Aeromonas popoffii sp. nov., a Mesophilic Bacterium Isolated from Drinking Water Production Plants and Reservoirs

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We examined the taxonomic position of seven *Aeromonas* isolates, recovered from Flemish and Scottish drinking water production plants and reservoirs, which were previously recognized by numerical analysis of genomic AFLP fingerprints as members of an unknown *Aeromonas* taxon that most closely resembled the species *Aeromonas bestiarum* (DNA hybridization group [HG] 2). The new phenotypic and DNA-DNA hybridization data obtained in this study show that the *A. bestiarum*-like strains constitute a separate *Aeromonas* species, for which the name *Aeromonas popoffii* sp. nov. is being proposed. The new species exhibited an internal DNA relatedness ranging from 79 to 100% and was 22 to 63% related to the type or reference strains of other *Aeromonas* spp. The highest DNA binding values were determined with *A. bestiarum* (51 to 63%), followed by *Aeromonas hydrophila* sensu stricto (HG1; 50 to 60%) and *Aeromonas salmonicida* (HG3; 39 to 55%). Although fingerprints generated by ribotyping and cellular fatty acid analysis often were highly similar, minor differences between the respective fingerprints were of significance for the differentiation of *A. popoffii* from its closest taxonomic neighbors, HG1, HG2, and HG3. Phenotypically, all seven strains of *A. popoffii* were positive for acid and gas production from D-glucose and glycerol, growth in KCN broth, arginine dihydrolase, DNase, Voges-Proskauer reaction, and resistance to vibriostatic agent O/129 and ampicillin but displayed negative reactions for production of urease, tryptophan deaminase, ornithine decarboxylase, and lysine decarboxylase (LDC). None of the strains displayed strong hemolytic activity. The lack of D-sucrose fermentation and LDC production and the ability to utilize DL-lactate as the sole energy and carbon source were useful characteristics for the biochemical separation of *A. popoffii* from *A. bestiarum*. Other *Aeromonas* spp. could be differentiated phenotypically from the new species by at least two features. The chromosomal G+C content of *A. popoffii* ranges from 57.7 to 59.6 mol%. Strain LMG 17541 is proposed as the type strain.

Members of the genus *Aeromonas* belong to the autochthonous microbiota of most freshwaters, raw drinking waters, and municipal sewage effluents (24). However, numerous reports on the incidence of aeromonads in cold-blooded animals (40), human clinical specimens (14, 35, 47), food (26, 41), and soil (51) clearly demonstrate that the natural habitats of these organisms are not strictly limited to aquatic environments. In general, microbiologists have primarily focused on *Aeromonas* in relation to the furunculosis-causing fish pathogen *Aeromonas salmonicida* (50, 57) or because of the steadily growing notion that some mesophilic *Aeromonas* species are opportunistic human pathogens (13, 34). Nonetheless, and in spite of the increasing number of medical cases describing *Aeromonas*-associated extraintestinal infections and gastroenteritis, none of the known species has yet been recognized as a primary pathogen for humans (33).

Apart from the clinical relevance of *Aeromonas*, the taxonomy of this genus appears to be continuously changing due to the addition of newly described species (2, 17) and the reclassification or extended description of existing taxa (27, 28). In addition, the combined use of the terms phenospecies (i.e., taxon delineated on the basis of phenotypic characteristics)

and genomospecies (i.e., DNA hybridization group [HG]) in the current *Aeromonas* classification seems highly confusing to many taxonomists outside the field (8). At least 13 *Aeromonas* species have been validated so far (2, 3, 7, 9, 17, 22, 23, 46, 53, 56), of which some encompass several HGs (e.g., *Aeromonas caviae* HG4, HG5A, and HG5B, the latter also including *Aeromonas media*) or are further subdivided in genotypically identical biogroups (e.g., *Aeromonas veronii* biogroup *sobria* [HG8] and *A. veronii* biogroup *veronii* [HG10]).

In a recent study (29) of the genotypic diversity among 168 *Aeromonas* isolates, obtained from Flemish drinking water production plants, using the DNA fingerprinting technique AFLP a group of 24 strains remained unidentified against the AFLP-based identification library representing all currently described taxa in the genus *Aeromonas*. A numerical analysis of individual AFLP fingerprints revealed that the unidentified strains constituted a separate genotypic cluster that was most closely related to but clearly distinct from *Aeromonas bestiarum*, formerly referred to as *A. hydrophila* HG2 (2). In the course of a subsequent AFLP study (31), additional *A. bestiarum*-like strains were also discovered among a collection of mesophilic *Aeromonas* isolates originating from Scottish drinking water supplies.

