Inclusion of *Aeromonas* DNA Hybridization Group 11 in *Aeromonas encheleia* and Extended Descriptions of the Species *Aeromonas eucrenophila* and *A. encheleia*

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The recently reported chemotaxonomic and genotypic description of two well-separated subgroups (I and II) in *Aeromonas eucrenophila* and their affiliation to *Aeromonas encheleia* and the unnamed *Aeromonas* DNA hybridization group (HG) 11 (G. Huys, M. Altwegg, M.-L. Hänninen, M. Vancanneyt, L. Vauterin, R. Coopman, U. Torck, J. Lüthy-Hottenstein, P. Janssen, and K. Kersters, *Syst. Appl. Microbiol.* 19:616–623, 1996) has questioned the original species descriptions of *A. eucrenophila* and *A. encheleia*. In order to elucidate the unclear taxonomic status of these taxa in the genus *Aeromonas*, we have further investigated a collection of 14 reference strains and 14 related isolates encompassing the taxa *A. eucrenophila* subgroups I and II, *A. encheleia*, and HG11 by DNA-DNA hybridization (on 17 of the 28 strains) and phenotypic characterization (on all 28 strains). Genotypically, the investigated strains could be grouped into two DNA hybridization groups that exhibited between-group homologies ranging from 42 to 52%. The members of DNA homology group I (DNA binding, 76 to 100%) were strains of *A. eucrenophila* subgroup I, including the type strain LMG 3774, and two *A. eucrenophila*-like isolates, leading to the conclusion that these strains should be considered true representatives of the species *A. eucrenophila*. The strains of *A. eucrenophila* subgroup II, HG11, and *A. encheleia*, on the other hand, were closely joined in DNA homology group II (DNA binding, 74 to 105%) together with two presumptive *A. encheleia* isolates. The fact that strain LMG 16330^T of *A. encheleia* was the only type strain residing in DNA homology group II implies that HG11 and *A. eucrenophila* subgroup II should be classified in the species *A. encheleia*. Except for the somewhat aberrant phenotypic positions of HG11 strains LMG 13075 and LMG 13076, the establishment of DNA homology groups I and II was supported by the delineation of phena 1 and 2 (level of correlation, 90%), respectively, as revealed by numerical analysis of 136 phenotypic test results. These data indicate that *A. eucrenophila* and *A. encheleia* are phenotypically highly related but can be easily separated by testing the production of acid from D-cellobiose and lactose and the assimilation of D-cellobiose. Extended descriptions of both species are given.

The taxonomy of the genus *Aeromonas* has, since its description by Popoff in *Bergey's Manual of Systematic Bacteriology* (33), undergone a large number of structural and nomenclatural amendments. In spite of these changes, the current classification of aeromonads (8) still fails to deal with the striking lack of congruence between groups delineated on the basis of phenotypic characteristics and groups delineated on the basis of DNA-DNA hybridizations. As a result, new *Aeromonas* isolates are now being identified at two different levels, i.e., phenospecies and genomospecies or DNA hybridization groups (HG), respectively, depending on the technique and the collection of reference strains used.

For many years, the confounding *Aeromonas* taxonomy harbored two unnamed HGs, i.e., HG2 and HG11. Despite the significant amount of taxonomic evidence demonstrating that these taxa constituted two homogeneous DNA hybridization groups, the nomenclatural recognition of HG2 and HG11 as new *Aeromonas* species could not be justified for a long time due to the lack of stable phenotypic markers that would have distinguished them from their respective taxonomic neighbors (4, 23). Only recently, Ali et al. (2) managed to shed more light

on the dubious status of HG2 as a new species and proposed to name it *Aeromonas bestiarum*. In a recent study by us (22), new insights were also reported on the relative taxonomic position of *Aeromonas* HG11. Formerly, this taxon was often referred to as *Aeromonas veronii*-like because one of the two representative HG11 strains (i.e., LMG 13075) produced ornithine decarboxylase, a typical biochemical feature of the species *A. veronii* (20) (now *A. veronii* biogroup *veronii*). From our own findings (22), however, we previously concluded from new genotypic and chemotaxonomic evidence that the two HG11 reference strains were highly related to two other *Aeromonas* taxa, namely *Aeromonas encheleia* and *Aeromonas eucrenophila* subgroup II. The delineation of the latter group was derived from the recent finding that the species *A. eucrenophila*, as represented by the eight original reference strains, does not constitute a homogeneous *Aeromonas* taxon as previously reported by Schubert and Hegazi (35), but instead encompasses two discrete subgroups (I and II) which can be easily separated from each other by AFLP analysis, ribotyping, electrophoretic fingerprinting of whole-cell proteins, and cellular fatty acid analysis (22). Likewise, the data presented in the recent description of *A. encheleia* (16) demonstrated that this species constitutes a phenotypically and genotypically homogeneous taxon. This study (16) also showed that all of the examined *A. encheleia* strains exhibited only a very limited degree of DNA relatedness with HG11 strain LMG 13075.

