Aeromonas molluscorum sp. nov., isolated from bivalve molluscs

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Five *Aeromonas* strains (848T^T, 93M, 431E, 849T and 869N), which were isolated from bivalve molluscs and were recognized previously by numerical taxonomy as members of an unknown *Aeromonas* taxon, were subjected to a polyphasic taxonomic study. DNA–DNA hybridization experiments showed that DNA of strain 848T^T was <70% similar (27–45%) to that of the type/reference strains of the current *Aeromonas* hybridization groups (HGs), but 93% similar to that of strain 93M. The DNA G + C content of the five strains ranged from 59.0 to 59.4 mol%. 16S rRNA gene sequence analysis confirmed that the strains belonged to the genus *Aeromonas* and showed high similarity to *Aeromonas encheleia*. Amplified fragment length polymorphism fingerprinting clustered the novel strains in a homogeneous group with low genotypic relatedness to other *Aeromonas* species. Useful phenotypic features for differentiating the five isolates from other *Aeromonas* species include their negative reactions in tests for indole production, lysine decarboxylase, gas from glucose and starch hydrolysis. From the results of this study, the name *Aeromonas molluscorum* sp. nov. is proposed for these strains, with the type strain 848T^T (= CECT 5864^T = LMG 22214^T).

The genus *Aeromonas* Stanier 1943 comprises a collection of Gram-negative, rod-shaped, non-spore-forming, oxidase- and catalase-positive, glucose-fermenting, facultatively anaerobic bacteria that are resistant to vibriostatic agent O/129 and are generally motile by means of polar flagella (Popoff, 1984; Euzéby, 1998). The genus *Aeromonas* belongs to the family *Aeromonadaceae* within the γ -*Proteobacteria*. Aeromonads are autochthonous to aquatic environments worldwide and are usual microbiota (as well as primary or secondary pathogens) of fish, amphibians and other animals. Some motile species (mainly *Aeromonas caviae*, *Aeromonas hydrophila* and *Aeromonas veronii* bv. *Sobria*) are opportunistic pathogens of humans (Janda & Abbott, 1998).

The taxonomy of the genus *Aeromonas* appears to change

continually, due to the addition of newly described species and the reclassification or extended description of existing taxa. Since the first DNA–DNA hybridization study that was performed on *Aeromonas* (Popoff *et al.*, 1981) and the description of the genus with four phenospecies (*A. caviae*, *A. hydrophila*, *Aeromonas salmonicida* and *A. sobria*) in *Bergey's Manual of Systematic Bacteriology* (Popoff, 1984), 18 *Aeromonas* species have been described: *A. hydrophila*, *Aeromonas bestiarum*, *A. salmonicida*, *A. caviae*, *Aeromonas media*, *Aeromonas eucrenophila*, *Aeromonas sobria*, *Aeromonas jandaei*, *A. veronii*, *Aeromonas* sp. HG11, *Aeromonas schubertii*, *Aeromonas* sp. HG13 (enteric Group 501), *Aeromonas trota*, *Aeromonas allosaccharophila*, *Aeromonas encheleia*, *Aeromonas popoffii*, *Aeromonas culicicola* and the most recently described species, *Aeromonas simiae* (Huys *et al.*, 2002; Pidiyar *et al.*, 2002; Esteve *et al.*, 2003; Harf-Monteil *et al.*, 2004).

The present investigation was initiated to determine the taxonomic position of a group of five strains that were isolated from bivalve molluscs, which clustered together as a separate phenon (phenon VI) in a previous phenotypic study (Miñana-Galbís *et al.*, 2002). We have completed the phenotypic study and subjected this group of strains to amplified fragment length polymorphism (AFLP) analysis, 16S rRNA gene sequencing, DNA G + C content determination and DNA–DNA homology determination. In this genotypic study, we included the reference strains

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Abbreviations: ADH, arginine dehydrolase; AFLP, amplified fragment length polymorphism; HG, hybridization group; LDC, lysine decarboxylase; MR, methyl red; ODC, ornithine decarboxylase; VP, Voges–Proskauer.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains 93M, 431E, 848T^T, 849T and 869N are AY532688–AY532692, respectively.

A dendrogram of AFLP profiles and tables showing 16S rRNA gene sequence similarities and DNA–DNA hybridization data are available as supplementary material in IJSEM Online.

of the *Aeromonas* DNA hybridization groups (HGs) that are currently described. The data indicated that our strains are genotypically and phenotypically distinct from the *Aeromonas* species that have been accepted until now and that they constitute a homogeneous taxon, for which the name *Aeromonas molluscorum* sp. nov. is proposed.

