salmonicida* subspecies strains grew from room temperature up

**Table 1.** Type and reference strains of *Aeromonas* spp. included in the present study

Strain designation as received:	Correspondence to other reference numbers	16S rDNA-RFLP identification
<i>A. hydrophila</i> CECT 839 <sup>T</sup>	ATCC 7966 <sup>T</sup> ; CDC 9079-79; LMG 2844; NCIMB 9240; Popoff 543, MDC35	<i>A. hydrophila</i>
<i>A. bestiarum</i> CECT 4227 <sup>T</sup>	ATCC 51108 <sup>T</sup> ; CDC 9533-76; LMG 3751; Popoff 218, MDC11	<i>A. bestiarum</i>
<i>A. bestiarum</i> LMG 13448	A169, MDC4	<i>A. salmonicida</i>
<i>A. bestiarum</i> LMG 13662	A295, MDC34	<i>A. bestiarum/A. salmonicida</i>
<i>A. hydrophila</i> LMG 13451 <sup>R</sup>	Popoff 316; CECT 5173; A308; CDC 0434-84, MDC148	<i>A. salmonicida</i>
<i>A. salmonicida</i> subsp. <i>salmonicida</i> CECT 894 <sup>T</sup>	ATCC 33658; LMG 3780; NCIMB 1102; Smith 20, MDC19	<i>A. salmonicida</i>
<i>A. salmonicida</i> subsp. <i>salmonicida</i> CECT 4235	ATCC 14174; CDC RH39; CDC 960-60; CDC 9087-79; NCIMB 833; Veron 29	<i>A. salmonicida</i>
<i>A. salmonicida</i> subsp. <i>salmonicida</i> CECT 4236	NCIMB 1104	<i>A. salmonicida</i>
<i>A. salmonicida</i> subsp. <i>salmonicida</i> CECT 4237	NCIMB 1103; Smith 2, MDC5	<i>A. salmonicida</i>
<i>A. salmonicida</i> subsp. <i>achromogenes</i> CECT 895 <sup>R</sup>	ATCC 33659; LMG 3781; NCIMB 1110; Smith 6263/4/5, MDC25	<i>A. bestiarum</i>
<i>A. salmonicida</i> subsp. <i>achromogenes</i> CECT 4238	ATCC 19261; ATCC 23310; NCIMB 1109	<i>A. bestiarum</i>
<i>A. salmonicida</i> subsp. <i>achromogenes</i> CECT 4239	NCIMB 2112	<i>A. bestiarum</i>
<i>A. salmonicida</i> subsp. <i>masoucida</i> CECT 896 <sup>R</sup>	ATCC 27013; CCM 4124; Kimura I-A-1; LMG 3782; NCIMB 2020, MDC44	<i>A. bestiarum</i>
<i>A. salmonicida</i> subsp. <i>smithia</i> NCIMB 13210 <sup>R</sup>	ATCC 49393; CECT 5179	<i>A. bestiarum</i>
Atypical <i>A. salmonicida</i> AS4 <sup>a</sup>	CECT 5200	<i>A. bestiarum</i>
Atypical <i>A. salmonicida</i> AS203 <sup>a</sup>	CECT 5203	<i>A. bestiarum</i>
Atypical <i>A. salmonicida</i> AS222 <sup>a</sup>	CECT 5204	<i>A. bestiarum/A. salmonicida</i>
Atypical <i>A. salmonicida</i> AS167 <sup>a</sup>	CECT 5202	<i>A. bestiarum</i>
Atypical <i>A. salmonicida</i> AS24 <sup>a</sup>	CECT 5201	<i>A. bestiarum</i>
<i>A. salmonicida</i> subsp. <i>pectinolytica</i> 34MEL <sup>Rb</sup>	DSMZ 12609, MDC26	<i>A. bestiarum/A. salmonicida</i>
<i>A. salmonicida</i> subsp. <i>pectinolytica</i> 85MEL <sup>b</sup>		<i>A. bestiarum/A. salmonicida</i>
<i>A. salmonicida</i> subsp. <i>pectinolytica</i> 93MEL <sup>b</sup>		<i>A. bestiarum/A. salmonicida</i>
<i>A. popoffii</i> LMG 17541 <sup>T</sup>	CECT 5176 <sup>T</sup> , MDC149	<i>A. popoffii</i>
<i>A. popoffii</i> LMG 17543	MDC9	
<i>A. popoffii</i> LMG 17546	MDC15	
<i>A. popoffii</i> LMG 17545	MDC23	
<i>A. popoffii</i> LMG 17544	MDC30	

<sup>T</sup>Type strain. <sup>R</sup>Reference strain.