In view of the inconsistencies observed between our recent

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TABLE 1. Description of the *A. eucrenophila* and *A. encheleia* strains used

Species ^a	Strain ^b	Designation as received ^{c,d} (other designation)	Former classification (reference[s])	Source of isolation (geographical origin)
<i>A. eucrenophila</i>	LMG 3774 ^T	NCMB 74 ^{Tc} (A 311 ^T , CCUG 30340 ^{Tf})	<i>A. eucrenophila</i> subgroup I (22)	Ascites of carp
	LMG 13057	A 914 (Popoff 546 ^c)	<i>A. eucrenophila</i> subgroup I (22)	Ascites of carp
	LMG 13058	A 1650 (NCMB 73 ^c , CCUG 30342 ^f)	<i>A. eucrenophila</i> subgroup I (22)	Ascites of carp
	LMG 13060	A 1652 (37/20b ^c , CCUG 30344 ^f)	<i>A. eucrenophila</i> subgroup I (22)	Rural well (Germany)
	LMG 13687	A 1651 (K 2232/1 ^c , CCUG 30343 ^f)	<i>A. eucrenophila</i> subgroup I (22)	Urban well (Germany)
	LMG 16179	A 1763	<i>A. eucrenophila</i> -like (22)	Human wound (Switzerland)
	LMG 17059	IK-O-c-4-2	<i>A. eucrenophila</i> -like (22)	Drinking water plant (Flanders, Belgium)
	LMG 17061 ^g	IK-O-c-10-3	<i>A. eucrenophila</i> -like (22)	Drinking water plant (Flanders, Belgium)
<i>A. encheleia</i>	LMG 17062 ^g	IK-O-c-11-5	<i>A. eucrenophila</i> -like (22)	Drinking water plant (Flanders, Belgium)
	LMG 13061	A 1653 (BPE 113 ^c , CCUG 30345 ^f)	<i>A. eucrenophila</i> subgroup II (22)	Artesian well (United Kingdom)
	LMG 13062	A 1654 (1/II ^c , CCUG 30346 ^f)	<i>A. eucrenophila</i> subgroup II (22)	Infiltration well (Germany)
	LMG 13075	A 902 ^f (ATCC 35941 ^f , CCUG 30364 ^f)	<i>Aeromonas</i> HG11 (20, 25)	Ankle fracture (New Zealand)
	LMG 13076	A 926 ^f (CDC 3136-78, CCUG 30365 ^f)	<i>Aeromonas</i> HG11 (25)	Water (Mohawk river, New York)
	LMG 13691	A 1655 (23/II ^c , CCUG 30347 ^f)	<i>A. eucrenophila</i> subgroup II (22)	Infiltration well (Germany)
	LMG 16328	CECT 4340 ^f (S 177)	<i>A. encheleia</i> (16)	European eel (Valencia, Spain)
	LMG 16329	CECT 4341 ^f (S 176)	<i>A. encheleia</i> (16)	European eel (Valencia, Spain)
	LMG 16330 ^T	CECT 4342 ^{Tf} (S 181 ^T)	<i>A. encheleia</i> (16)	European eel (Valencia, Spain)
	LMG 16331	CECT 4343 ^f (S 191)	<i>A. encheleia</i> (16)	European eel (Valencia, Spain)
	LMG 16398 ^g	A 9 (NE 59)	<i>A. encheleia</i> -like (22)	Hospital environment (Germany)
	LMG 16402 ^g	KV 21 (A 1782)	<i>A. encheleia</i> -like (22)	Drinking water well (Finland)
	LMG 16403 ^g	KV 42 (A 1783)	<i>A. encheleia</i> -like (22)	Drinking water well (Finland)
	LMG 16405	KV 78 (A 1785)	<i>A. encheleia</i> -like (22)	Drinking water well (Finland)
	LMG 16406 ^g	KV 88 (A 1786)	<i>A. encheleia</i> -like (22)	Drinking water well (Finland)
	LMG 17058 ^g	IK-K-r-5-1	<i>A. encheleia</i> -like (22)	Drinking water plant (Flanders, Belgium)
	LMG 17060 ^g	IK-O-c-10-2	<i>A. encheleia</i> -like (22)	Drinking water plant (Flanders, Belgium)
	LMG 17063 ^g	AG-5	<i>A. encheleia</i> -like (22)	Drinking water supply (Scotland, United Kingdom)
	LMG 17064 ^g	AG-6	<i>A. encheleia</i> -like (22)	Drinking water supply (Scotland, United Kingdom)
	LMG 17065	AG-11	<i>A. encheleia</i> -like (22)	Drinking water supply (Scotland, United Kingdom)

^a Species as redefined on the basis of the data reported in this study.

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

^c DNA-DNA hybridizations and phenotypic characterizations were performed using subcultures of the strains as originally received.

^d A, Institute of Medical Microbiology, Zürich, Switzerland; AG, School of Applied Sciences, Faculty of Science and Technology, Robert Gordon University, Aberdeen, Scotland, United Kingdom; ATCC, American Type Culture Collection, Rockville, Md.; CDC, Centers for Disease Control, Atlanta, Ga.; CECT, Colección Española de Cultivos Tipo, Universitat de València, Valencia, Spain; CCUG, Culture Collection University of Göteborg, Göteborg, Sweden; IK, Laboratorium voor Microbiële Ecologie, Universiteit Gent, Ghent, Belgium; KV, Department of Food and Environmental Hygiene, Faculty of Veterinary Medicine, University of Helsinki, Helsinki, Finland; NCMB, National Collection of Marine Bacteria, Aberdeen, Scotland, United Kingdom; NE, Institute of Hygiene, University of Heidelberg, Heidelberg, Germany; S, Departamento de Microbiología y Ecología, Universitat de València, Valencia, Spain.