The five strains 848T^T (=CECT 5864^T=LMG 22214^T), 93M (=CECT 5865=LMG 22215), 431E (=CECT 5866=LMG 22216), 849T (=CECT 5867=LMG 22217) and 869N (=CECT 5868=LMG 22218) were isolated from wedge-shells (*Donax trunculus*), mussels (*Mytilus* sp.), cockles (*Cardium* sp.), wedge-shells (*Donax trunculus*) and razor-shells (*Ensis* sp.), respectively, from various retail markets in Barcelona (Spain) between April and October 1997. Bivalves without their shells (25 g) were suspended 1/10 (w/v) in 1/4 Ringer's solution for homogenization in a Masticator (IUL Instruments). Samples and serial dilutions were cultured on m-*Aeromonas* selective agar base Havelaar (Biolife) and the same medium with 10 mg ampicillin l⁻¹ for 24–48 h at 25 °C. Samples were also enriched in tryptone soy broth (TSB) and alkaline peptone water (pH 9.0) overnight and then inoculated on the above-mentioned media. Isolates were grown on tryptone soy agar (TSA) for purification and further study. Strains were maintained in TSB with 20 % (v/v) glycerol at –40 °C.

Cell size, morphology and flagellar arrangement were determined by transmission electron microscopy (JEOL 1010). Cells were grown on TSA supplemented with 0.5 % (w/v) NaCl for 48 h at 25 °C and, after further suspension in MilliQ water, were examined by negative staining with 2 % (w/v) uranyl acetate.

Physiological and biochemical characterization, unless otherwise stated, was performed at 25 °C and all media contained 1 % (w/v) NaCl (Miñana-Galbis *et al.*, 2002). Gram-staining, motility, glucose oxidation–fermentation test, oxidase and catalase activity, nitrate reduction, indole production, susceptibility to vibriostatic agent O/129, swarming motility, production of a brown diffusible pigment, gas production from D-glucose, methyl red (MR) and Voges–Proskauer (VP) reactions, β -galactosidase activity (ONPG), hydrogen sulfide production from cysteine and thiosulfate, growth on MacConkey agar, salt tolerance, pH and temperature ranges for growth, acid production from carbohydrates, hydrolysis of arbutin, DNA, elastin, aesculin, starch, urea and xanthine, utilization of substrates as sole carbon and energy sources and sensitivity to antibiotics were determined as described previously (Miñana-Galbis *et al.*, 2002). Arginine dehydro-lase (ADH), lysine decarboxylase (LDC) and ornithine decarboxylase (ODC) activity (Moeller's method) and gelatin hydrolysis by using tannic acid (1 %) as the gelatin-precipitating reagent were determined as described by Smbert & Krieg (1994).

For 16S rRNA gene sequencing and phylogenetic analysis,

DNA was extracted by using a REALPURE genomic DNA extraction kit (RBMEG03; Durviz). Oligonucleotide primers used for PCR amplification and sequencing of the 16S rRNA gene were those described by Martínez-Murcia *et al.* (1999). DNA was subjected to PCR amplification in a total volume of 50 μ l that contained 50 mM KCl, 15 mM Tris/HCl (pH 8.0), 1.5 mM MgCl₂, 0.2 mM each deoxy-ribonucleotide (dATP, dCTP, dGTP, dTTP; Amersham Biosciences), 1.25 U AmpliTaq Gold DNA polymerase (Applied) and 25 pmol each primer. The reaction mixtures were subjected to the following thermal cycling: an initial single step of 95 °C for 9 min, 30 cycles of 94 °C for 60 s, 51 °C for 30 s and 72 °C for 90 s, and a final single step of 72 °C for 10 min. PCR products were purified by using Montage PCR centrifugal filter devices (Millipore) and prepared for sequencing by employing a BigDye Terminator v3.1 cycle sequencing kit (Applied). The amplified 16S rRNA genes were sequenced with an ABI PRISM 3730 DNA analyser by the Scientific and Technical Services of Barcelona University.

The sequences obtained were aligned with 16S rRNA gene sequences of the type strains of all members of the genus *Aeromonas* that were available in GenBank by using the CLUSTAL_X program version 1.8 (Thompson *et al.*, 1997). Distances and clustering with the neighbour-joining and maximum-parsimony methods (pairwise deletion and Kimura two-parameter model) were determined by using the MEGA program version 2.1 (Kumar *et al.*, 2001). Stability of the relationships was assessed by bootstrapping (1000 replicates).