CECT, Spanish Type Culture Collection, University of Valencia, Valencia, Spain; NCIMB, Nationals Collections of Industrial and Marine Bacteria, Torry Research Station, Aberdeen, Scotland, UK; LMG, BCCM/LMG Culture Collection, Laboratorium voor Microbiologie, Universiteit Gent, Gent, Belgium. ATCC, American Type Culture Collection, Manassas, USA. CDC, Centers for Disease Control and Prevention, Atlanta, Georgia, USA. DSMZ, Deutsche Sammlung von Mikro-organismen und Zellkulturen GmbH, Braunschweig, Germany. MDC, Molecluar Diagnostics Center, Orihuela, Alicante, Spain.

<sup>a</sup>Strains provided by B. Austin. <sup>b</sup>Strains provided by J.M. Janda.

to 37°C, and “atypical” *A. salmonicida* isolates up to 30°C, with the exception of strain AS167, which could grow also at 37°C. Brown diffusible pigment production was observed in two strains of *A. salmonicida* subsp. *pectinolytica*, all strains of *A. salmonicida* subsp. *salmonicida*, and in three “atypical” isolates, but this response was temperature-dependent. All strains from our collection grew from 20–22°C up to 37°C, while only some grew also at 4°C or at 44.5°C (data not shown). Brown diffusible pigment production was observed in one *A. bes-*

*tiarum* isolate, one *A. bestiarum/A. salmonicida* isolate, and one of the *A. salmonicida* isolates (data not shown).

Type strains of *A. hydrophila* and *A. bestiarum* and the reference strain “*A. hydrophila*” LMG 13451 (Popoff 316) were motile and indole-positive, while the type and reference strains of the subspecies of *A. salmonicida* and the “atypical” isolates were nonmotile and indole-negative (with the exception of two *A. salmonicida* subsp. *Achromogenes*, which were indole-positive). The 51 strains of *A. salmonicida*, *A. bes-*

**Table 2.** Biochemical response of the type and reference strains of *Aeromonas* spp. used in the present study

Type and reference strains	Urocanic acid	Gluconate oxidation	DL-lactate	Sucrose	Rhamnose	Sorbitol	Lactose	Esculin hydrolysis	Motility	Salicin	N-acetyl-glucosamine	Citrate	Lysine decarboxylase	Elastase	Indole	Growth at 4°C	Growth at 20–22°C	Growth at 30°C	Growth at 37°C	Growth at 44.5°C	Pigmentation at 4°C	Pigmentation at 20–22°C	Pigmentation at 30°C	Pigmentation at 37°C	Pigmentation at 44.5°C
<i>A. hydrophila</i> CECT 839 <sup>T</sup>	-	+	+	+	-	-	-	+	+	+	+	-	-	+	+	-	+	+	+	+	-	-	-	-	-
<i>A. bestiarum</i> CECT 4227 <sup>T</sup>	+	-	-	+	+	-	-	+	+	+	+	+	+	+	+	-	+	+	+	-	-	-	-	-	-
<i>A. bestiarum</i> LMG 13448	+	-	-	+	+	-	-	+	+	-	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-
<i>A. bestiarum</i> LMG 13662	+	+	-	+	-	-	-	+	+	-	+	-	-	+	+	-	+	+	+	-	-	-	-	-	-
<i>A. hydrophila</i> LMG 13451 <sup>R</sup>	+	+	-	+	-	+	+	+	+	-	+	+	-	+	+	+	+	+	+	-	-	-	-	-	-
<i>A. salmonicida</i> subsp. <i>salmonicida</i> CECT 894 <sup>T</sup>	-	-	-	-	-	-	-	+	-	-	-	-	+	+	-	+	+	+	+	-	-	+	-	-	-
<i>A. salmonicida</i> subsp. <i>salmonicida</i> CECT 4235	-	-	-	-	-	-	-	+	-	-	+	+	-	-	-	-	+	+	+	-	-	+	-	-	-
<i>A. salmonicida</i> subsp. <i>salmonicida</i> CECT 4236	-	-	-	-	-	-	-	+	-	-	+	+	-	-	-	-	+	+	+	-	-	+	-	-	-
<i>A. salmonicida</i> subsp. <i>salmonicida</i> CECT 4237	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	+	+	+	-	-	+	+	-	-
<i>A. salmonicida</i> subsp. <i>achromogenes</i> CECT 895 <sup>R</sup>	-	-	-	+	+	-	-	+	-	-	-	-	+	-	+	-	+	+	+	-	-	-	-	-	-
<i>A. salmonicida</i> subsp. <i>achromogenes</i> CECT 4238	-	-	-	+	-	-	-	-	-	-	-	-	+	-	+	-	+	+	+	-	-	-	-	-	-
<i>A. salmonicida</i> subsp. <i>achromogenes</i> CECT 4239	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-
<i>A. salmonicida</i> subsp. <i>masoucida</i> CECT 896 <sup>R</sup>	-	-	-	+	+	-	-	-	-	-	-	+	+	-	-	-	+	+	+	-	-	-	-	-	-
<i>A. salmonicida</i> subsp. <i>smithia</i> NCIMB 13210 <sup>R</sup>	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-
Atypical <i>A. salmonicida</i> AS4 <sup>a</sup>	-	-	-	+	-	-	-	+	-	+	+	-	-	-	-	-	+	+	-	-	-	+	-	-	-
Atypical <i>A. salmonicida</i> AS203 <sup>a</sup>	-	-	-	+	-	-	-	-	-	-	-	+	-	+	-	-	+	+	-	-	-	+	+	-	-
Atypical <i>A. salmonicida</i> AS222 <sup>a</sup>	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-
Atypical <i>A. salmonicida</i> AS167 <sup>a</sup>	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-
Atypical <i>A. salmonicida</i> AS24 <sup>a</sup>	-	-	-	+	-	-	-	+	-	+	-	-	-	-	-	-	+	+	-	-	-	+	-	-	-
<i>A. salmonicida</i> subsp. <i>pectinolytica</i> 34 MEL <sup>Rb</sup>	+	-	-	+	-	+	+	+	-	-	+	+	+	+	-	+	+	+	+	-	-	+	+	+	-
<i>A. salmonicida</i> subsp. <i>pectinolytica</i> 85 MEL <sup>b</sup>	+	-	-	+	-	+	+	+	-	-	+	+	+	+	-	+	+	+	+	-	-	-	-	-	-
<i>A. salmonicida</i> subsp. <i>pectinolytica</i> 93 MEL <sup>b</sup>	+	-	-	+	-	+	+	+	-	-	+	+	+	+	-	+	+	+	+	-	-	+	+	+	-
<i>A. popoffii</i> LMG 17541 <sup>T</sup>	+	+	+	-	-	-	-	-	+	-	+	-	-	-	nd <sup>c</sup>	nd <sup>c</sup>	nd	nd	nd	nd	nd	nd	nd	nd	nd