^e Original designation of the eight *A. eucrenophila* reference strains as described by Schubert and Hegazi (35).

^f Duplicate strains included in an additional AFLP fingerprinting study (see Discussion) to verify the authenticity of the originally received type and reference strains.

^g Strains were not included in the DNA-DNA hybridization study. Their respective taxonomic assignments were based on the results of polyphasic fingerprinting (22) and phenotypic characterization (Fig. 1).

findings (22) and previous species descriptions (16, 35), it is clear that the exact positioning of HG11 in the genus *Aeromonas* cannot be conclusive without a thorough analysis of the taxonomic relationships among *A. eucrenophila* subgroup II, *A. encheleia*, and HG11. In this study, we continued our previous work (22) by performing DNA-DNA hybridizations and phenotypic screening on a representative set of type and reference strains encompassing the taxa *A. eucrenophila*, *A. encheleia*, and HG11. The taxonomic consequences of our findings have prompted us to propose *Aeromonas* genomospecies 11 to be included in the species *A. encheleia* and to extend the current descriptions of the species *A. eucrenophila* and *A. encheleia*.

MATERIALS AND METHODS

Strains. All *A. eucrenophila* and *A. encheleia* strains that were used in this study are listed in Table 1. These strains were either obtained from the Culture Collection of the Laboratorium voor Microbiologie Gent, Ghent, Belgium, or were kindly donated by H. K. Geiss (Institute of Hygiene, University of Heidelberg, Heidelberg, Germany), M.-L. Hänninen (Department of Food and Environmental Hygiene, Faculty of Veterinary Medicine, University of Helsinki, Helsinki, Finland), I. Kersters (Laboratorium voor Microbiële Ecologie, Univer-

siteit Gent, Ghent, Belgium), or A. Lamb (School of Applied Sciences, Faculty of Science and Technology, Robert Gordon University, Aberdeen, Scotland, United Kingdom). In addition, the type strains of *Aeromonas hydrophila* (LMG 2844), *Aeromonas caviae* (LMG 3775), and *Aeromonas sobria* (LMG 3783) were included as references in the DNA-DNA hybridizations. Unless otherwise stated, all strains were cultured aerobically on Trypticase soy agar (TSA) containing 3% (wt/vol) Trypticase soy broth (TSB; BBL) and 1.5% (wt/vol) bacteriological agar no. 1 (Oxoid) at 28°C for 24 h.

Physiological and biochemical characterization. Cell shape and Gram-staining characteristics of the 28 strains described in Table 1 were determined with cultures grown overnight on TSA medium (36). The oxidation-fermentation test was performed in O/F basal medium supplemented with 1% (wt/vol) glucose as described by Hugh and Leifson (21). Production of a brown diffusible pigment was determined after 7 days on TSA medium.

All strains in Table 1 were further tested for 136 physiological and biochemical properties. Indole and hydrogen sulphide production; esculin hydrolysis; tryptophan deaminase, arginine dihydrolase, lysine and ornithine decarboxylase, urease, citrate and malonate alkalization; hydrolysis of *ortho*-nitrophenyl- β -D-galactopyranoside, *p*-nitrophenyl (*p*NP)- β -D-glucuronide, and *p*NP- β -D-xyloside; and acid production from adonitol, D-glucose, inositol, L-rhamnose, D-sorbitol, D-sucrose, and D-xylose were tested with the Microaut-E System (Merlin Diagnostica, Bornheim-Hersel, Germany), formerly the Titertek-Enterobac-Automated System (24). Kovacs cytochrome oxidase was tested by using commercially available test strips (Merck, Darmstadt, Germany). The following tests were performed as described previously (36): catalase activity (method 1), casein hydrolysis, gas production from D-glucose and glycerol, gelatinase activity (meth-

FIG. 1. Phenogram showing the delineation of phenon 1 and 2 (delineation level, 91%, as indicated by the arrow) from numerical analysis of 136 phenotypic test results by using the simple matching coefficient (S_{SM}) and UPGMA. *A. ench.*, *A. encheleia*; *A. eucr.*, *A. eucrophila*; *A. sp.*, *Aeromonas* sp.

od 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 0/129, nitrate and nitrite reduction, and the Voges-Proskauer reaction. Hydrolysis of chitin was performed according to the method of Lingappa and Lockwood (26). Growth in KCN medium was performed as described by Edwards and Fife (13), 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) TSB. The following tests were performed as described previously (25): 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-ribose, 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, α -D-melibiose, methyl-D-glucoside, raffinose, salicin, and 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-phenyl-phosphonate, 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. The test results were scored as plus (1) or minus (0) and clustered by the unweighted pair group method with arithmetic averages (UPGMA) with the simple-matching coefficient (S_{SM}) and the Jaccard coefficient (S_J) (37).

Antibiotic susceptibility testing. Susceptibilities to 72 different antibiotics were tested in 96-well microplates by using the Micronaut-S kit (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.

DNA extraction. Chromosomal DNA of high molecular weight was isolated according to the method of Marmur (27).

