Aeromonas bivalvium sp. nov., isolated from bivalve molluscs

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A polyphasic study was performed to determine the taxonomic position of two *Aeromonas* strains, 665N and 868E^T, isolated from bivalve molluscs, that could not be identified at the species level in a previous numerical taxonomy study. The DNA G + C content of these isolates was 62.3 and 62.6 mol%, respectively. Sequence analysis of the 16S rRNA gene showed that the two new strains were closely related to members of the genus *Aeromonas*. Fluorescence amplified fragment length polymorphism fingerprinting revealed that strains 665N and 868E^T clustered together with a similarity of 78 % but did not cluster with any of the *Aeromonas* genomospecies. DNA–DNA hybridization experiments revealed a high level of relatedness between the two new isolates (76 %) but low levels of relatedness between these and phylogenetically most closely related *Aeromonas* genomospecies (30–44 %). Useful tests for the phenotypic differentiation of strains 665N and 868E^T from other mesophilic *Aeromonas* species included those for gas from glucose, lysine decarboxylase, Voges–Proskauer reaction, acid from L-arabinose, hydrolysis of aesculin and utilization of L-lactate. On the basis of genotypic and phenotypic evidence, strains 665N and 868E^T are considered to represent a novel species of the genus *Aeromonas*, for which the name *Aeromonas bivalvium* sp. nov. is proposed. The type strain is 868E^T (= CECT 7113^T = LMG 23376^T).

Members of the genus *Aeromonas*, belonging to the class *Gammaproteobacteria*, are Gram-negative, non-spore-forming bacilli or coccobacilli, and are facultatively anaerobic, chemo-organotrophic, oxidase- and catalase-positive, resistant to the vibriostatic agent O/129 (2,4-diamino-6,7-diisopropylpteridine), generally motile by means of a single polar flagellum and are able to reduce nitrate to nitrite. Aeromonads are primarily aquatic, widespread in environmental habitats, frequently isolated from foods and often associated with aquatic animals, and some species are primary or opportunistic pathogens in invertebrates and vertebrates including humans (Martin-Carnahan & Joseph, 2005).

At the time of writing, 17 *Aeromonas* species and 20 DNA–DNA hybridization groups (HGs) have been described: *Aeromonas hydrophila* (HG1), *A. bestiarum* (HG2), *A. salmonicida* (HG3), *A. caviae* (HG4), *A. media* (HG5), *A.*

eucrenophila (HG6), *A. sobria* (HG7), *A. veronii* bv. *Sobria* (HG8/10), *A. jandaei* (HG9), *A. veronii* bv. *Veronii* (HG10/8), *Aeromonas* sp. HG11, *A. schubertii* (HG12), *Aeromonas* sp. group 501 (HG13), *A. trota* (HG14), *A. allosaccharophila* (HG15), *A. encheleia* (HG16), *A. popoffii* (HG17), *A. culicicola* (HG18), *A. simiae* (HG19) and *A. molluscorum* (HG20) (Pidiyar *et al.*, 2002; Harf-Monteil *et al.*, 2004; Miñana-Galbis *et al.*, 2004; Martin-Carnahan & Joseph, 2005). In addition to the continuous description of novel species, the complexity of *Aeromonas* taxonomy results from the isolation of motile and non-motile, mesophilic and psychrophilic, pigmented and non-pigmented strains within several *Aeromonas* species (Altwegg *et al.*, 1990; Martin-Carnahan & Joseph, 2005), description of novel subspecies of *A. hydrophila* (Huys *et al.*, 2002, 2003) and *A. salmonicida* (Pavan *et al.*, 2000), taxonomic rearrangement of *Aeromonas* sp. HG11 (Huys *et al.*, 1997) and *A. culicicola* (Huys *et al.*, 2005) and extended descriptions of *A. eucrenophila* and *A. encheleia* (Huys *et al.*, 1997) and *A. jandaei* (Esteve *et al.*, 2003). In this context, several authors have proposed useful tables for the phenotypic differentiation of *Aeromonas* species based on a limited number of key tests (Carson *et al.*, 2001; Miñana-Galbis *et al.*, 2002, 2004; Valera & Esteve, 2002; Abbott *et al.*, 2003; Martin-Carnahan & Joseph, 2005). Genotypic classification of aeromonads at the genus level is currently recommended based on DNA G + C content and for species delineation based on 16S

Abbreviations: FAFLP, fluorescence amplified fragment length polymorphism; HG, hybridization group.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains 868E^T and 665N are DQ504429 and DQ504430, respectively.