AFLP analysis, which included DNA extraction and purification (Pitcher *et al.*, 1989), AFLP fingerprinting, data processing and numerical analysis (Huys & Swings, 1999), was performed by the BCCM/LMG (Belgian Coordinated Collections of Microorganisms/Laboratorium voor Microbiologie from Universiteit Gent) Identification Service.

For DNA–DNA hybridization and determination of DNA G + C content, genomic DNA of bacterial strains was prepared according to a modification of the procedure of Wilson (1987). The G + C content of each DNA sample was determined by three independent analyses using the HPLC technique (Mesbah *et al.*, 1989). DNA–DNA hybridizations were performed in four replicates at 47 °C according to a modification of the method described by Ezaki *et al.* (1989). These analyses were performed by the BCCM/LMG Identification Service.

The five strains isolated from bivalve molluscs (848T^T, 93M, 431E, 849T and 869N) were identified as belonging to the genus *Aeromonas* as they were Gram-negative, rod-shaped, motile by one polar flagellum, oxidase-positive, facultatively anaerobic, glucose-fermentative and resistant to vibriostatic agent O/129 and could grow in the absence of NaCl, but not at 6 % (w/v) NaCl. Cells of all isolates were about 1.2–2.2 $\times$ 0.5–1.0 μ m in size. Their antibiotic-resistance pattern was similar to that of all *Aeromonas*

species (Kämpfer *et al.*, 1999). These strains formed non-pigmented, circular colonies with a diameter of 3–4 mm on TSA when incubated at 25 °C. The growth-temperature range was 4–37 °C and optimal growth occurred at 25–30 °C.

The novel mesophilic species showed several differentiating phenotypic features in relation to other mesophilic *Aeromonas* species (Table 1). Four or more tests allowed differentiation of *A. molluscorum* from all *Aeromonas* species except *A. caviae*, *A. media* and *A. simiae*. Negative reactions in the tests for indole production and starch hydrolysis allowed the separation of *A. molluscorum* from *A. caviae*. Brown pigment production and the above-mentioned tests discriminated *A. molluscorum* from *A. media*. *A. molluscorum* could be differentiated from *A. simiae* by lysine decarboxylation and acid production from L-arabinose and D-mannitol. These results allowed the phenotypic discrimination of *A. molluscorum* from all *Aeromonas* taxa that have been described to date.

Phylogenetic analysis of 16S rRNA gene sequences of isolates of *A. molluscorum* showed an intraspecies range of zero to three nucleotide differences (Table 2). These sequences

were compared with 16S rRNA gene sequences of all type/reference strains of *Aeromonas* species and subspecies, including the recently described *A. simiae* (Harf-Monteil *et al.*, 2004). The phylogenetic tree constructed by the neighbour-joining method is shown in Fig. 1. *A. encheleia* was the closest neighbour of *A. molluscorum*, with an interspecies range of one to four nucleotide differences (Table 2). Species and subspecies of the genus *Aeromonas* exhibited high levels of overall 16S rRNA gene sequence similarity to each other; the number of nucleotide differences ranged from 0 to 48 (see Supplementary Table A in IJSEM Online). 16S rRNA gene sequence similarity between *A. molluscorum* and *A. simiae* was below 97 %, indicating that they represent different species (Stackebrandt & Goebel, 1994). On the other hand, it is noteworthy that the 16S rRNA gene sequence of *A. hydrophila* subsp. *dhakensis* exhibited a significant number of differences (12–13 nt) from the sequences of the other *A. hydrophila* subspecies and was related closely to *A. caviae* and *A. trota*. This result was unexpected, as *A. hydrophila* subsp. *dhakensis* shows high relatedness to *A. hydrophila* subsp. *hydrophila* and *A. hydrophila* subsp. *ranae* by AFLP fingerprinting and DNA–DNA hybridization (Huys *et al.*, 2003) and, at

Table 1. Key tests for the phenotypic differentiation of *A. molluscorum* from other mesophilic *Aeromonas* species