Determination of DNA base compositions. The moles percent guanine-plus-cytosine 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 (28) and reexamined by De Ley (11).

DNA-DNA hybridization experiments. DNA-DNA hybridizations were performed by using the optical renaturation method of De Ley et al. (12). Prior to thermal denaturation, the concentrations of the DNA solutions were brought to 0.057 mM in 0.1 $\times$ SSC. Hybridization experiments were performed in 2 $\times$ SSC at an optimal renaturation temperature of 78.3°C (17).

RESULTS

Numerical analysis of phenotypic data. Because cluster analyses using the two different coefficients S_{SM} and S_J led to essentially the same grouping, only results of the UPGMA- S_{SM} analysis are described in detail. Clustering analysis of the data obtained from 136 physiological and biochemical tests resulted in the delineation of two phenon at a similarity level of 91% (Fig. 1). Phenon 1 contained the five strains previously assigned to *A. eucrophila* subgroup I together with four *A. eucrophila*-like isolates, whereas phenon 2 comprised the four original reference strains of *A. encheleia* joined by the three representatives of *A. eucrophila* subgroup II and 10 *A. encheleia*-like isolates (Table 1). Strains LMG 13075 and LMG 13076 of HG11 were closely related to phenon 1 and 2, respectively, but nevertheless remained unclustered (Fig. 1). As shown in Table 2, 3 of the 136 characters examined were useful for the differentiation of both phenon. The separation of phenon 1 from phenon 2 strongly relied on the inability of the latter taxon to produce acid from lactose and D-cellobiose and to utilize D-cellobiose. Interestingly, the HG11 strains LMG 13075 and LMG 13076 also showed negative reactions for these tests. However, it was found that both strains behaved somewhat atypically in a number of other tests (Table 2). The separation of phenon 1 and 2 from the previously described *Aeromonas* species (Table 3) is discussed below.

Antibiotic susceptibility testing. On the whole, the antibiotic resistance patterns of *A. eucrophila* and *A. encheleia*, as defined in Table 1, were highly similar (data not shown). For a

TABLE 2. Typical phenotypic characteristics of *A. eucrophila* and *A. encheleia*^a

Characteristic	<i>A. eucrophila</i> (% positive)	<i>A. encheleia</i> (% positive)		
		Phenon 2	LMG 13075	LMG 13076
Acid and gas from D-glucose	89	100	+ ^b	—
Arginine dihydrolase	100	100	—	+
Lysine decarboxylase	0	0	+	—
Ornithine decarboxylase	0	0	+	—
Utilization of:				
D-Cellobiose	100	0	—	—
Glutarate	0	0	+	—
3-Hydroxybenzoate	0	0	—	+
Inositol	0	0	+	—
Acid from:				
D-Cellobiose	100	0	—	—
Glycerol	100	100	—	—
Lactose	100	0	—	—
Hydrolysis of:				
Casein	100	100	+	—
Lecithin	100	100	+	—

^a Phenotypic grouping as shown in Fig. 1. All strains of *A. eucrophila* are classified as phenon 1. The results shown are for 9 strains in phenon 1 and 17 strains in phenon 2.

^b +, presence of characteristic; —, absence of characteristic.

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

Species	Motility	Gas from glucose	Utilization of DL-lactate	Acid from salicin	Decarboxylation of:	
					Ornithine	Lysine
<i>A. encheleia</i> ^b	+	+	—	+	—	—
<i>A. eucrenophila</i> ^b	+	+	—	+	—	—
<i>A. hydrophila</i> ^c	+	v+	+ ^d	v+	—	+
<i>A. bestiarum</i> ^e	+	v+	—	v+	—	+
<i>A. caviae</i> ^f	v+	—	v+	+	—	—
<i>A. media</i> ^g	—	—	+	v+	—	—
<i>A. sobria</i> ^h	+	v+	v+	—	—	+
<i>A. veronii</i> biogroup <i>sobria</i> ⁱ	+	v+	—	—	—	+
<i>A. veronii</i> biogroup <i>veronii</i> ^j	v+	+	ND	+	+	+
<i>A. jandaei</i> ^k	+	+	+	—	—	+
<i>A. schubertii</i> ^l	+	—	v+	—	—	v+
<i>A. trota</i> ^m	+	v+	ND	—	—	+
<i>A. allosaccharophila</i> ⁿ	+	+	ND	—	v—	+

^a +, ≥90% of the strains are positive; —, ≥90% of the strains are negative; v+, 50 to 90% of the strains are positive; v—, 50 to 90% of the strains are negative; ND, no data found.

^b Data from this study.

^c Data from references 1, 2, 5, 6, 10, 14, and 25.

^d The substrate DL-lactate is used by *A. hydrophila* HG1 but not by *A. hydrophila* HG3. The latter taxon can be further differentiated from *A. encheleia* and *A. eucrenophila* by testing the acid formation from D-sorbitol (6).

^e Data from references 2, 5, and 6.

^f Data from references 1, 5, 6, 10, 14, and 25. The species *A. caviae* includes HG4 and HG5A/B.

^g Data from references 3, 5, 6, and 10.

^h Data from references 1, 5, 10, and 25.

ⁱ Data from references 1, 6, and 10.

^j Data from references 10, 20, and 25.

^k Data from references 1, 5, 7, 10, and 25.