The purpose of the present study was to examine the genomic and phenotypic relationships of the Flemish and Scottish *A. bestiarum*-like isolates to *A. bestiarum* and to other previously identified *Aeromonas* species. Based on the reported findings, it was concluded that this unknown group

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TABLE 1. *A. popoffii* strains used in this study^a

Strain ^b	Original designation	Source of isolation	Geographical origin
LMG 17541 ^T	IK-O-a-10-3	Drinking water production plant (sampled after flocculation-decantation)	Oelegem (Belgium)
LMG 17542	IK-B-r-15-1	Drinking water production plant (sampled from raw surface water)	De Blankaart (Belgium)
LMG 17543	IK-S-a-10-2	Drinking water production plant (sampled after flocculation-decantation)	Snellegem (Belgium)
LMG 17544	IK-E-a-14-1	Drinking water production plant (sampled after flocculation-decantation)	Eeklo (Belgium)
LMG 17545	IK-S-b-8-1	Drinking water production plant (sampled after rapid sand filtration)	Snellegem (Belgium)
LMG 17546	AG-7	Drinking water service reservoir	Udny Station (Scotland)
LMG 17547	AG-9	Drinking water treatment plant (sampled after rapid sand filtration)	Turriff (Scotland)
LMG 17548 ^c	IK-E-b-3-1	Drinking water production plant (sampled after rapid sand filtration)	Eeklo (Belgium)

^a In addition to the 7 *A. popoffii* strains shown, 16 *A. bestiarum*-like isolates (as delineated by numerical analysis of AFLP fingerprints [29]) were included for discriminatory phenotypic characterization (see Table 3).

^b LMG, BCCM/LMG Culture Collection, Laboratorium voor Microbiologie, Universiteit Gent, Ghent, Belgium.

^c Intermediate strain between *A. bestiarum* and *A. popoffii* (see Results and Discussion).

represents a new mesophilic member of the genus *Aeromonas*, for which the name *Aeromonas popoffii* is proposed. Furthermore, we also investigated the usefulness of ribotyping and fatty acid analysis as rapid and reliable techniques for the differentiation of *A. popoffii* from its closest taxonomic neighbors.

MATERIALS AND METHODS

Strains. For the description of the species *A. popoffii*, a total of seven representative strains were selected from the original collection of 24 Flemish (29) and 3 Scottish (31) isolates of the *A. bestiarum*-like group. Five of these seven strains were recovered in the period September 1992 to October 1993 from water samples taken during various treatment steps at four Flemish drinking water production plants (Table 1) (30, 39). The remaining two strains originated from two water supplies located in northeast Scotland that were routinely sampled from April 1995 to December 1996 to determine the incidence of mesophilic *Aeromonas* spp. in distributed potable waters (18). Strain LMG 17548 (Table 1), which was classified by numerical analysis of AFLP patterns as an isolate intermediate between HG2 and the HG2-like cluster (29), was also included for further taxonomic investigation. For a further verification of the discriminatory phenotypic characterizations (see Table 3), 16 additional members of the *A. bestiarum*-like AFLP cluster (29) were also included with the 7 *A. popoffii* strains in Table 1. In addition, type and reference strains of other *Aeromonas* species were used for DNA-DNA hybridization (see Table 2), antimicrobial susceptibility testing (data not shown), ribotyping (see Fig. 1), and fatty acid analysis (see Table 4). Unless otherwise stated, all strains were cultured aerobically on trypticase soy agar (TSA) containing 3% (wt/vol) Trypticase soy broth (BBL) and 1.5% (wt/vol) bacteriological agar no. 1 (Oxoid) at 28°C for 24 h.

Physiological and biochemical characterization. All of the phenotypic characters listed below were tested for the first seven strains in Table 1. In addition, the discriminatory tests shown in Table 3 were also performed for 16 other members of the *A. bestiarum*-like AFLP cluster (29) and strain LMG 17548.

Cell shape and Gram-staining characteristics were determined with cultures grown overnight on TSA medium (58). The oxidation-fermentation test was performed in O/F basal medium supplemented with 1% (wt/vol) glucose as described by Hugh and Leifson (25). Production of a brown diffusible pigment was determined after 7 days on TSA medium. Hydrogen sulfide production; esculin hydrolysis, tryptophan deaminase, arginine dihydrolase, lysine and ornithine decarboxylase, urease, citrate and malonate alkalization; hydrolysis of *o*-nitrophenyl- β -D-galactopyranoside, *p*-nitrophenyl (*p*NP)- β -D-glucuronide, and *p*NP- β -D-xyloside; and acid formation from D-glucose, L-rhamnose, D-sucrose, D-xylose, adonitol, inositol, and D-sorbitol were tested with the Micronaut-E System (Merlin Diagnostica, Bornheim-Hersel, Germany), formerly the Titer-tek-Enterobac-Automated System (36). Kovacs cytochrome oxidase was tested by using commercially available test strips (Merck, Darmstadt, Germany). The following tests were performed as described previously (58): indole production (method 1), catalase activity (method 1), casein hydrolysis, gas production from D-glucose and glycerol, gelatinase activity (method 1), hemolysis of sheep blood (method 1), lecithinase activity, Tween 80 hydrolysis (method 2), motility, DNase activity (method 1), starch hydrolysis, susceptibility to vibriostatic agent O/129, nitrate and nitrite reduction, and the Voges-Proskauer reaction. Hydrolysis of chitin was performed according to the method of Lingappa and Lockwood (42). Growth in KCN medium was performed as described by Edwards and Fife (15), whereas salt tolerance was determined in nutrient broth containing 3, 6, 8, or 10% (wt/vol) NaCl. Growth at 37°C was determined in 3% (wt/vol) Trypticase soy broth. The following tests were performed as described previously (37): utilization of carbon sources (acetate, *N*-acetyl-D-glucosamine, *cis*-aconitate, *trans*-aconitate, adipate, adonitol, β -alanine, L-alanine, 4-aminobutyrate, L-arabinose, arbutin, L-arginine, L-aspartate, azelate, D-cellobiose, citrate, citrullin,