Data were taken from Carson *et al.* (2001), Miñana-Galbis *et al.* (2002) and Valera & Esteve (2002) unless otherwise indicated. Taxa are identified as: 1, *A. molluscorum* (data from this study); 2, *A. hydrophila* (Carson *et al.*, 2001; Huys *et al.*, 2002; Miñana-Galbis *et al.*, 2002; Valera & Esteve, 2002); 3, *A. bestiarum*; 4, *A. salmonicida* (Miñana-Galbis *et al.*, 2002); 5, *A. caviae*; 6, *A. media*; 7, *A. eucrenophila*; 8, *A. sobria*; 9, *A. veronii* bv. *Sobria*; 10, *A. jandaei* (Carson *et al.*, 2001; Miñana-Galbis *et al.*, 2002; Valera & Esteve, 2002; Esteve *et al.*, 2003); 11, *A. veronii* bv. *Veronii*; 12, *A. schubertii*; 13, *A. trota*; 14, *A. allosaccharophila*; 15, *A. encheleia*; 16, *A. popoffii* (Huys *et al.*, 1997; Esteve *et al.*, 2003); 17, *A. culicicola* (Pidiyar *et al.*, 2002); 18, *A. simiae* (Harf-Monteil *et al.*, 2004). Abbreviations: +, 85–100 % of strains positive; –, 0–15 % of strains positive; v+, 50–84 % of strains positive; v–, 16–49 % of strains positive; d, different results depending on above-mentioned references; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Production of:																		
Brown pigment	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
Gas from D-glucose	–	+	+	+	–	–	+	+	+	+	+	–	+	+	+	+	+	–
H ₂ S from cysteine	–	+	+	+	–	–	+	+	+	+	d	–	+	+	+	ND	ND	ND
Indole	–	+	+	+	+	+	+	+	+	+	+	–	+	+	+	v+	+	–
LDC	–	+	+	+	–	–	–	+	+	+	+	+	+	+	–	–	+	+
ODC	–	–	–	–	–	–	–	–	–	–	+	–	–	v–	–	–	–	–
VP	–	+	+	+	–	–	–	+	+	+	+	+	–	–	v–	+	+	–
Acid from:																		
L-Arabinose	+	+	+	+	+	+	+	–	d	–	–	–	–	+	–	–	–	–
D-Cellobiose	v+	v–	v–	ND	+	+	+	+	v+	–	v+	–	+	+	–	–	–	+
D-Mannitol	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	–
Sorbitol	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
D-Sucrose	+	+	+	+	+	+	d	+	+	v+	+	–	–	+	v+	–	+	+
Hydrolysis of:																		
Aesculin	+	+	+	+	+	+	+	–	–	–	+	–	–	+	+	–	ND	v+
Arbutin	+	+	+	+	+	+	+	–	–	–	+	–	d	–	+	–	ND	ND
Elastin	–	+	v+	+	–	–	–	–	–	d	–	–	–	–	–	ND	ND	ND
Starch	–	+	+	+	+	+	+	+	–	+	+	+	–	–	+	+	+	ND
Utilization of L-lactate	v+	+	–	–	v+	+	–	–	–	–	–	+	+	d	–	+	ND	ND

Table 2. Base differences in 16S rRNA gene sequence between the type strain of *A. encheleia* and *A. molluscorum* strains

Strain	GenBank accession number	Position*					
		258	471	1011	1018	1355	1367
<i>A. encheleia</i> CECT 4342 ^T	AJ224309	G	T	T	A	R	Y
<i>A. molluscorum</i> 93M	AY532688	A	Y	C	G	G	C
<i>A. molluscorum</i> 431E	AY532689	A	C	Y	R	G	C
<i>A. molluscorum</i> 848T ^T	AY532690	A	T	T	A	G	C
<i>A. molluscorum</i> 849T	AY532691	A	C	C	G	G	C
<i>A. molluscorum</i> 869N	AY532692	A	C	C	R	G	C

*Referring to the *Escherichia coli* sequence numbering system described by Brosius *et al.* (1978).

Fig. 1. Phylogenetic relationships of *A. molluscorum* to type/reference strains of the genus *Aeromonas*. The phylogenetic tree was constructed by using 1544 nt of 16S rRNA gene sequence by the neighbour-joining method in the MEGA program version 2.1. Bootstrap values (> 50 %) after 1000 replicates are shown. Bar, distance value of 0.002 (calculated in MEGA).

present, *Aeromonas* strains that belong to the same species have never shown a phylogenetic divergence of 12–13 nucleotide positions, constituting different phylogenetic lines (Martínez-Murcia, 1999).

The AFLP banding patterns of our five strains were compared with the laboratory database AEROLIB, which comprises well-characterized strains of all *Aeromonas* DNA HGs that are currently described (Huys & Swings, 1999; Huys *et al.*, 2003). This comparison revealed that the five isolates form a homogeneous cluster, delineated at a correlation level of 72 %. The simplified dendrogram (see Supplementary Figure in IJSEM Online) shows clearly that *A. molluscorum* constitutes a new AFLP group with low genotypic relatedness to other members of the genus *Aeromonas*. These results allow the genotypic discrimination of *A. molluscorum* from other *Aeromonas* species.