^l Data from references 1, 5, 19, and 25.

^m Data from references 5, 9, and 25.

ⁿ Data from reference 29.

selection of 20 relevant antibiotics, results of the susceptibility testing have been reported in the extended descriptions of both species (see Discussion).

DNA-DNA hybridization. Of the 28 strains described in Table 1, a total of 17 strains were selected for the DNA homology study. Hybridization of these strains with the type strains of *A. eucrenophila* (LMG 3774) and *A. encheleia* (LMG 16330) resulted in the delineation of two DNA homology groups (I and II), a finding that was readily confirmed by additional within-group and between-group hybridizations (Table 4). DNA homology group I consisted of four representatives of *A. eucrenophila* subgroup I, including the type strain LMG 3774, and two *A. eucrenophila*-like strains, LMG 16179 and LMG 17059. In DNA homology group II, the four original reference strains of *A. encheleia* were joined by the three representatives of *A. eucrenophila* subgroup II, strains LMG 13075 and LMG 13076 of HG11, and two *A. encheleia*-like isolates (LMG 16405 and LMG 17065). The degrees of DNA binding in DNA homology group I ranged from 76 to 100%, whereas the strains in DNA homology group II displayed 74 to 105% genomic relatedness with each other. Between both groups, levels of DNA homology ranged from 42 to 52% (Table 4). For the 57 reported percentages of DNA binding (Table 4), which were each determined from at least two experimental values, standard deviations ranged from 0.0 to 6.5% with a mean standard deviation of 2.7%.

DNA base compositions. The G+C ratios, determined from the midpoint of thermal denaturation in 1× SSC (Table 4), ranged from 58.8 to 60.7 mol% for DNA homology group I (*A. eucrenophila*) and from 59.2 to 61.7 mol% for DNA homology group II (*A. encheleia*). From duplicate experiments using strains LMG 16405, LMG 16330^T, and LMG 16331, we determined that the standard deviations did not exceed 0.2 mol%.

DISCUSSION

On the basis of the new phenotypic and DNA relatedness data reported in this study, it is clear that part of the current *Aeromonas* taxonomy has to be revised. In what follows, new findings are analyzed and evaluated with respect to previously published data, ultimately leading to extended descriptions of the species *A. eucrenophila* and *A. encheleia*.

As illustrated in Table 4, it was found that the genomic relatedness between members of *A. eucrenophila* subgroup I (residing in DNA homology group I) and *A. eucrenophila* subgroup II (residing in DNA homology group II) did not exceed 52%, whereas the DNA relatedness determined within both subgroups was at least 74%. As mentioned before, these new results are not in line with the current genotypic classification of the genus *Aeromonas*, in which members of the species *A. eucrenophila* are allocated to one single DNA hybridization group, i.e., HG6 (8). Likewise, the finding that the two strains of HG11 were closely linked to representative strains of *A. eucrenophila* subgroup II and of *A. encheleia* in DNA homology group II questions the widely accepted notion that HG11 represents a new but currently unnamed *Aeromonas* species (4). The fact that the type strains of the species *A. hydrophila*, *A. caviae*, and *A. sobria* displayed DNA binding degrees as high as 44 and 41% (Table 4) with the type strains of *A. eucrenophila* (LMG 3774) and *A. encheleia* (LMG 16330), respectively, suggests that DNA homology groups I and II deserve the status of separate species rather than subspecies rank.

In comparison with data in the literature, it is clear that the DNA hybridization results presented in this study (Table 4) do not corroborate the levels of DNA relatedness originally reported by other workers. Schubert and Hegazi (35) demonstrated that the eight strains constituting *A. eucrenophila* (Table 1) exhibited 80 to 100% genomic relatedness with the type

TABLE 4. Results of DNA-DNA hybridizations and determination of DNA base compositions in the *A. eucrenophila*-*A. encheleia*-HG11 complex

Taxon	Strain	G+C (mol%)	% DNA homology by cross-hybridization ^a																
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
DNA homology group I (<i>A. eucrenophila</i>)	1. LMG 3774 ^T	59.1	100																
	2. LMG 13058	59.2	97	100															
	3. LMG 17059		89		100														
	4. LMG 13060	60.7	87	81	85	100													
	5. LMG 16179	58.8	77				100												
	6. LMG 13687	58.8	76			84		100											
DNA homology group II (<i>A. encheleia</i>)	7. LMG 16330 ^T	59.2	46					42	100										
	8. LMG 16329	60.9	52			52			105	100									
	9. LMG 16331	61.2	45	48					105		100								
	10. LMG 13061	60.3	50	49				48	95			100							
	11. LMG 13062	60.1	46						94	85	82	87	100						
	12. LMG 16405	60.8	45		46				89		88			100					
	13. LMG 13076	61.3	51						87	98					100				
	14. LMG 13075	61.0	52						84			96		90	94	100			
	15. LMG 17065	61.7	48			45			83								100		
	16. LMG 16328	61.1	52					42	82	87			89			83	90	100	
	17. LMG 13691	61.2	50									74		87	83				100
<i>A. hydrophila</i>	18. LMG 2844 ^T	41							41										
<i>A. caviae</i>	19. LMG 3775 ^T	44							36										
<i>A. sobria</i>	20. LMG 3783 ^T	33							37										

^a Data are means of at least two determinations.