dulcitol, erythritol, ethanol, D-fructose, fumarate, D-galactose, D-gluconate, D-glucose, D-glucuronate, glutarate, L-glutamate, L-glutamine, glycerol, glycine, L-histidine, 3-hydroxybenzoate, 4-hydroxybenzoate, DL-3-hydroxybutyrate, inositol, itaconate, DL-lactate, lactose, L-leucine, L-malate, maltitol, D-maltose, D-mannose, D-mannitol, α -D-melibiose, mesaconate, L-ornithine, oxoglutarate, phenylacetate, L-phenylalanine, L-proline, propionate, putrescine, pyruvate, D-raffinose, L-rhamnose, D-ribose, salicin, L-serine, D-sorbitol, suberate, succinate, D-sucrose, D-trehalose, L-tryptophan, L-tyrosine, and D-xylose), fermentation of carbohydrates (acid production from L-arabinose, D-arabitol, D-cellobiose, dulcitol, erythritol, D-galactose, glycerol, lactose, D-maltose, D-mannitol, D-mannose, melibiose, methyl-D-glucoside, D-raffinose, salicin, and D-trehalose), and qualitative enzyme tests (using the chromogenic substrates L-alanine-*p*-nitroanilide [*p*NA], 2-deoxythymidine-5'-*p*NP-phosphate, L-glutamate- γ -3-carboxy-*p*NA, *p*NP- α -D-glucopyranoside, *p*NP- β -D-glucopyranoside, *p*NP-phenylphosphonate, bis-*p*NP-phosphate, *p*NP-phosphoryl choline, and L-proline-*p*NA). All biochemical tests were read after 48 h of incubation with the exception of the chitin hydrolysis test which was read after 7 days.

Antibiotic susceptibility testing. Susceptibilities to 72 different antibiotics were tested in 96-well microplates with Micronaut-S kits (Merlin Diagnostika). Nine microplates contained the 72 freeze-dried antibiotics in 12 different concentrations. From a 24 ($\pm$ 2)-h-old culture that was grown on Sheep Blood Agar Base (Oxoid) at 30°C, single colonies were picked and suspended in 3 to 5 ml of a standardized sterile saline medium (Braun, Melsungen, Germany). This suspension was photometrically adjusted to a McFarland standard of 0.5, which equals an A_{560} value of about 0.12, and 10 μ l of this suspension was then added to 10 ml of Iso-Sensitest broth (Oxoid). After thorough mixing, 100 μ l of this suspension was added to each well of the Micronaut-S microplates. The sealed plates were read after an incubation of 24 $\pm$ 2 h at 30°C by using a microplate reader (Labsystems, Multiscan Multisoft). An A_{540} value of >0.1 was considered a positive growth response.

Mol% G+C determination and DNA-DNA hybridization. Chromosomal DNA of high molecular weight was isolated according to the method of Marmur (43). The moles percent guanine-plus-cytosine (mol% G+C) values were determined in 1 $\times$ SSC (sodium saline citrate; 0.15 M NaCl, 0.015 M sodium citrate [pH 7.0]) from the midpoint of thermal denaturation as first described by Marmur and Doty (44) and reexamined by De Ley (11).

DNA-DNA hybridizations were performed by using the optical renaturation method of De Ley et al. (12). Prior to thermal denaturation in a boiling-water bath for 10 min, the DNA solutions were brought to a concentration of 0.054 mM in 0.1 $\times$ SSC. The reassociation reactions were performed in 2 $\times$ SSC by quickly cooling the DNA solutions to an optimal renaturation temperature (T_{OR}) of 76.9°C. This temperature was calculated from the mean mol% G+C of the *A. bestiarum*-like strains (see Table 2) by using the equation $T_{OR} = 0.51 \times (\text{mol\% G+C}) + 47.0$ (19). The degree of binding was calculated from the renaturation rates determined from the decrease in A_{260} over a period of 30 min according to De Ley et al. (12).

Ribosomal DNA gene restriction fragment analysis. For ribotyping purposes, strains were cultured overnight in Luria-Bertani broth, containing 1% (wt/vol) tryptone (Oxoid), 0.5% (wt/vol) yeast extract (Oxoid), and 1% (wt/vol) NaCl, at 28°C under continuous shaking. Total genomic DNA was extracted by using the standard miniprep procedure (6). Approximately 5 μ g of DNA was digested with the restriction endonuclease *Sma*I according to the manufacturer's instructions (Boehringer GmbH, Mannheim, Germany). Restriction fragments were separated in a 1.2% agarose gel submerged in TBE buffer (89 mM Tris, 89 mM boric acid, 0.2 mM EDTA [pH 8.0]), stained with ethidium bromide, and transferred to a nylon 66 membrane (BiodyneA; Pall Biosupport) according to the Southern blot procedure (59).