A table giving levels of DNA–DNA hybridization between strains 868E^T and 665N and strains of phylogenetically related *Aeromonas* species is available as supplementary material in IJSEM Online.

rRNA gene sequence analyses and DNA–DNA reassociation experiments, although other genomic methods, such as DNA profiling or *rpoD* and *gyrB* gene sequencing, are useful for *Aeromonas* species discrimination (Stackebrandt *et al.*, 2002; Soler *et al.*, 2004; Martin-Carnahan & Joseph, 2005; Morandi *et al.*, 2005).

In the present study, a polyphasic approach was used in order to determine the taxonomic position of two *Aeromonas* strains isolated from bivalve molluscs that clustered together as a separate phenon (phenon VII) but could not be identified at the species level in a previous phenotypic study of the genus *Aeromonas* (Miñana-Galbis *et al.*, 2002). For this purpose, an extended phenotypic characterization, 16S rRNA gene sequencing, DNA G + C content determination, fluorescence amplified fragment length polymorphism (FAFLP) analysis and DNA–DNA hybridization experiments were performed for the two isolates. Based on the results obtained, these strains are considered to represent a novel species of the genus *Aeromonas*.

Strains 868E^T and 665N were isolated from cockles (*Cardium* sp.) and razor shells (*Ensis* sp.), respectively, from retail markets in Barcelona (Spain). Isolation, growth and preservation of strains were performed as described previously (Miñana-Galbis *et al.*, 2004).

Cell size, morphology and flagellar arrangement were determined by transmission electron microscopy (JEOL 1010). Cells were grown on trypticase soy agar (TSA) supplemented with 0.5 % (w/v) NaCl for 24 h at 25 °C and, following further suspension in MilliQ water, were examined by negative staining with 2 % (w/v) uranyl acetate.

Physiological and biochemical characterization experiments, unless stated otherwise, were performed at 25–30 °C and all media contained 1 % (w/v) NaCl. Tests for the following were determined as described previously (Miñana-Galbis *et al.*, 2002): Gram-staining, motility, glucose oxidation–fermentation, oxidase and catalase activity, nitrate reduction, indole production, susceptibility to O/129, swarming motility, production of a brown diffusible pigment, gas production from D-glucose, methyl red and Voges–Proskauer reactions, ONPG, hydrogen sulfide production from cysteine and thiosulfate, growth on MacConkey agar and m-*Aeromonas*-selective agar base Havelaar, salt tolerance, pH and temperature ranges for growth, acid production from carbohydrates, hydrolysis of aesculin, arbutin, DNA, elastin, erythrocytes, starch, urea and xanthine, utilization of substrates as sole carbon and energy sources and sensitivity to antibiotics. Arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase activity (Moeller's method) and hydrolysis of gelatin by using tannic acid (1 %) as the gelatin-precipitating reagent were determined as described by Smibert & 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 µl that contained 50 mM KCl, 15 mM Tris/HCl (pH 8.0), 1.5 mM MgCl₂, 0.2 mM each dNTP (Amersham Biosciences), 1.25 U AmpliTaq Gold DNA polymerase (Applied Biosystems) 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, 35 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 Biosystems). The amplified 16S rRNA genes were sequenced with an ABI PRISM 3730 DNA analyser by the Unitat de Genòmica of the Serveis Científicotècnics of the Universitat de Barcelona.

The sequences obtained were aligned with 16S rRNA gene sequences of the type or reference strains of the *Aeromonas* DNA HGs by using the DNASTAR Lasergene software. Distances and clustering with the neighbour-joining and maximum-parsimony methods (pairwise deletion and Kimura two-parameter model) were determined by using MEGA version 2.1 (Kumar *et al.*, 2001). Stability of the relationships was assessed by bootstrapping (1000 replicates).