strain LMG 3774, whereas Esteve et al. (16) showed that the DNA homologies among representatives of *A. encheleia* (i.e., strains LMG 16329 and LMG 16330^T) and HG11 strain LMG 13075 were not higher than 37%. To decide about the taxonomical impact of the present investigation, it seems appropriate to evaluate the general approach followed throughout the different parallel studies involved rather than to speculate on the possible cause of the observed discrepancies in the DNA hybridization results. The reports of Schubert and Hegazi (35) and Esteve et al. (16) differ essentially from the present investigation in two ways. First, our approach was not limited to DNA hybridizations that solely included the type strains and one or two reference strains but also concentrated on cross-hybridization experiments using DNAs of representative strains other than strains LMG 3774^T, LMG 13075, LMG 16329, and LMG 16330^T. As discussed before (15), the inclusion of additional reference strains and related isolates is highly warranted especially when the type strain of a species does not seem to be representative for all other members of that species, which is apparently the case for *A. eucrenophila* as proposed by Schubert and Hegazi (35). Moreover, we also checked the authenticity of most type and reference strains used in this study by comparing their DNA fingerprints with those of duplicate cultures obtained from various international culture collections (Table 1). This comparative survey was performed by the high-resolution genomic fingerprinting technique AFLP with identical protocols as those described previously (22) and clearly showed that different subcultures of the original *A. eucrenophila* strains, the *A. encheleia* strains, and the two HG11 reference strains displayed highly similar if not identical band patterns within the same strain (results not shown). In our opinion, these findings provide sufficient evidence to rule out the possibility that deviating or wrongly labeled strains were used in the current study. Second, the phenotypic (see below) and genomic data reported in this

paper are fully supported by a polyphasic framework of chemotaxonomic and genotypic fingerprinting results (22), which essentially means that the new taxonomic findings are now confirmed by six different methods. Therefore, this study again emphasizes the usefulness of rapid and powerful screening techniques in addition to conventional DNA-DNA hybridization and phenotypic characterization in polyphasic taxonomy (38). In summary, we believe that the DNA hybridization data presented in the current study should be regarded as the taxonomic basis to extend the species descriptions of *A. eucrenophila* and *A. encheleia*. As such, the recovery of the *A. eucrenophila* type strain LMG 3774 in DNA homology group I implies that the five members of *A. eucrenophila* subgroup I together with four isolates previously identified as *A. eucrenophila*-like (Table 1) constitute the genotypical core of the species *A. eucrenophila*. Similarly, the finding that the type strain LMG 16330 of *A. encheleia* belonged in DNA homology group II justifies the classification of *A. eucrenophila* subgroup II, *Aeromonas* HG11, and 10 *A. encheleia*-like isolates (Table 1) in the species *A. encheleia*.

The species *A. eucrenophila* and *A. encheleia*, as delineated by the DNA hybridization data shown in Table 1, were shown to correspond to phenon 1 and to phenon 2 (plus strains LMG 13075 and LMG 13076), respectively, in numerical analysis of 136 phenotypic characteristics (Fig. 1). The tests for acid production from D-cellobiose and lactose and for the assimilation of D-cellobiose constitute the typical biochemical profile that allowed differentiation of members of phenon 2 from phenon 1 (Table 2). In this context, it should be mentioned that the majority of the phenotypic test results previously reported by Esteve et al. (16) were confirmed in the current study. A few test results were not confirmed (e.g., use of L-arginine, D-gluconate, and L-glutamine), which is probably due to the different methods used. Surprisingly, HG11 strains LMG 13075 and LMG 13076 were not recovered in phenon 2 (Fig. 1),

although both strains also uniformly displayed negative reactions in the three key tests mentioned above. In agreement with our phenotypic results (Table 2), Noterdaeme et al. (32) also recently determined that strain ATCC 35941 (= LMG 13075) was ornithine decarboxylase positive, arginine dihydrolase negative, and did not produce acid from lactose, D-cellobiose, and glycerol. The reported levels of DNA relatedness (Table 4, 84 to 96%), on the other hand, leave no doubt about the taxonomic allocation of strain LMG 13075 to the species *A. encheleia*. Therefore, whether the atypical reactions of strain LMG 13075 in six phenotypic tests (Table 2) should be regarded strain-specific features remains open for discussion. Likewise, the fact that the position of HG11 strain LMG 13076 was somewhat deviating from phenon 2 (Fig. 1) can be explained by five atypical test results (Table 2). Most likely, it is this rather aberrant biochemical behavior of strains LMG 13075 and LMG 13076 that has caused a long-time confusion concerning the precise taxonomic position of HG11 in the genus *Aeromonas*.