The construction of plasmid pGML1, which contains a 567-bp fragment of the *mbf* gene of *Escherichia coli*, has been described previously by Martinetti Lucchini and Altwegg (45). Purification of pGML1 DNA from *E. coli* was performed by cesium chloride-ethidium bromide gradient centrifugation, and the purified

plasmid DNA was labeled by nick translation with biotin-11-UTP (GIBCO-Bethesda Research Laboratories) (45). Nylon blots were hybridized with labelled plasmid pGML1 following standard protocols. The BluGene kit (GIBCO-Bethesda Research Laboratories) was used for visualizing the resulting hybrids according to the manufacturer's instructions. Reconstructed images of the visualized ribotyping band patterns in the low-molecular-weight zone (0.8 to 4.0 kb) were scanned with a Scan Jet 4c/T document scanner (Hewlett Packard). Further processing, calculation of the band-matching Dice coefficient, and cluster analysis by the unweighted pair-group method using arithmetic averages were carried out by using GelCompar software, version 3.1 (Applied Maths, Kortrijk, Belgium).

Gas-liquid chromatographic analysis of cellular FAMES. Strains were inoculated on TSA medium containing Bacto agar (Difco) instead of bacteriological agar no. 1 (Oxoid) according to the quadrant streak technique and were incubated for 48 h at 28°C. Cell harvesting, saponification of lipids, methylation of fatty acids, extraction of fatty acid methyl esters (FAMES), and washing of extracts were done as described in the standardized protocol for the Microbial Identification System (MIDI; Microbial ID Inc.). Extracts were analyzed using a gas chromatograph (model HP5890A, Hewlett-Packard) equipped with a flame ionization detector, an automatic sampler, an integrator, and a personal computer (52). Identification and quantification of individual FAME profiles and calculation of mean profiles were performed by using the Microbial Identification System software package, version 3.9.

RESULTS AND DISCUSSION

DNA relatedness. The DNA hybridization data presented in Table 2 demonstrate the existence of a clear gap between the internal relatedness of the *A. bestiarum*-like group and their relatedness to the type and reference strains of all known *Aeromonas* taxa. Based on DNA-DNA hybridization experiments with strains LMG 17541^T and LMG 17542, the first seven strains in Table 1 exhibited an intragroup relatedness ranging from 79 to 100% (Table 2). The eighth strain in Table 1, i.e., the intermediate isolate LMG 17548 (29), is discussed further. DNA hybridization of strains LMG 17541^T and LMG 17542 with the type and reference strains of the 13 species currently recognized in *Aeromonas* revealed that the two *A. bestiarum*-like isolates were 22 to 63% related to other *Aeromonas* spp. (Table 2). As would be expected from our previously reported AFLP data (29), high proportional degrees of DNA binding ranging from 51 to 63% were obtained with the type strain and two reference strains of the species *A. bestiarum*. Furthermore, Table 2 shows that strain LMG 2844^T of *A. hydrophila* HG1 (DNA binding, 50 to 60%) and strain LMG 3780^T of *A. salmonicida* HG3 (DNA binding, 39 to 55%) also displayed relatively high levels of genomic relatedness with strains LMG 17541^T and LMG 17542. The finding of such high levels with representatives of HG1 (phenotypically *A. hydrophila*) and HG3 (phenotypically *A. hydrophila* or *A. salmonicida*) was not very surprising keeping in mind that several workers previously have experienced major difficulties in separating HG2 (*A. bestiarum*) from *A. hydrophila* HG1 and HG3 solely on the basis of DNA-DNA hybridization experiments. For instance, Hänninen (20) reported levels of DNA relatedness between strains of HG2 and HG3 that were as high as 87% at an optimal reassociation temperature of 60°C, whereas Ali and coworkers (2) showed that the type strain of *A. bestiarum* was 78 (divergence in melting temperature [ΔT_m], 6.0°C) and 71% (ΔT_m , 3.3°C) related at 60°C with the type strain of HG1 and the reference strain of HG3, respectively. As for the latter study, it should be mentioned that ΔT_m values within *A. bestiarum* did not exceed 2.0°C (2). Unfortunately, the optical renaturation method used in the current DNA hybridization study does not allow the determination of ΔT_m values. In conjunction with the previous findings of Hänninen (20) and Ali et al. (2), however, the new DNA relatedness data shown in Table 2 clearly indicate that the first seven strains in Table 1 are sufficiently different from *A. bestiarum* and from all other

TABLE 2. Mol% G+C of *A. popoffii* and DNA relatedness of *A. popoffii* strains LMG 17541^T and LMG 17542 to other strains of *A. popoffii* and *Aeromonas* spp.