<sup>T</sup>Type strain. <sup>R</sup>Reference strain. <sup>a</sup>Strains provided by B. Austin. <sup>b</sup>Strains provided by J.M. Janda. <sup>c</sup>nd, not determined.

*tiarum/A. salmonicida* and *A. bestiarum* showed variable motility (67, 69, and 86%, respectively) (Table 3) and indole production (86, 75, and 25 %, respectively) (data not shown).

The analysis of DL-lactate utilization allowed separation of 78% of the strains of *A. hydrophila* from *A. bestiarum* and *A. salmonicida*. None of the 14 biochemical tests could unequivocally distinguish *A. bestiarum* from *A. salmonicida*, nor from

the subspecies of *A. salmonicida*. However, positive acid production from sorbitol and lactose was more prevalent for strains of *A. salmonicida* than for those of *A. bestiarum*. The analysis of DL-lactate utilization, acid production from sucrose, esculin hydrolysis, and elastase allowed discrimination between *A. popoffii* and the rest of the species of the “*A. hydrophila*” complex.

**Table 3.** Biochemical response of the *Aeromonas* spp. strains used in the present study

	No. tested strains	Urocanic acid	Gluconate oxidation	DL-lactate	Sucrose	Rhamnose	Sorbitol	Lactose	Esculin hydrolysis	Motility	Salicin	N-acetyl-glucosamine	Citrate	Lysine Decarboxylase	Elastase
<i>A. hydrophila</i>	18	11 <sup>a</sup>	13	14	17	3	2	4	17	18	10	16	15	14	14
<i>A. bestiarum</i>	7	7	5	1	7	4	2	1	7	6	3	6	6	5	6
<i>A. bestiarum/A. salmonicida</i>	26	25	16	1	26	5	18	7	26	18	15	22	25	17	22
<i>A. salmonicida</i>	18	16	12	0	18	2	15	13	16	12	14	18	17	12	16
<i>A. popoffii</i>	24	19	18	22	1	1	0	1	1	24	1	11	24	1	1