Phenotypic differentiation of *A. eucrenophila* and *A. encheleia* from other *Aeromonas* species. The differentiation of *A. eucrenophila* and *A. encheleia* from the sole psychrophilic *Aeromonas* species, i.e., *Aeromonas salmonicida*, is based on the inability of the latter taxon to grow in broth at 37°C and on its nonmotile character (33). By further comparing our test results with the phenotypic data available from the literature, a total of six key tests were found for the phenotypic separation of *A. eucrenophila* and *A. encheleia* from previously described mesophilic *Aeromonas* species (Table 3). Both species can be easily differentiated from all other mesophilic members of the genus *Aeromonas* except *A. caviae* and *Aeromonas media* by their lack of lysine decarboxylase activity. The phenotypic separation of *A. eucrenophila* and *A. encheleia* from the latter two species relies on differences in the abilities to produce gas from D-glucose (aerogenicity) and to utilize DL-lactate as the sole energy and carbon source (Table 3). In addition, *A. media* can be readily distinguished from other mesophilic aeromonads by its lack of motility (3). Also, the assimilation of DL-lactate by *A. hydrophila* HG1 (but not by *A. hydrophila* HG3) (Table 3) and *Aeromonas jandaei* and the anaerogenic character of *Aeromonas schubertii* can be used as additional key tests to differentiate these species from *A. eucrenophila* and *A. encheleia*. Other useful characteristics for the phenotypic identification of *A. eucrenophila* and *A. encheleia* are the production of acid from salicin (negative for *A. sobria*, *A. veronii* biogroup *sobria*, *A. jandaei*, *A. schubertii*, *Aeromonas trota*, and *Aeromonas allosaccharophila*) and a negative reaction in the ornithine decarboxylase test (positive for *A. veronii* biogroup *veronii*) (Table 3).

Extended description of *Aeromonas eucrenophila*. Cells are gram-negative, straight, motile rods. Optimal growth occurs after 24 h at 28°C on TSA medium, but all strains also grow in TSB broth at 37°C. No brown water-soluble pigment is produced on TSA medium. Chemoorganotrophic, with both oxidative and fermentative metabolism. Acid and gas are produced from D-glucose by all strains except strain LMG 13057. Acid, but no gas, is produced from glycerol. Cytochrome oxidase and catalase positive. Nitrate is reduced to nitrite without the production of gas. Resistant to vibriostatic agent O/129. Growth occurs in KCN broth, but not in the presence of 6, 8, or 10% (wt/vol) NaCl. Seven of nine strains grow in the presence of 3% (wt/vol) NaCl. Arginine dihydrolase, DNase, and indole are positive, but Voges-Proskauer is negative. No production of H₂S, urease, tryptophan deaminase, and ornithine and lysine decarboxylase. No alkalization of citrate and malonate.

The following substrates are used by all nine *A. eucrenophila*

strains as the sole carbon and energy sources: acetate (except strain LMG 3774^T), N-acetyl-D-glucosamine, arbutin (except strain LMG 13687), L-arginine, L-aspartate, D-cellobiose, D-fructose (except strain LMG 17061), fumarate, D-galactose, D-gluconate (except strain LMG 13687), D-glucose, L-glutamate (except strain LMG 17061), L-glutamine, glycerol, L-histidine, L-malate, D-maltose, D-mannitol, D-mannose, L-ornithine, L-proline (except strain LMG 17059), putrescine, pyruvate, D-ribose, salicin, succinate, L-serine, D-trehalose, and L-tyrosine. None of the strains use *cis*-aconitate, *trans*-aconitate, adipate, adonitol, β-alanine, L-alanine (except strains LMG 17061 and LMG 17062), azelate, citrate (except strain LMG 16179), citrullin, dulcitol, ethanol, erythritol, D-glucuronate, glutarate, glycine (except strain LMG 13058), 3-hydroxybenzoate, 4-hydroxybenzoate, inositol, itaconate, DL-lactate, lactose, maltitol, α-D-melibiose, mesaconate (except strain LMG 16179), oxoglutarate, phenylacetate, L-phenylalanine, propionate, D-raffinose, D-sorbitol, suberate, D-sucrose (except strain LMG 13057), L-tryptophan, and D-xylose.

Acid is uniformly produced from D-cellobiose, D-galactose, lactose, D-maltose, D-mannitol, D-mannose, salicin, and D-trehalose but not from adonitol, D-arabitol, dulcitol (except strain LMG 13058), erythritol, inositol, α-D-melibiose, methyl-D-glucoside, raffinose, D-sorbitol, D-sucrose (except strain LMG 13057), and D-xylose.

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

All *A. eucrenophila* strains are resistant to amoxicillin, ampicillin, cefetamet, 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 cut-off levels defined by the National Committee for Clinical Laboratory Standards (30, 31).

The G+C content ranges from 58.8 to 60.7 mol%. Isolated from fresh water and virus-infected carp (34) (Table 1). One isolate (strain LMG 16179) was recovered from a human wound. Four of the nine strains (44%) were hemolytic.

The type strain is strain LMG 3774 (= NCMB 74). The G+C content of this strain is 59.1 mol% (this study) or 61.8 mol% (35).

Extended description of *Aeromonas encheleia*. Cells are gram-negative, straight, motile rods. Optimal growth occurs after 24 h at 28°C on TSA medium, but all strains also grow in TSB broth at 37°C. No brown water-soluble pigment is produced on TSA medium. Chemoorganotrophic, with both oxidative and fermentative metabolism. Acid and gas are produced from D-glucose by all strains except strain LMG 13076. Except for strains LMG 13075 and LMG 13076, all strains produce acid, but no gas, from glycerol. Cytochrome oxidase and catalase positive. Nitrate is reduced to nitrite without the production of gas. Resistant to vibriostatic agent O/129. Growth occurs in KCN broth but not in the presence of 6, 8, or 10% (wt/vol) NaCl. Eight of 19 strains (42%) grow in the presence of 3% (wt/vol) NaCl. Arginine dihydrolase (except strain LMG 13075), DNase, and indole are positive, but Voges-Proskauer is negative. No production of H₂S, urease, and tryptophan deaminase. Except for strain LMG 13075, ornithine and lysine

decarboxylase are not produced. No alkalization of citrate and malonate.

