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 16330T 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-cellulobiose and lactose and the assimilation of d-cellulobiose. 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 acronomads (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 (9). 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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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-like (22), and A. encheleia-like (22).

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, Germany), M.-L. Hanninen (Department of Food and Environmental Hygiene, Faculty of Veterinary Medicine, University of Helsinki, Finland), I. Kersters (Laboratorium voor Microbiële Ecologie, Universiteit Gent, Ghent, Belgium), and M.-L. Hanninen (Department of Food and Environmental Hygiene, Faculty of Veterinary Medicine, University of Helsinki, Finland). The following tests were performed as described previously (36): catalase activity (method 1), casein "Downloaded from www.microbiologyresearch.org by
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DNA extraction. Chromosomal DNA of high molecular weight was isolated according to the method of Marmur (27).

Determination of DNA base compositions. The mol
cents percent guanine-plus-

cytosine values were determined in 1× 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× SSC. Hybridization experiments were performed in 2× 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 the 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 phena at a similarity level of 91% (Fig. 1). Phenon 1 contained the five strains previously assigned to A. eucrenophila subgroup I together with four A. eucrenophila-like isolates, whereas phenon 2 comprised the four original reference strains of A. encheleia joined by the three representatives of A. eucrenophila subgroup II and 10 A. eucrenophila-like isolates (Table 1). Strains LMG 13075 and LMG 13076 of HG11 were closely related to phena 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 phena. The separation of phena 1 from pheno 2 strongly relied on the abilities 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 phena 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. eucrenophila and A. encheleia, as defined in Table 1, were highly similar (data not shown). For a

![FIG. 1. Phenogram showing the delineation of phena 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. eucrenophila; A. sp., Aeromonas sp.](image)
A. eucrenophila subgroup I, including the type strain LMG 17059, is a member of DNA homology group I, and A. eucrenophila subgroup II, including the type strain LMG 3774, is a member of DNA homology group II. Within group I, the four original reference strains of A. encheleia were joined by the three representatives of A. eucrenophila subgroup I and the type strain LMG 3774. The six strains described in Table 1, including the type strain and one additional strain, were determined to be members of DNA homology group I. Of the 28 strains described in Table 1, including the type strains, 57% of the strains are positive; 50 to 90% of the strains are positive; and 50 to 90% of the strains are negative; and ND, no data found.

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). 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 1x 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, 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
A suggested 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 (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 I, 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-cellulobiose and lactose and for the assimilation of D-cellulobiose 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),

<table>
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<th>Taxon</th>
<th>Strain</th>
<th>G+C (%) (mol%)</th>
<th>% DNA homology by cross-hybridization*</th>
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<tr>
<td>DNA homology group I (A. eucrenophila)</td>
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<td>1. LMG 3774</td>
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<td>100</td>
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<td>2. LMG 13058</td>
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<td>87</td>
<td>81 85 100</td>
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<td>77</td>
<td>84 100</td>
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<td>46</td>
<td>42 100</td>
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<td>48 105 100</td>
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<td>15. LMG 17065</td>
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<td>48</td>
<td>45 83 100</td>
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<td>16. LMG 16328</td>
<td>61.1</td>
<td>52</td>
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</tr>
<tr>
<td>17. LMG 13691</td>
<td>61.2</td>
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<td>A. sobria</td>
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* Data are means of at least two determinations.
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 Aeromonas jandaei and the anaerogenic character of Aeromonas except A. caviae and Aeromonas media by their lack of A. eucrenophila and A. encheleia from the latter two species is 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 HGI 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 HGI (but not by A. hydrophila HG3) (Table 3) and Aeromonas jandaei and the anaerogenic character of Aeromonas schuberti 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. jan-
daei, A. schuberti, Aeromonas trota, and Aeromonas alloschuberti) 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, trypothan 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-glucuronate (except strain LMG 13687), D-glucose, l-glutamate (except strain LMG 17061), l-glutamine, glycerol, L-histidine, L-malate, D-maltose, l-mannitol, D-mannose, l-ornithine, L-proline (except strain LMG 17059), putrescine, pyruvate, D-ribose, salicin, sucinate, l-serine, D-trehalose, and l-tyrosine. None of the strains use cis-aconitate, trans-aconitate, adipate, adonitol, β-alanine, L-alaile (except strain 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, l-lactate, lactose, maltilitol, α-D-melibiose, mesonate (except strain LMG 16179), oxoglutarate, phenylacetate, t-phenylalanine, propionate, d-raffinose, d-sorbitol, suberate, D-sucrose (except strain LMG 13057), t-tryptophan, and D-xylene.

Acid is uniformly produced from D-cellobiose, D-galactose, lactose, D-maltose, l-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, suberate, D-sucrose (except strain LMG 13057), and D-xylene.

All A. eucrenophila strains hydrolyze the following substrates: L-alanine-pNA, casein, 2-deoxyxymyidine-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-xylene.

All A. eucrenophila strains are resistant to amoxicillin, ampicillin, cefotetam, oxacillin, penicillin G, teicoplanin, and vancomycin but are sensitive to amikacin, cefotaxin, ceftazidin, chloramphenicol, gentamicin, kanamycin, mezlocillin, netilmicin, piperacillin, spectinomycin, tetraclycin, tobramycin, and trimethoprim according to the cut-off levels defined by the National Committee for Clinical Laboratory Standards (30, 31).

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).
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, t-arginine (except strains LMG 16405 and LMG 16328), fumarate, d-galactose, d-glucuronate, d-glucose, l-glutamine (except strain LMG 16405), glycercol, l-bistidine, l-malate, d-malose, d-mannitol (except strain LMG 17058), d-mannose, l-ornithine (except strain LMG 16330B), 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-gluconurate, glutarate (except strain LMG 13075), glycine (except strains LMG 13061 and LMG 17058), 3-hydroxybenzoate (except strain LMG 13076), 4-hydroxybenzoate, d,L-3-hydroxybutyrate, isonitrosothiol, 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-D-glucuronide, and pNP-D-xylрose.

All *A. encheleia* strains are resistant to amoxicillin, ampicillin, d-mannose, d-mannitol (except strains LMG 16330B and LMG 17058), salicin, and d-trehalose but not from adonitol, d-arabitol, d-cellobiose, dulcitol, erythritol, isonitrosothiol, lactose, α-d-melibiose, methyl-d-glucoside, raffinose, d-sorbitol, and xylose.

Acid is uniformly produced from d-galactose, d-maltose, d-mannose, d-mannitol (except strains LMG 16330B and LMG 17058), L-proline, D-ribose, L-serine, and L-trehalose. 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 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 (HGS), 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.

**ACKNOWLEDGMENTS**

This research was carried out in the framework of contract G.O.A. 91/96-2 of the Ministerie van de Vlaamse Gemeenschap, Bestuur Wetenschappelijk Onderzoek (Belgium).

We thank R. H. W. Schubert for providing detailed descriptions of the original *A. eucrenophila* reference strains and V. Schmidt and K. Grebing for excellent technical assistance. P.K. thanks Merlin Diagnostika GmbH (Bornheim-Hersel, Germany) for supplying the kits for antibiotic resistance testing.

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