<sup>a</sup>No. strains with positive response



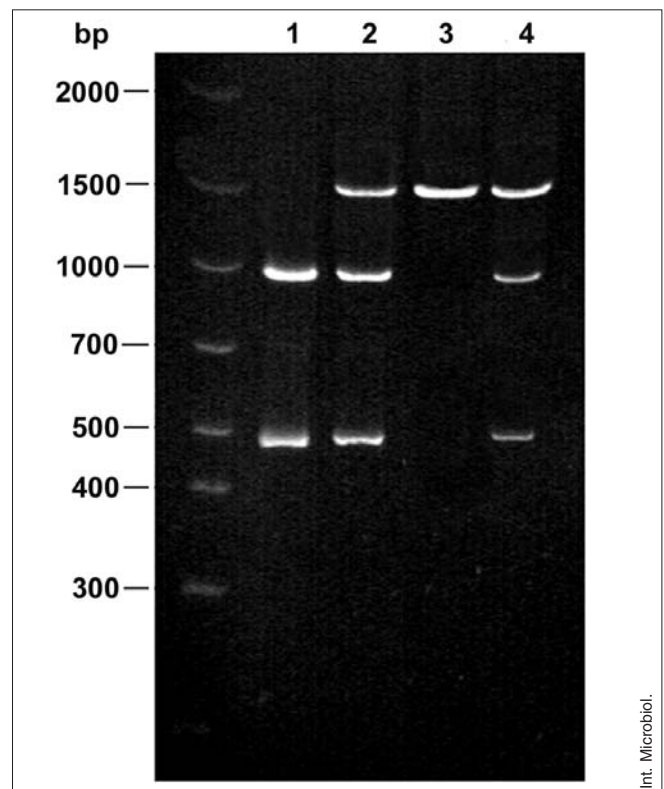
**16S rDNA-RFLP and 16S rDNA sequencing.**

The typical 16S rDNA-RFLP profiles of the type strains of *A. bestiarum* (fragments of 1005 bp and 497 bp), *A. salmonicida* (fragment of 1502 bp), and of strains referred to as *A. bestiarum/A. salmonicida*, with a mixture of these two patterns, are shown in Fig. 1. Variation in band intensity was observed in the latter as: (i) similar intensity of the three bands (i.e., strain J4N-98) and (ii) a relatively higher intensity of the 1502-bp band (i.e., strain LMG 13662).

Three subspecies of *A. salmonicida*, i.e., *achromogenes*, *masoucida*, and *smithia*, and four “atypical” *A. salmonicida* isolates yielded the 16S-RFLP pattern of *A. bestiarum*, while strain “*A. hydrophila*” LMG 13451 (Popoff 316) showed the pattern of *A. salmonicida*. Furthermore, of the 26 strains from our collection included in the study, one “atypical” *A. salmonicida* strain (AS222), three *A. salmonicida* subsp. *pectinolytica* strains (34 MEL, 85 MEL and 93 MEL), and the strain LMG 13662 used in the description of the species *A. bestiarum* [3] showed the mixed *A. bestiarum/A. salmonicida* 16S rDNA-RFLP pattern (see Table 1).

The sequences of the 16S rDNA gene of the type strains showed the expected nucleotides at the variable positions 1011 and 1018, which correspond to T and A for *A. salmonicida*, and C and G for *A. bestiarum*. However, the 14 *A. bestiarum/A. salmonicida* strains selected for sequencing gave ambiguities suggesting micro-heterogeneity. Visual inspection of the chromatograph showed a double sequencing signal at these positions, although secondary peaks were slightly smaller. Two types of double sequencing peaks were discerned and they were in accordance with the obtained RFLP intensity profiles. When the 16S rDNA-RFLP pattern showed equal intensity of the three bands, sequencing analysis showed C/T and G/A at positions 1011 and 1018, respectively, while T/C and A/G were obtained with higher intensity of the 1502 bp band (Fig. 1 and Table 4).

**DNA-DNA hybridization.** DNA-DNA hybridization results are shown in Table 5. The strain “*A. hydrophila*” HG3 (Popoff 316) hybridized 88.3% with the type strain of *A. salmonicida* (*A. salmonicida* subsp. *salmonicida*, CECT 894<sup>T</sup>) and with a similarity ranging from 79.6 to 83.8% with



**Fig. 1.** Acrylamide gel showing the RFLP patterns, obtained by using the endonuclease *Pst*I, of 16S rRNA gene amplified by PCR. Lane 1 pattern typical of *A. bestiarum* (strain CECT 4227<sup>T</sup>), lane 3 pattern typical of *A. salmonicida* (strain CECT 894<sup>T</sup>), lanes 2 and 4, mixture of the two previous patterns (strains J4N-98 and LMG13662, respectively).

the reference strains of the other subspecies. In addition, the type strain of *A. bestiarum* (CECT 4227<sup>T</sup>) showed 75.6% similarity with the type strain of *A. salmonicida* (*A. salmonicida* subsp. *salmonicida*, CECT 894<sup>T</sup>) and similarities ranging from 70.5 to 83.5% with reference strains of other subspecies.