DNA fingerprinting using FAFLP, which included DNA extraction and purification (Gevers *et al.*, 2001), FAFLP fingerprinting, data processing and numerical analysis (Huys & Swings, 1999), was performed by the BCCMTM/LMG (Belgian Coordinated Collections of Microorganisms/Laboratorium voor Microbiologie from Universiteit Gent) Identification Service.

For DNA–DNA hybridization experiments 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 via the HPLC technique (Mesbah *et al.*, 1989). DNA–DNA hybridizations were performed in a minimum of four replicates at 47 °C according to a modification of the method described by Ezaki *et al.* (1989). These analyses were also performed by the BCCMTM/LMG Identification Service.

Strains 868E^T and 665N were identified as belonging to the genus *Aeromonas* as they were Gram-negative, rod-shaped, motile by means of one polar flagellum, oxidase-positive, facultatively anaerobic, glucose-fermentative and resistant to O/129 and they grew in the absence of NaCl but not at 7 % (w/v) NaCl. Cells of the two isolates were coccoid to rod-shaped (0.3–1.0 × 0.5–2 µm). Their antibiotic-resistance pattern was similar to that of most recognized *Aeromonas* species (Kämpfer *et al.*, 1999). The two strains were resistant to ampicillin and strain 665N was also resistant to cephalothin, whereas strain 868E^T was not. These strains

formed non-pigmented, circular colonies with a diameter of 3–4 mm on TSA when incubated at 25–30 °C. The growth temperature range was 4–37 °C, although strain 868E^T was able to grow at 40.5 °C. Optimal growth occurred at 30–37 °C. The two isolates showed several differential phenotypic features with regard to other mesophilic *Aeromonas* species (Table 1). Three or more tests allowed differentiation of strains 665N and 868E^T from all recognized *Aeromonas* species except *A. caviae* and *A. media*. A positive reaction in the lysine decarboxylase test allowed the separation of these strains from *A. caviae* and *A. media*. In addition, they could be differentiated from *A. media* based on the following tests: production of ONPG and brown pigment, fermentation of lactose and mannose and utilization of lactose, mannose and raffinose. In contrast to *A. caviae*, strains 665N and 868E^T were able to use glycerol and to produce acid from it. These results allowed the phenotypic discrimination of strains 665N and 868E^T from all recognized *Aeromonas* taxa.

Comparative analysis of 16S rRNA gene sequences of strains 665N and 868E^T and those of all type or reference strains of *Aeromonas* species confirmed that the new isolates belonged to the genus *Aeromonas*. 16S rRNA gene sequence similarity between strains 665N and 868E^T and other *Aeromonas* species ranged from 96.75 % (to *A. simiae*) to 99.71 % (to *A. popoffii*). Dendrograms generated by the maximum-parsimony and

neighbour-joining methods were similar. The phylogenetic tree constructed by the neighbour-joining method is shown in Fig. 1. The sequences of strains 665N and 868E^T differed by 2 nt from each other and by 4 and 6 nt differences, respectively, with regard to that of *A. popoffii* (Table 2).

The FAFLP banding patterns of strains 665N and 868E^T were compared with the laboratory database AEROLIB, which comprises well-characterized strains of all *Aeromonas* DNA HGs (Huys & Swings, 1999; Huys *et al.*, 2003; Miñana-Galbis *et al.*, 2004). This comparison revealed that isolates 665N and 868E^T clustered together with a similarity of 78 % but did not cluster within any of the 20 *Aeromonas* DNA HGs currently described. A dendrogram of the cluster analysis of FAFLP fingerprints is shown in Fig. 2. These results indicated that strains 665N and 868E^T probably belonged to the same species and represented a novel *Aeromonas* species.