Strain ^a	% DNA binding with ^b :		mol% G+C
	LMG 17541 ^T	LMG 17542	
<i>A. popoffii</i>			
LMG 17541 ^T	100		57.7
LMG 17547	100	90	
LMG 17542	93	100	59.0
LMG 17546	87	89	58.6
LMG 17545	86	91	57.7
LMG 17544	83	80	59.6
LMG 17543	79	86	58.6
<i>Aeromonas</i> sp. LMG 17548	60	63	
<i>A. hydrophila</i> LMG 2844 ^T (=ATCC 7966 ^T)	50	60	
<i>A. bestiarum</i>			
LMG 13444 ^T (=ATCC 51108 ^T)	53	63	
LMG 13447 (=A 39)	60	60	
LMG 13448 (=A 169)	51	57	
<i>A. salmonicida</i> LMG 3780 ^T (=ATCC 33658 ^T) ^c	39	55	
<i>A. caviae</i> LMG 3775 ^T (=ATCC 15468 ^T)	39	54	
<i>A. media</i> LMG 9073 ^T (=ATCC 33907 ^T)	37	39	
<i>A. eucrenophila</i> LMG 3774 ^T (=NCMB 74 ^T)	42	41	
<i>A. sobria</i> LMG 3783 ^T (=CIP 7433 ^T)	40	46	
<i>A. veronii</i> LMG 9075 ^T (=ATCC 35604 ^T) ^c	45	43	
<i>A. jandaei</i> LMG 12221 ^T (=ATCC 49568 ^T)	22	29	
<i>A. encheleia</i> LMG 16330 ^T (=CECT 4342 ^T)	47	48	
<i>A. schubertii</i> LMG 9074 ^T (=ATCC 43700 ^T)	39	43	
<i>A. trota</i> LMG 12223 ^T (=ATCC 49657 ^T)	44	43	
<i>A. allosaccharophila</i> LMG 14059 ^T (=CECT 4199 ^T)	36	40	

^a A, Institute of Medical Microbiology, Zürich, Switzerland; ATCC, American Type Culture Collection, Rockville, Md.; CECT, Colección Española de Cultivos Tipo, Universidad de Valencia, Valencia, Spain; NCMB, National Collection of Marine Bacteria, Aberdeen, Scotland.

^b Most of the reported values are the means from at least two determinations.

^c In the current genotypic classification of the genus *Aeromonas*, the nonmotile psychrophilic species *A. salmonicida* resides in HG3 together with a group of motile, mesophilic *A. hydrophila*-like strains. However, both phenotypic groups behave identically in DNA hybridization (5). Similarly, the species *A. veronii* comprises two biogroups (i.e., *sobria* and *veronii*) that cannot be separated on the basis of DNA-DNA hybridization studies (5).

Aeromonas spp. to assign them to a new species, for which the name *Aeromonas popoffii* is proposed (see below).

Phenotypic differentiation of *A. popoffii* from other *Aeromonas* spp. The new mesophilic species can be distinguished from the psychrophilic fish pathogen *A. salmonicida* by its motility and its ability to grow in broth at 37°C (53). The differential test results of *A. popoffii* in relation to other mesophilic *Aeromonas* spp. reported in Table 3 were further confirmed for 16 other isolates of the *A. bestiarum*-like AFLP cluster (29) that can also be considered representative for the new species. Negative reactions in the tests for D-sucrose fermentation and lysine decarboxylation (LDC⁻) are both essential biochemical features for the differentiation of *A. popoffii* from the most closely related species, *A. hydrophila* (HG1 and HG3) and *A. bestiarum*; in addition, these two characteristics also allowed differentiation from the taxa *A. sobria* and *A. veronii* biogroups *sobria* and *veronii*. A third distinguishing feature for *A. bestiarum* was found in the assimilation of DL-lactate (Table 3). The species *A. caviae* and *A. media* can be separated phenotypically from *A. popoffii* by their lack of gas production from glucose (Gfg⁻) and their ability to ferment D-sucrose, whereas utilization of DL-lactate and acid production from salicin are useful

TABLE 3. Key tests for the phenotypic differentiation of *A. popoffii* from other mesophilic *Aeromonas* species^a

Species	Motility	Production of gas from glucose	Utilization of DL-lactate	Production of acid from:				Decarboxylation of:		Found in clinical specimens
				Salicin	D-Cellobiose	D-Mannitol	D-Sucrose	Ornithine	Lysine	
<i>A. popoffii</i> ^b	+	+	+	—	—	+	—	—	—	No
<i>A. hydrophila</i> ^c	+	v+	+ ^d	v+	v—	+	+	—	+	Yes
<i>A. bestiarum</i> ^e	+	v+	—	v+	v—	+	+	—	+	Yes
<i>A. caviae</i> ^f	v+	—	v+	+	v+	+	+	—	—	Yes
<i>A. media</i> ^g	—	—	+	v+	+	+	+	—	—	No
<i>A. eucrenophila</i> ^h	+	+	—	+	+	+	—	—	—	Yes
<i>A. sobria</i> ⁱ	+	v+	v+	—	+	+	+	—	+	No
<i>A. veronii</i> biogroup <i>sobria</i> ^j	+	v+	—	—	v+	+	+	—	+	Yes
<i>A. veronii</i> biogroup <i>veronii</i> ^k	v+	+	ND	+	v+	+	+	+	+	Yes
<i>A. jandaei</i> ^l	+	+	+	—	—	+	—	—	+	Yes
<i>A. encheleia</i> ^h	+	+	—	+	—	+	v—	—	—	Yes
<i>A. schubertii</i> ^m	+	—	v+	—	—	—	—	—	v+	Yes
<i>A. trota</i> ⁿ	+	v+	ND	—	+	+	v—	—	+	Yes
<i>A. allosaccharophila</i> ^o	+	+	ND	—	+	+	+	v—	+	Yes

^a +, ≥85% of the strains are positive; —, ≥85% of the strains are negative; v+, 50 to 84% of the strains are positive; v—, 50 to 84% of the strains are negative; ND, no data found.