The following substrates are used by all 19 *A. encheleia* strains as the sole carbon and energy sources: *N*-acetyl-D-glucosamine, 4-aminobutyrate, arbutin, L-arginine (except strains LMG 16405 and LMG 16328), fumarate, D-galactose, D-glucuronate, D-glucose, L-glutamine (except strain LMG 16405), glycerol, L-histidine, L-malate, D-maltose, D-mannitol (except strain LMG 17058), D-mannose, L-ornithine (except strain LMG 16330^T), L-proline (except strain LMG 16328), pyruvate, D-ribose, salicin, succinate, L-serine, and D-trehalose. None of the strains use *cis*-aconitate, *trans*-aconitate, adipate, adonitol, β -alanine, azelate, D-cellobiose, citrate, citrullin, dulcitol, ethanol, erythritol, D-glucuronate, glutarate (except strain LMG 13075), glycine (except strains LMG 13061 and LMG 17058), 3-hydroxybenzoate (except strain LMG 13076), 4-hydroxybenzoate, DL-3-hydroxybutyrate, inositol (except strain LMG 13075), itaconate, DL-lactate, lactose, L-leucine (except strain 17060), maltitol, α -D-melibiose, mesaconate, oxoglutarate, phenylacetate, L-phenylalanine, propionate, D-raffinose, L-rhamnose, D-sorbitol, suberate, L-tryptophan, and D-xylose.

Acid is uniformly produced from D-galactose, D-maltose, D-mannose, D-mannitol (except strains LMG 16330^T and LMG 17058), salicin, and D-trehalose but not from adonitol, D-arabitol, D-cellobiose, dulcitol, erythritol, inositol, lactose, α -D-melibiose, methyl-D-glucoside, raffinose, D-sorbitol, and D-xylose.

All *A. encheleia* strains hydrolyze the following substrates: L-alanine-*p*NA, casein (except strain LMG 13076), 2-deoxythymidine-5'-*p*NP-phosphate (except strain LMG 13062), gelatin, lecithin (except strain LMG 13076), bis-*p*NP-phosphate, *ortho*-nitrophenyl- β -D-galactopyranoside, *p*NP- β -D-glucopyranoside, *p*NP-phenylphosphonate, *p*NP-phosphoryl choline, L-proline-*p*NA, starch, and Tween 80. None of the strains are able to hydrolyze chitin, 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. encheleia* strains are resistant to amoxicillin, ampicillin, cefetamet, 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 cut-off levels defined by the National Committee for Clinical Laboratory Standards (30, 31).

The G+C content ranges from 59.2 to 61.7 mol%. Isolated from fresh water and healthy European eels (16) (Table 1). One isolate (strain LMG 13075) was of human origin (20). Four of 19 strains (21%) were hemolytic.

The type strain is strain LMG 16330 (= CECT 4342). The G+C content of this strain is 59.2 mol% (this study) or 60.0 mol% (16).

Prevalence and pathogenicity of *A. eucrenophila* and *A. encheleia*. The fact that most isolates of *A. eucrenophila* and *A. encheleia* have so far only been recovered from freshwater or from freshwater fish (Table 1) may be highly significant in establishing the relative pathogenicity of both species. In a recent study (18), Hänninen et al. included a group of taxonomically diverse aeromonads from clinical and environmental sources for determining their maximum growth temperature ($t_{\max}$), which is a useful parameter for predicting whether an organism has the potential to colonize, multiply, and cause infections in animals and humans. The authors reported that, together with HG7, strains of HG6 (*A. eucrenophila*) and HG11 (now *A. encheleia*) exhibited the lowest $t_{\max}$ (ranging from 33 to 37°C) among the mesophilic members of *Aeromonas* (18). The apparent correlation between the typical prevalence of *A. eucrenophila* and *A. encheleia* in aquatic environments and their relatively low $t_{\max}$ is also fully consistent with

previous reports demonstrating the nonpathogenic character of these organisms for mammals (35) and fish (16). On the other hand, the recovery of strains LMG 13075 (*A. encheleia*) and LMG 16179 (*A. eucrenophila*) from human material (Table 1) may indicate that representatives of these species can also persist outside freshwater habitats. Interestingly, the latter strain was isolated from a human wound infection without other pathogenic flora except for a second *Aeromonas* strain that was presumptively assigned to *A. veronii* biogroup *sobria* (HG8), which is one of the most pathogenic taxa in *Aeromonas*. Although no extensive virulence testing was performed in the present study, the various data cited above clearly suggest that the redefined species *A. eucrenophila* and *A. encheleia* probably belong to the least pathogenic members of the genus *Aeromonas*. Nonetheless, keeping in mind the relatively high level of genomic and phenotypic relatedness between many *Aeromonas* taxa, it is beyond doubt that a better understanding of the taxonomy of etiologically less important aeromonads would also greatly benefit the proper classification of clinically relevant *Aeromonas* species.

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