**16S-23S ISR-RFLP analyses.** Using *Hinf*I-*Cfo*I, digestions of the 16S-23S ISR produced five to nine bands per strain, while with *Hinf*I-*Taq*I, four to 12 bands were obtained; all the bands were smaller than 500 bp. The UPGMA dendrogram derived from the analysis of the combined profiles of

**Table 4.** Nucleotides present at the variable positions of the 16 rDNA sequences in the *A. bestiarum*, *A. bestiarum/A. salmonicida* and *A. salmonicida* sequenced strains

Strains	16S rDNA-RFLP identification	Nucleotides present at the variable positions	
		1011	1018
<i>A. salmonicida</i> CECT 894 <sup>T</sup>	<i>A. salmonicida</i>	T	A
<i>A. bestiarum</i> CECT 4227 <sup>T</sup>	<i>A. bestiarum</i>	C	G
A7, A99, LMG 13662, 118p	<i>A. bestiarum/A. salmonicida</i>	T/C	A/G
22, 26, 37, 107f, 130f, 116p, 117p, 361c, 852T, J4N-98	<i>A. bestiarum/A. salmonicida</i>	C/T	G/A

**Table 5.** DNA–DNA relatedness between type and reference strains of *A. bestiarum* and *A. salmonicida*

Strain	Labelled DNA	
	<i>A. bestiarum</i> CECT 4227 <sup>T</sup>	“ <i>A. hydrophila</i> ” HG3 LMG 13451 (Popoff 316)
<i>A. salmonicida</i> subsp <i>salmonicida</i> CECT 894 <sup>T</sup>	75.6	88.3
<i>A. salmonicida</i> subsp <i>achromogenes</i> CECT 895 <sup>R</sup>	nd	81.1
<i>A. salmonicida</i> subsp <i>mausoucida</i> CECT 896 <sup>R</sup>	80.4	83.8
<i>A. salmonicida</i> subsp <i>smithia</i> NCIMB 13210 <sup>R</sup>	83.5	79.6
<i>A. salmonicida</i> subsp <i>pectinolitica</i> 34 MEL <sup>R</sup>	70.5	83.4

<sup>T</sup>Type strain; <sup>R</sup>Reference strain; nd, not determined. Results are expressed as mean of 2 determinations.

*HinfI*–*CfoI* and *HinfI*–*TaqI* is shown in Fig. 2. The two main clusters, one containing the *A. popoffii* strains and the other the rest of the strains, were separated by a coefficient value of <20%. The latter cluster split into two subclusters, one that

encompassed the strains of *A. hydrophila* (at a similarity level of 52%) and the other containing the strains of *A. bestiarum*, *A. salmonicida*, and *A. bestiarum/A. salmonicida* interspersed (at 62% profile similarity).



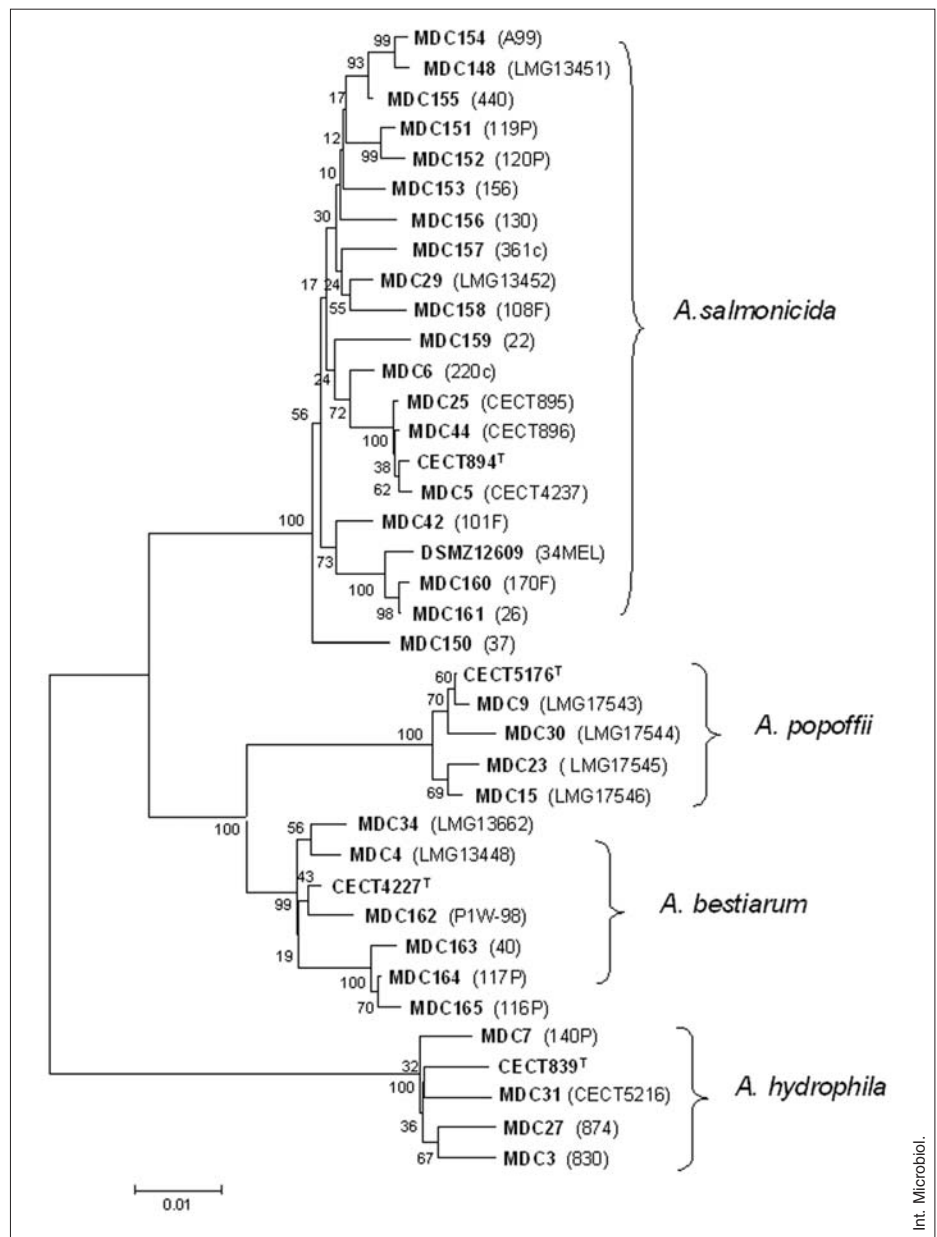
**Fig. 2.**  $S_0$ /UPGMA cluster analysis based on the combined *HinfI*–*CfoI* and *HinfI*–*TaqI* restriction patterns of amplified 16S–23S ISR.