The DNA G + C content of strains 665N and 868E^T was 62.3 and 62.6 mol%, respectively, which agrees with the range described for the genus *Aeromonas* (57–63 mol%; Martin-Carnahan & Joseph, 2005). Levels of DNA–DNA relatedness were determined between the type strain of the presumptively novel *Aeromonas* species (868E^T) and the type or reference strains of the phylogenetically most closely related *Aeromonas* HGs (Fig. 1) and between strains 868E^T and

Table 1. Key tests for the phenotypic differentiation of strains 665N and 868E^T from other mesophilic *Aeromonas* species

Data were taken from Miñana-Galbis *et al.* (2004) and Martin-Carnahan & Joseph (2005) unless indicated otherwise. Taxa: 1, strains 665N and 868E^T (data from this study); 2, *A. hydrophila*; 3, *A. bestiarum*; 4, *A. salmonicida*; 5, *A. caviae*; 6, *A. media*; 7, *A. eucrenophila*; 8, *A. sobria*; 9, *A. veronii* bv. *Sobria*; 10, *A. jandaei*; 11, *A. veronii* bv. *Veronii*; 12, *A. schubertii*; 13, *A. trota*; 14, *A. allosaccharophila*; 15, *A. encheleia*; 16, *A. popoffii*; 17, *A. culicicola*; 18, *A. simiae*; 19, *A. molluscorum*. +, 85–100% of strains positive; d, 16–84% of strains positive; –, 0–15% of strains positive; ND, no data available. Results obtained in this study for reference strains are given in parentheses.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Production of:																			
Brown pigment	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
Gas from D-glucose	–	+	+	+	–	–	+	+	+	+	+	–	+	+	+	+	+	–	–
H ₂ S from cysteine	–	+	+	+	–	–	+	+	+	+	d	–	+	+	+	(+)	(+)	(–)	–
Indole	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	(+)	+	–	–
Lysine decarboxylase	+	+	+	+	–	–	–	+	+	+	+	+	+	+	–	–	+	+	–
Ornithine decarboxylase	–	–	–	–	–	–	–	–	–	–	+	–	–	d	–	–	–	–	–
Voges–Proskauer reaction	–	+	+	+	–	–	–	+	+	+	+	+	–	–	–	+	+	–	–
Acid from:																			
L-Arabinose	+	d	+	+	+	+	+	–	d	–	–	–	–	+	–	d	–	–	+
D-Mannitol	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	–	+
Sorbitol	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Sucrose	+	+	+	+	+	+	d	+	+	d	+	–	–	+	d	–	+	+	+
Hydrolysis of:																			
Aesculin	+	+	+	+	+	+	+	–	–	–	+	–	–	+	+	(–)	(–)	(–)	+
Arbutin	+	+	+	+	+	+	+	–	–	–	+	–	d	–	+	(–)	(–)	(+)	+
Elastin	–	+	d	+	–	–	–	–	–	d	–	–	–	–	–	–	(–)	(–)	–
Starch	+	+	+	+	+	+	+	+	–	+	+	+	–	–	+	+	+	(+)	–
Utilization of L-lactate	+	+	–	–	d	+	–	–	–	–	–	+	+	d	–	+	ND	ND	d

Fig. 1. Phylogenetic relationships of strains 665N and 868E^T to type or reference strains of the genus *Aeromonas*. The phylogenetic tree was constructed by using 1544 nt of the 16S rRNA gene sequence by the neighbour-joining method in MEGA version 2.1. Bootstrap values (>50%) based on 1000 replicates are shown. Bar, 0.5% sequence divergence. **A. culicicola* is a later heterotypic synonym of *A. veronii* (Huys *et al.*, 2005).

665N. Strains 868E^T and 665N showed a level of DNA–DNA relatedness of 76%, above the level of 70% that is accepted as the limit for species delineation (Wayne *et al.*, 1987). Strain 868E^T clearly showed <70% DNA reassociation (30–44%) with any of the type or reference *Aeromonas* strains analysed (see Supplementary Table S1 in IJSEM Online).

Based on the results of DNA–DNA hybridization, phenotypic characterization, FAFLP analysis and 16S rRNA gene sequencing, we consider that strains 868E^T and 665N represent a single novel species of the genus *Aeromonas*, for which the name *Aeromonas bivalvium* sp. nov. is proposed.

Description of *Aeromonas bivalvium* sp. nov.

Aeromonas bivalvium (bi.val'vi.um. N.L. pl. neut. n. *Bivalvia* scientific name of a class of molluscs; N.L. neut. gen. pl. n. *bivalvium* of bivalves of the class Bivalvia).