^b Data from this study are based on results obtained with 23 strains, including the first 7 strains in Table 1, of the *A. bestiarum*-like AFLP cluster (29).

^c Data from references 1, 2, 4, 5, 10, 16, and 37.

^d The substrate DL-lactate is used by *A. hydrophila* HG1 but not by *A. hydrophila* HG3 (5).

^e Data from references 2, 4, and 5.

^f Data from references 1, 4, 5, 10, 16, and 37. The species *A. caviae* includes HG4, HG5A, and HG5B.

^g Data from references 3, 4, 5, and 10.

^h Data from references 17, 28, and 55.

ⁱ Data from references 1, 4, 10, and 37.

^j Data from references 1, 5, and 10.

^k Data from references 10, 23, and 37.

^l Data from references 1, 4, 7, 10, and 37.

^m Data from references 1, 4, 22, and 37.

ⁿ Data from references 4, 9, and 37.

^o Data from reference 46.

characteristics to discriminate between the new species and the nonpathogenic species *A. eucrenophila* and *A. encheleia*. The clinically relevant species *A. jandaei* and *A. schubertii* are both LDC⁺ and can also be differentiated from *A. popoffii* by their hemolytic activity (7). In addition, members of *A. schubertii* are Gfg[—] and fail to ferment D-mannitol. A positive result in the LDC test and the ability to produce acid from D-cellobiose allow the separation of *A. popoffii* from *A. allosaccharophila* and *A. trota*. The latter species can also be separated from *A. popoffii* by its susceptibility to ampicillin (9).

The antibiotic resistance patterns of *A. popoffii* and *A. bestiarum* were generally very similar (data not shown). In the species description of *A. popoffii*, results of susceptibility testing are reported for a selection of 20 frequently used antibiotics.

Ribotyping and fatty acid profiling of *A. popoffii*. Despite the availability of several discriminatory biochemical tests, strains of HG1, HG2, and HG3 (phenotype *A. hydrophila*) were often very difficult to separate in routine identification because of their close phenotypic relationship (1, 5, 20). In this context, the definition of *A. popoffii* as a new *Aeromonas* taxon that is closely related to these three HGs (Table 2) might give rise to additional problems concerning the correct identification of *Aeromonas* populations at the species level in ecotaxonomic surveys. In addition to the high discriminative power displayed by AFLP analysis (29), we also decided to evaluate two more frequently used fingerprinting methods as rapid and reliable tools for differentiating *A. popoffii* from its closest taxonomic neighbors.

A visual comparison of reconstructed ribotyping patterns clearly shows that the seven *A. popoffii* strains can be readily

distinguished from the type strains of *A. hydrophila* (HG1) and *A. bestiarum* (HG2) and the reference strain of *A. hydrophila* HG3 by their typical lack of bands in the molecular weight region between 0.8 and 1.6 kb (Fig. 1). Numerical analysis of the reconstructed images using a band-based similarity coefficient (Fig. 1) confirmed the status of *A. popoffii* as a separate taxon in relation to members of the former *A. hydrophila* complex and further indicated that *A. bestiarum* and *A. hydrophila*

FIG. 1. Clustering analysis (unweighted pair-group method using arithmetic averages) of reconstructed ribotyping patterns generated from seven *A. popoffii* strains, type and reference strains of *A. hydrophila* (HG1 and HG3, respectively), and the type strain of *A. bestiarum* (HG2). Similarities are expressed as the band-matching Dice coefficient (S_D). Positions of the low-molecular-weight markers are indicated by arrows.

TABLE 4. Major fatty acids of *A. popoffii*, *A. hydrophila* (HG1 and HG3), and *A. bestiarum* (HG2)

Fatty acid ^a	Mean % composition (SD) ^b			
	<i>A. popoffii</i> (n = 7)	<i>A. hydrophila</i> (HG1) (n = 5)	<i>A. bestiarum</i> (HG2) (n = 4)	<i>A. hydrophila</i> (HG3) (n = 5)
Straight				
12:0	6.41 (0.36)	6.65 (0.90)	7.10 (0.53)	7.29 (0.35)
14:0	2.49 (0.47)	4.38 (0.72)	3.19 (0.39)	1.68 (0.66)
15:0	1.02 (0.21)	1.80 (0.58)	1.48 (0.26)	1.03 (0.46)
16:0	17.2 (0.91)	17.9 (1.64)	15.5 (1.11)	19.4 (2.45)
17:1 ω 8 ^c	1.01 (0.28)	1.56 (0.63)	1.73 (0.40)	1.51 (0.63)
17:0	0.77 (0.30)	1.35 (0.63)	1.15 (0.29)	1.30 (0.55)
Branched				
13:0 iso	1.41 (0.15)	0.92 (0.24)	0.81 (0.16)	0.75 (0.23)
15:0 iso	0.94 (0.32)	1.44 (0.58)	1.02 (0.35)	tr
16:0 iso	tr	1.19 (0.77)	1.05 (0.71)	0.93 (0.39)
iso 17:1 ω 9 ^c	2.14 (1.00)	1.22 (0.44)	1.27 (0.29)	1.61 (0.57)
17:0 iso	3.06 (0.50)	1.39 (0.64)	1.73 (0.41)	3.68 (1.24)
17:0 10 methyl	0.85 (0.36)	1.42 (1.06)	1.49 (0.38)	tr
Branched hydroxy, 15:0 iso 3OH	3.47 (0.39)	1.56 (0.42)	2.43 (0.33)	2.42 (0.79)
Unknown 14.503 ^d	0.60 (0.08)	ND	ND	0.73 (0.12)
Summed feature ^e				
3	7.92 (0.54)	8.42 (1.67)	8.78 (1.54)	7.54 (0.35)
4	35.3 (2.34)	30.6 (1.74)	33.5 (1.59)	35.9 (3.53)
7	8.71 (1.37)	12.0 (1.46)	9.76 (0.78)	10.4 (2.46)
9	1.02 (0.39)	ND	ND	ND