The DNA G+C content of strains 93M, 431E, 848T^T, 849T and 869N was 59.0–59.4 mol%, which agrees with the range described for the genus *Aeromonas* (57–63 mol%; Holt *et al.*, 1994). Results of DNA–DNA hybridization experiments are summarized in Supplementary Table B in IJSEM Online. We chose two representative isolates from the novel *Aeromonas* species, the type strain (848T^T) and strain 93M. These two strains showed 93 % DNA–DNA similarity, clearly above the level of 70 % that is accepted as the limit for species delineation (Wayne *et al.*, 1987). The type strain of *A. molluscorum* clearly showed <70 % DNA–DNA similarity (27–45 %) with any of the type/reference strains of the current *Aeromonas* HGs. Therefore, *A. molluscorum* constitutes a new *Aeromonas* HG.

Based on the results of DNA–DNA hybridization, phenotypic characterization, AFLP analysis and 16S rRNA gene sequencing, we propose that strains 93M, 431E, 848T^T, 849T and 869N represent a novel species within the genus *Aeromonas*, for which we propose the name *Aeromonas molluscorum* sp. nov.

Description of *Aeromonas molluscorum* sp. nov.

Aeromonas molluscorum (mol.lus.co'rum. N.L. pl. n. *Mollusca* a zoological phylum; N.L. gen. pl. n. *molluscorum* of molluscs classified in the phylum *Mollusca*).

Cells are Gram-negative, straight, motile rods with a polar flagellum, 1.2–2.2 µm long and 0.5–1.0 µm wide. Colonies on TSA are 3–4 mm in diameter, translucent to opaque, circular and beige in colour after 48 h at 25 °C. Growth occurs at 4–37 °C, but not at 40–5 °C; optimal growth occurs at 25–30 °C. Oxidase- and catalase-positive, reduces nitrate to nitrite and is resistant to vibriostatic agent O/129 (150 µg). Indole, brown diffusible pigment, swarming, gas from D-glucose and hydrogen sulfide from cysteine and thiosulfate are not produced. Positive for glucose oxidation–fermentation, ADH, MR and ONPG tests. Negative for VP, LDC and ODC tests. Grows on MacConkey agar. Able to grow at 0–3 % NaCl and pH 9.0, but not at 6 % NaCl or

pH 4.5. All strains hydrolyse arbutin, DNA, aesculin and gelatin, but not starch, elastin, urea or xanthine. All strains produce acid from L-arabinose, arbutin (except strain 93M), D-cellobiose (except strain 431E), dextrin, D-galactose, glycerol, D-mannitol, D-mannose, D-sucrose and D-trehalose, but not from lactose, D-melibiose, D-raffinose, L-rhamnose, sorbitol or D-xylose. The following substrates are used as sole carbon and energy sources: acetate (except strain 93M), L-arabinose, arbutin, L-arginine, D-cellobiose (except strain 431E), citrate, aesculin, D-fructose, D-galactose, D-glucose, glycerol, L-histidine, maltose, D-mannitol, D-mannose, N-acetylglucosamine, D-sucrose, salicin (except strain 93M) and D-trehalose. None of the strains uses adonitol, dulcitol, inositol, inulin, lactose, D-melezitose, D-melibiose, D-raffinose, L-rhamnose, sorbitol, L-sorbose or D-xylose. All strains are resistant to ampicillin (10 µg) (except strain 431E), erythromycin (15 µg) and penicillin G (10 µg). All strains show intermediate sensitivity to streptomycin (10 µg) and are sensitive to amikacin (30 µg), amoxycillin + clavulanic acid (30 µg) (intermediate sensitivity for strains 93M and 869N), cefoxitin (30 µg) (except strain 93M), ceftriaxon (30 µg), cefuroxime (30 µg), cephalothin (30 µg) (except strains 93M and 869N), ciprofloxacin (5 µg), colistin (50 µg), gentamicin (10 µg), imipenem (10 µg), polymyxin B (300 U), tetracycline (30 µg), tobramycin (10 µg) and trimethoprim + sulfamethoxazole (1.25 µg + 23.75 µg). DNA G+C content is 59.0–59.4 mol%.

The type strain, 848T^T (= CECT 5864^T = LMG 22214^T), was isolated from wedge-shells (*Donax trunculus*) obtained from a retail market in Barcelona (Spain) in 1997.

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