Cells are Gram-negative, straight, motile coccoids/rods with a polar flagellum, 0.5–2.0 µm long and 0.3–1.0 µm wide. Colonies on TSA are 3–4 mm in diameter, opaque, circular and beige in colour after 48 h at 25 °C. Optimal growth

occurs at 30–37 °C but growth occurs at 4–37 °C and strain 868E^T grows at up to 40.5 °C. Oxidase- and catalase-positive, reduces nitrate to nitrite, produces indole from tryptophan and is resistant to the vibriostatic agent O/129 (150 µg). Positive for glucose oxidation–fermentation, lysine decarboxylase, methyl red and ONPG tests. Negative for arginine dihydrolase, ornithine decarboxylase and Voges–Proskauer tests. Negative for brown diffusible pigment, swarming, and production of gas from D-glucose and hydrogen sulfide from cysteine and thiosulfate. Grows on MacConkey agar and m-*Aeromonas*-selective agar base Havelaar. Able to grow at pH 9.0 and 0–3% NaCl, grows weakly at 6% NaCl but does not grow at pH 4.5 or 7% NaCl. Hydrolyses arbutin, DNA, aesculin, gelatin and starch, but not elastin, erythrocytes, urea or xanthine. Produces acid from L-arabinose, arbutin, dextrin, D-galactose, glycerol, D-mannitol, salicin, sucrose and D-trehalose but not from lactose, D-mannose, D-melibiose, D-raffinose, L-rhamnose, sorbitol or D-xylose. The following substrates are used as sole carbon and energy sources: acetate, N-acetylglucosamine, aesculin, L-arabinose, p-arbutin, L-arginine, D-cellobiose, citrate, D-fructose, D-galactose, D-glucose, glycerol, L-histidine, L-lactate, maltose, D-mannitol, salicin, starch, sucrose and

Table 2. Differences in 16S rRNA gene sequences among *A. popoffii* LMG 17541^T and strains 665N and 868E^T

Positions refer to the *Escherichia coli* sequence numbering system (Brosius *et al.*, 1978).

Strain	GenBank accession no.	Position					
		456	459	469	476	1011	1018
<i>A. popoffii</i> LMG 17541 ^T	AJ224308	T	T	C	A	C	G
665N	DQ504430	A	C	T	T	C	G
868E ^T	DQ504429	A	C	T	T	T	A

Fig. 2. Simplified dendrogram showing cluster analysis of the FAFLP fingerprints of strains 665N and 868E^T and reference strains of the *Aeromonas* DNA hybridization groups. The *Aeromonas* reference strains from the AEROLIB database are listed in Huys & Swings (1999), Huys *et al.* (2003) and Miñana-Galbis *et al.* (2004), except for *A. simiae* LMG 22269^T and strains 665N and 868E^T, which were added to the database in the framework of the current study. The dendrogram was constructed with the UPGMA clustering method by using the Pearson product-moment correlation coefficient (expressed as a percentage).

D-trehalose. Does not use adonitol, dulcitol, inositol, lactose, D-mannose, D-melezitose, D-melibiose, D-raffinose, L-rhamnose, sorbitol, L-sorbose or D-xylose. Resistant to amoxycillin/clavulanic acid (30 µg), ampicillin (10 µg), erythromycin (15 µg) and penicillin G (10 µg), shows intermediate sensitivity to cefuroxime (30 µg) and streptomycin (10 µg) and is sensitive to amikacin (30 µg), ceftriaxone (30 µg), 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 (1.25/23.75 µg). Strain 665N is resistant to cephalothin (30 µg), cefoxitin (30 µg) and ticarcillin (75 µg), whereas strain 868E^T is not. The DNA G+C content is 62.3 mol% for strain 665N and 62.6 mol% for strain 868E^T.

The type strain, 868E^T (=CECT 7113^T=LMG 23376^T), was isolated from cockles (*Cardium* sp.) obtained from a retail market in Barcelona (Spain). Strain 665N (=CECT 7112=LMG 23377) is a second strain of the species.

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