^a The fatty acids 12:0 iso, unknown 11.798, 13:0, 12:0 3OH, 14:1 iso E, 14:0 iso, unknown 13.691, 14:0 iso 3OH, 15:0 3OH, 17:0 anteiso, 17:1 ω 6c, 18:0, and summed feature 2 had mean values of less than 0.6% and are therefore not listed.

^b Means (and standard deviations) were calculated regardless of the fact that a particular fatty acid was not produced by all strains within a taxon. *n*, number of strains included per taxon. In addition to the seven *A. popoffii* strains (Table 1), the following strains were also used for FAME analysis: LMG 2844^T (=ATCC 7966^T), LMG 13443, LMG 13440, LMG 13658, and LMG 13660 of *A. hydrophila* (HG1); LMG 13444^T (=ATCC 51108^T), LMG 13447, LMG 13448, and LMG 13446 of *A. bestiarum* (HG2); and LMG 13449, LMG 13450, LMG 13451, LMG 13453, and LMG 13675 of *A. hydrophila* (HG3). Detailed descriptions of these strains have been reported elsewhere (2, 32). ND, not detected; tr, trace amount (<0.6%).

^c For unsaturated fatty acids, the position of the double bond can be located by counting from the ω or methyl end of the carbon chain. *cis* isomers are indicated by the suffix *c*.

^d Unknown fatty acids have no name listed in the Peak Library File of the MIDI system and can therefore only be indicated by their equivalent chain length.

^e Summed features represent groups of two or three fatty acids which cannot be separated by gas-liquid chromatography with the MIDI system. Summed feature 3 contains 16:1 iso 1 and/or 14:0 3OH, summed feature 4 comprises 15:0 iso 2OH, 16:1 ω 7*t*, and/or 16:1 ω 7*c*, and summed feature 7 contains one or more of the following isomers: 18:1 ω 7*c*, 18:1 ω 9*t*, and/or 18:1 ω 12*r* (the suffix *t* indicates a *trans* isomer). Summed feature 9 represents unknown 18.846, unknown 18.858, and/or the cyclopropane fatty acid 19:0 cyclo ω 10*c*. In this context, it should be mentioned that cyclic fatty acids only rarely occur in *Aeromonas* (21, 32, 38).

HG3 were its closest taxonomic neighbors. Interestingly, Martinetti Lucchini and Altwegg (45) found that all 20 strains of HG2 and HG3 included in their ribotyping study displayed a major band in the zone from 0.8 to 1.6 kb. To a lesser extent, the presence or absence of the bands with an approximate molecular weight of 1.8 and 3.2 kb may also be considered as possible discriminating features for the new species.

Following gas-liquid chromatographic analysis of their FAMES, the mean cellular fatty acid composition of *A. popoffii* was compared with the mean profiles obtained with representative strains of the taxa HG1, HG2, and HG3. As shown in Table 4, only one minor compound was found to be of qualitative significance for the chemotaxonomic characterization of *A. popoffii*. Summed feature 9, a component that contains the cyclic fatty acid 19:0 cyclo ω 10*c* and/or one or both unknown fatty acids with equivalent chain lengths, 18.846 and 18.858 (Table 4), was produced by all seven strains of *A. popoffii* (range, 0.50 to 1.55%) but remained undetected in strains of the other three HGs examined. The fatty acids that were present in all four *Aeromonas* taxa generally showed very little quantitative variation. Repeated FAME analyses of three strains of each HG tested clearly confirmed that summed feature 9 should be regarded as the most significant diagnostic compound for the separation of *A. popoffii* from HG1, HG2, and HG3 by cellular fatty acid analysis.

Intermediate strain LMG 17548. In the course of a previous AFLP study (29), it was suggested that the drinking water isolate LMG 17548 might represent a genotypic intermediate between *A. bestiarum* and the HG2-like group now proposed as *A. popoffii*. This is now substantiated by new DNA hybridization data showing that strain LMG 17548 is 60 to 63% (Table 2) and 66 to 67% (data not shown) related to representatives of *A. popoffii* and *A. bestiarum*, respectively. Phenotypically, strain LMG 17548 could be easily excluded from *A. popoffii* (Table 3) by its ability to produce acid from D-sucrose and by its negative reaction in the DL-lactate assimilation test, whereas a positive result in the fermentation of D-sorbitol and the lack of gas production from glucose allowed the differentiation of this strain from *A. bestiarum* (2) (individual data not shown). In addition, the intermediate strain also exhibited an atypical ribotyping pattern (comprising a band of 1.4 kb) and fatty acid profile (no production of summed feature 9) (results not shown). The possibility that strain LMG 17548 might belong to one of the other existing *Aeromonas* species was ruled out by comparison of its AFLP fingerprint with the genotypic *Aeromonas* database AERO94 (29). In view of these findings, it can be expected that the accumulation of more atypical *A. bestiarum* and *A. popoffii* strains might stimulate further systematic research on the relative position of this isolate in the genus *Aeromonas*.