**gyrB and rpoD phylogenetic analysis.** Nucleotide sequences of *gyrB* and *rpoD* amplicons were determined from representative strains of these four *Aeromonas* species. The corresponding sequences of reference strains were previously published [49,54]. Experiments were repeated at least twice from single colonies of original cultures to confirm readings and resolve ambiguities. The derived *gyrB* sequences comprised between 960 and 1100 nucleotides and the *rpoD* sequences between 813 and 825 nucleotides. The unrooted phylogenetic tree, constructed by using the combined *gyrB-rpoD* gene sequences (1770 bp; Fig. 3), showed a clear clustering of these species.

### Discussion

Although several approaches have been attempted aimed at clarifying the phylogenetic relationships among the species of the genus [24,36,38], the taxonomy of *Aeromonas* remains difficult. One of the most evident problems lies in the delimitation of the species of the “*A. hydrophila*” complex, since DNA–DNA reassociation results between *A. bestiarum* and *A. salmonicida* [3,4,19] do not provide conclusive information.

An interesting aspect observed in the present study was that *A. salmonicida* showed mesophilic behavior in being



**Fig. 3.** Phylogenetic tree based on the combined *gyrB-rpoD* gene sequences.

able to grow at 37°C. This species had been traditionally considered psychrophilic, with optimal growth at 22°C–25°C [6,7,32,33,44]. Its response to temperature is identical to that of *A. bestiarum* [3].

Classical tests used to separate the subspecies of *A. salmonicida* from each other are motility and indole production [43,44,52]. However, these properties did not prove useful for that purpose in our study. Motility only yielded the expected results for the type and reference strains, whereas it was very variable in other strains. Similarly, indole production was also a highly variable character and therefore inappropriate for subspecies differentiation. Another feature traditionally used to differentiate *A. salmonicida* subsp. *salmonicida* is the production of brown diffusible pigment, which is lacking in the other subspecies [7,32,33,40,52]. However, in agreement with Austin et al. [6], the results of this test were temperature-dependent and not specific for *A. salmonicida* subsp. *salmonicida*. This was also observed by Dalsgaard et al. [12], who found that *A. salmonicida* subsp. *achromogenes* was able to produce a brown diffusible pigment. Urocanic acid utilization was tested for the first time on reference strains of the different subspecies of *A. salmonicida* and all of them were negative.

Other biochemical tests, such as gluconate oxidation, acid production from lactose and sucrose, esculin hydrolysis, *N*-acetyl-glucosamine utilization, and elastase production, which have been proposed by different authors to separate subspecies of *A. salmonicida* [6,7,42,43,52], were unable to discriminate such subspecies—as was demonstrated previously by Noterdaeme et al. [41] using 128 biochemical and physiological characters.

Similar difficulties in the biochemical identification of the *A. salmonicida* subspecies have been found by other authors, who then had to consider numerous isolates of *A. salmonicida* as “atypical” [6,7,22,35,50,52]. Although the synonymization of the subspecies of *A. salmonicida* has been under discussion for several years [7, 8, McCarthy DH, 1978, PhD thesis], no consensus has ever been reached, and even a new subspecies, *A. salmonicida* subsp. *pectinolytica*, was proposed recently [43]. Our results demonstrate that strains of the latter subspecies also share ribosomal operons from both *A. bestiarum* and *A. salmonicida*.

An extensive revision of the biochemical tests proposed by different authors to differentiate the species of the “*A. hydrophila*” complex [see Table 6 in the online version of this article] revealed the existence of numerous contradictory data, such as acid production from sorbitol [2], and rhamnose [2,3,19], lactose [1–3,31], urocanic acid, and DL-lactate utilization [3], within each of the species *A. hydrophila*, *A. bestiarum*, and *A. salmonicida*. These tests have previously been

considered useful to discriminate these three species [1,3,19,29,31]. Our results mostly agree with those of Abbott et al. [1], even though those authors did not include the type species of *A. salmonicida*, and the strains that we investigated were from more diverse origins.

In our study, DL-lactate utilization and acid production from sorbitol and lactose were the most useful tests to distinguish *A. hydrophila* (see Table 3), but these tests cannot be considered unequivocal criteria, in agreement with other authors [31,34]. Acid production from D-sucrose, DL-lactate utilization, and lysine decarboxylase activity were used by Huys et al. [25] to separate *A. popoffii* from species of the “*A. hydrophila*” complex. In the present study, the tests useful for this purpose were esculin hydrolysis and acid production from D-sucrose. DL-lactate utilization was useful for the separation of *A. popoffii* from *A. bestiarum* and *A. salmonicida* but not from *A. hydrophila*. Recently, Abbott et al. [1] indicated that *A. popoffii* is always elastase negative, while in our study only one strain was clearly positive for this test.

Sequencing of the 16S rRNA gene is considered a robust taxonomic tool and is now widely used in bacterial taxonomy. In *Aeromonas*, this gene provides also signature regions for the delineation and identification of most species [38], but for some strains of some species the sequence was found to be extremely conserved; for example, *A. salmonicida* and *A. bestiarum* type strains differ by only two nucleotides, at positions 1011 and 1018. In the present study, RFLP and/or sequencing of the 16S rDNA of a considerable number of the isolates tested (ca. 60% of strains investigated), including some reference strains, suggested that these isolates may share ribosomal operons from both species, *A. bestiarum* and *A. salmonicida*. Differences in the intensity of the 16S rDNA–RFLP bands and of the sequencing signals were consistent and may be related to a difference in the number of *rrn* operon copies of one species or another among strains. Furthermore, 16S–23S ISR-RFLP analysis showed that, while *A. hydrophila* and *A. popoffii* formed two different clusters, strains of *A. bestiarum*, *A. bestiarum*/*A. salmonicida*, and *A. salmonicida* clustered together in a single group.

DNA–DNA hybridization remains the standard method for species designation [15,51]. In the present study, we determined the DNA–DNA hybridization values for strains that had not previously been evaluated. The type strain for *A. bestiarum* showed 75.6% hybridization with the type strain for *A. salmonicida* (*A. salmonicida* subsp. *salmonicida* CECT 894<sup>T</sup>), which is consistent with difficulties found in separating strains of these two species by DNA–DNA pairing [19]. Ali et al. [3] described *A. bestiarum* as a new species following only a DNA–DNA hybridization with the reference strain of “*A. hydrophila*” HG3, named Popoff 316.



Discordances between different sets of DNA–DNA hybridization data published by different authors using different techniques, but dealing with the same *Aeromonas* reference strains, have been reported several times for other species of this genus. For instance, strains of *A. allosaccharophila* exhibited 0–40% DNA relatedness with *A. veronii* [16], although later DNA–DNA hybridization studies by Huys et al. [26] indicated a much higher range (78–84%) using the same reference strains, but also inconsistency with previous data obtained by Hickman-Brenner et al. [21]. The most pronounced disagreement so far is that regarding *A. encheleia/Aeromonas* sp. HG11/A. *eucrenophila*, reported by Martínez-Murcia [33], by comparing data from two previous studies. As suggested, the measure of DNA sequence homology by DNA–DNA hybridization may be too crude, or at least not fine enough to split highly similar species.

However, phylogenetic analysis of *gyrB* and *rpoD* housekeeping genes (see Fig. 3) showed that, although genetically related, the strains of *A. bestiarum* and *A. salmonicida* form two clearly separated clusters with considerable phylogenetic divergence, in agreement with previous studies based on AFLP fingerprinting [24]. Substitution rates of *rpoD* between strains of *A. salmonicida* and *A. bestiarum* were double those obtained from the corresponding *gyrB* sequences, as previously reported [49]. New isolates of these two species were also included in the present phylogenetic analysis and the tree topology (clustering and branch distances) was maintained. This result confirmed that *gyrB* and *rpoD* are excellent molecular markers for assessing the phylogeny of closely related *Aeromonas* species, but also indicated a new lack of congruence with results derived from DNA–DNA hybridization data and 16S rDNA sequencing.