Description of *Aeromonas popoffii* sp. nov. We propose the name *Aeromonas popoffii* (po.pof' f.i. M. L. gen. n. *popoffii*, of Popoff) for the first seven strains in Table 1. The species is named after Michel Y. Popoff, a French microbiologist who has greatly contributed to our current knowledge of the phenotypic and genotypic relationships among the mesophilic members of the genus *Aeromonas* (53–55).

All seven strains of *A. popoffii* (Table 1) display the following characteristics typical for the genus *Aeromonas*: gram-negative straight motile rods, chemoorganotrophic with both oxidative and fermentative metabolism, cytochrome oxidase and catalase positive, reduction of nitrate to nitrite without the production of gas, and resistance to vibriostatic agent O/129. Optimal growth occurs after 24 h at 28°C on TSA medium, but all strains also grow at 37°C in broth. No brown water-soluble pigment is produced on TSA medium. Acid and gas are produced from D-glucose and glycerol. Growth occurs in KCN broth but not in the presence of 3, 6, 8, and 10% (wt/vol) NaCl. Arginine dihydrolase, DNase, indole (except strains LMG 17541^T and LMG 17546), and Voges-Proskauer positive. No production of urease, tryptophan deaminase, ornithine and lysine decarboxylase. Production of H₂S by four of the seven strains. Alkalinization of citrate (except strains LMG 17544 and LMG 17546) and malonate (except strain LMG 17544).

The following substrates are used by all seven *A. popoffii* strains as the sole carbon and energy source: *N*-acetyl-D-glucosamine, L-alanine (except strain LMG 17547), L-aspartate, citrate (except strains LMG 17543 and LMG 17544), D-fructose (except strains LMG 17541^T and LMG 17545), fumarate, D-galactose, D-gluconate, D-glucose, L-glutamate, L-glutamine, glycerol, L-histidine, DL-lactate (except strain LMG 17546), L-malate, D-mannitol, D-mannose, D-maltose, putrescine, pyruvate, D-ribose, succinate, L-serine, D-trehalose, and L-tyrosine. None of the strains use acetate, *cis*-aconitate, *trans*-aconitate, adipate, adonitol, β-alanine, 4-aminobutyrate, arbutin, L-arginine (except strains LMG 17544 and LMG 17546), azelate, D-cellobiose, citrullin, dulcitol, ethanol, erythritol, D-glucuronate, glutarate, glycine, 3-hydroxybenzoate, 4-hydroxybenzoate, DL-3-hydroxybutyrate, inositol, itaconate, lactose (except strain LMG 17542), L-leucine, maltitol, α-D-melibiose, mesaconate, L-ornithine, oxoglutarate, phenylacetate, L-phenylalanine, L-proline, propionate, D-raffinose, L-rhamnose, salicin, D-sorbitol, suberate, D-sucrose, L-tryptophan, and D-xylose.

Acid is uniformly produced from D-galactose, D-maltose (except strain LMG 17546), D-mannitol, D-mannose, methyl-D-glucoside, and D-trehalose but not from adonitol, D-arabitol, D-cellobiose, dulcitol, erythritol, inositol, lactose, α-D-melibiose, D-raffinose, L-rhamnose, salicin, D-sorbitol, D-sucrose, and D-xylose.

All *A. popoffii* strains hydrolyze the following substrates: L-alanine-*p*NA, casein, 2-deoxythymidine-5'-*p*NP-phosphate, gelatin, lecithin, bis-*p*NP-phosphate, *ortho*-nitrophenyl-β-D-galactopyranoside, *p*NP-phenylphosphonate, *p*NP-phosphoryl choline, L-proline-*p*NA, starch, and Tween 80. None of the strains are able to hydrolyze chitin, esculin, L-glutamate-γ-3-carboxy-*p*NA, *p*NP-α-D-glucopyranoside, *p*NP-β-D-glucuronide, and *p*NP-β-D-xyloside.

All *A. popoffii* strains listed in Table 1 are resistant to amoxicillin, ampicillin, clarithromycin, oxacillin, penicillin G, teicoplanin, and vancomycin but are sensitive to amikacin, cefotaxim, ceftazidim, chloramphenicol, gentamicin, kanamycin, mezlocillin, netilmicin, piperacillin, spectinomycin, tetracycline, tobramycin, and trimethoprim according to the interpretive standards provided by the National Committee for Clinical Laboratory Standards (48, 49).

The G+C content ranges from 57.7 to 59.6 mol% (Table 2).

Isolated from drinking water production plants and reservoirs (Table 1). One isolate (strain LMG 17544) displayed weak hemolytic activity after 7 days of incubation.

The type strain is strain LMG 17541; it has been deposited in the BCCM/LMG Culture Collection of the Laboratorium voor Microbiologie, Universiteit Gent, Ghent, Belgium. The G+C content of this strain is 57.7 mol%.

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