In conclusion, the high DNA homology of *A. bestiarum* and *A. salmonicida*, the impossibility of separating the two species by biochemical tests, neither by 16S rDNA nor by 16S–23S ISR-RFLP, could be taken as preliminary evidence that they are a single taxon. However, by analysis of faster molecular chronometers (e.g., *rpoD*, *gyrB*), the resulting phylogenies indicated that these two species show a modest amount of divergence, considering the overall phylogenetic depth of the genus *Aeromonas*. This study confirms the limitations of 16S rDNA sequencing to split *Aeromonas* species and suggests that DNA–DNA hybridization is not reliable when dealing with closely related species. Finally, the use of conserved housekeeping genes is recommended to identify new *Aeromonas* isolates.

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### Discrepancias fenotípicas, genotípicas y filogenéticas para diferenciar *Aeromonas salmonicida* de *Aeromonas bestiarum*

**Resumen.** La taxonomía del complejo "Aeromonas hydrophila" (que comprende las especies *A. hydrophila*, *A. bestiarum*, *A. salmonicida*, y *A. popoffii*) ha sido controvertida, en particular las relaciones entre los dos patógenos de peces, *A. salmonicida* y *A. bestiarum*. De hecho, de las pruebas bioquímicas evaluadas en el presente estudio, ninguna fue capaz de separar estas dos especies. Ciento dieciséis cepas pertenecientes a las cuatro especies de este complejo se identificaron mediante el análisis del polimorfismo de la longitud de los fragmentos de restricción (RFLP) del 16S rDNA. La secuenciación del 16S rDNA y el análisis de grupos de RFLP de la región espaciadora intergénica (ISR) 16S–23S en cepas seleccionadas de *A. salmonicida* y *A. bestiarum* indicaron que las dos especies podrían compartir operones ribosómicos extremadamente conservados y demostraron que, debido a su elevado grado de conservación de secuencia, el 16S rDNA no puede utilizarse para diferenciar estas dos especies de relación tan estrecha. Además, la similitud de hibridación DNA–DNA entre las cepas tipo de *A. salmonicida* subsp. *salmonicida* y de *A. bestiarum* era del 75,6 %, lo que sugiere que pueden ser un único taxón. Sin embargo, el análisis simultáneo de las secuencias de los genes *gyrB* y *rpoD* puso de manifiesto una marcada divergencia filogenética entre *A. salmonicida* y *A. bestiarum*, lo cual aporta pruebas de la falta de congruencia de los resultados de 16S rDNA, ISR-RFLP, 16S–23S, apareamiento DNA–DNA y perfiles bioquímicos. [Int Microbiol 2005; 8(4):259-269]

**Palabras clave:** *Aeromonas* spp. · técnicas de ácidos nucleicos · gen *gyrB* · gen *rpoD*

### Discrepancias fenotípicas, genotípicas e filogenéticas para diferenciar *Aeromonas salmonicida* de *Aeromonas bestiarum*

**Resumo.** A taxonomia do complexo "Aeromonas hydrophila" (que compreende as espécies *A. hydrophila*, *A. bestiarum*, *A. salmonicida*, e *A. popoffii*) tem sido controversia, em particular a relacionada com as duas espécies patogênicas em peixes, *A. salmonicida* e *A. bestiarum*. De facto, com as provas bioquímicas efectuadas no presente estudo, observou-se que nenhuma foi capaz para separar estas duas espécies. Cento e dezasseis estirpes pertencentes às quatro espécies deste complexo identificaram-se por a análise do polimorfismo no tamanho dos fragmentos de restrição (RFLP) do 16S rDNA. A sequência do 16S rDNA e a análise dos grupos de RFLP da região espaciadora intergénica (ISR) 16S–23S em estirpes seleccionadas de *A. salmonicida* e de *A. bestiarum* indicaram que as duas espécies poderão partilhar operões ribossómicos extremamente conservados e demonstram que, devido ao seu elevado grau de conservação da sequência, o 16S rDNA não se pode utilizar para diferenciar estas duas espécies de relação tão estreita. Por outro lado, a semelhança de hibridação DNA–DNA entre estirpes tipo de *A. salmonicida* subsp. *salmonicida* e de *A. bestiarum* foi de 75,6% sugerindo que podem representar um único taxon. No entanto, a análise simultânea das sequências dos genes *gyrB* e *rpoD* apresenta uma notável divergência filogenética entre *A. salmonicida* e *A. bestiarum*, o qual evidencia uma falta de congruência no que diz respeito aos resultados de 16S rDNA, 16S–23S ISR-RFLP, emparelhamento DNA–DNA e perfis bioquímicos. [Int Microbiol 2005; 8(4):259-269]

**Palavras chave:** *Aeromonas* spp. · técnicas de ácidos nucleicos · gene *gyrB* · gene *rpoD*