

Alcaligenes aquatilis sp. nov., a novel bacterium from sediments of the Weser Estuary, Germany, and a salt marsh on Shem Creek in Charleston Harbor, USA

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Four nitrite-dissimilating strains, isolated from Weser Estuary sediments, were investigated using a polyphasic taxonomic approach. Phylogenetic analysis based on 16S rRNA gene sequences indicated that these strains belong to the 'Betaproteobacteria' and are related to the genus *Alcaligenes*. The highest level of sequence similarity (100 %) was found with strain M3A (= ATCC 700596), a dimethyl sulfide-producing marine isolate that was included in this study. DNA–DNA hybridizations between the five strains and related *Alcaligenes faecalis* strains confirmed that the former belong to a single and novel species within the genus *Alcaligenes*. The isolates are Gram-negative, motile, rod-shaped cells with a DNA G+C content of about 56 mol%. The whole-cell fatty acid profiles of the isolates were very similar and included C_{16:0}, C_{17:0} cyclo, C_{18:1}ω7c, summed feature 2 (comprising any combination of C_{12:0} aldehyde, an unknown fatty acid of equivalent chain length 10–928, C_{16:1} iso I and C_{14:0} 3-OH) and summed feature 3 (C_{15:0} iso 2-OH and/or C_{16:1}ω7c) as the major fatty acid components. On the basis of their phylogenetic, genomic and phenotypic properties, the five novel strains can be assigned to the genus *Alcaligenes* as a novel species, for which the name *Alcaligenes aquatilis* sp. nov. is proposed. The type strain is LMG 22996^T (= CCUG 50924^T).

The genus *Alcaligenes* has undergone several changes since its creation in 1919, and is now limited to the species *Alcaligenes faecalis* (type species), which has been subdivided into *A. faecalis* subsp. *faecalis* and *A. faecalis* subsp. *parafaecalis* (Schroll *et al.*, 2001), *Alcaligenes latus* (Palleroni & Palleroni, 1978) and *Alcaligenes defragrans* (Foss *et al.*, 1998). *A. faecalis* strains have been isolated from a wide variety of different habitats, e.g. soil, water and several clinical samples (Kerstens & De Ley, 1984; Schroll *et al.*, 2001). *A. defragrans* strains have been isolated from soil and are capable of using alkenoic monoterpenes as sole carbon sources (Foss *et al.*, 1998). *A. latus* is considered a *species incertae sedis* and recent data have shown

that this organism belongs to the family *Comamonadaceae* (Coenye *et al.*, 2003). Recently, another novel subspecies, '*A. faecalis* subsp. *phenolicus*', isolated from a greywater bioprocessor, has been described (Rehfuß & Urban, 2005).

A polyphasic taxonomic study was performed on five isolates from different aquatic habitats to elucidate their taxonomic position. The results of the genotypic and phenotypic analyses showed that they belong to a novel *Alcaligenes* species.

Four nitrite-dissimilating strains (LMG 22996^T, LMG 22997, LMG 22998 and LMG 22999) were isolated from Weser Estuary sediments in Germany (Rüger *et al.*, 1983). Strain M3A (= ATCC 700596) was isolated from salt-marsh sediment from the bank of Shem Creek in Charleston Harbor (South Carolina, USA) (de Souza & Yoch, 1995). The reference strains *A. faecalis* subsp. *faecalis* LMG 1229^T, *A. faecalis* subsp. *parafaecalis* LMG 22680^T and *A. defragrans* LMG 18538^T were included in some experiments. All strains were routinely cultivated on trypticase soy agar at 28 °C for 48 h, except when mentioned otherwise.

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain LMG 22996^T is AJ937889.

A dendrogram derived from the linkage of correlation coefficients between the protein patterns of the strains studied and a digitized representation of normalized rep-PCR profiles are available as supplementary figures in IJSEM Online.

SDS-PAGE of whole-cell proteins of the five strains was performed and strains were grown on buffered nutrient agar for 48 h at 28 °C. Preparation of whole-cell proteins and SDS-PAGE were performed as described previously (Pot *et al.*, 1994). Densitometric analysis, normalization and interpolation of the protein profiles, as well as numerical analysis using Pearson's product-moment correlation coefficient, were performed using the GELCOMP 4.2 software package (Applied Maths).

After numerical analysis and visual comparison of the profiles, one cluster could be delineated: strains LMG 22996^T, LMG 22997, LMG 22998 and LMG 22999 showed almost identical profiles, whereas strains ATCC 700596, LMG 1229^T (*A. faecalis* subsp. *faecalis*), LMG 18538^T (*A. defragrans*) and LMG 22680^T (*A. faecalis* subsp. *parafaecalis*) formed separate branches in the dendrogram (see Supplementary Fig. S1 available in IJSEM Online).

The five strains, together with the type strains of *A. faecalis* subsp. *faecalis* and *A. faecalis* subsp. *parafaecalis*, were arranged in similarity groups based upon the results of rep-PCR fingerprinting using the GTG₅ and BOX primers (Versalovic *et al.*, 1991; Rademaker & de Bruijn, 1997; Rademaker *et al.*, 2000). Numerical analysis was carried out using the BioNumerics 4.0 software package, as described by the same authors. Strains LMG 22996^T, LMG 22997, LMG 22998 and LMG 22999 showed virtually identical profiles, whereas strain ATCC 700596 and the type strains of the two *A. faecalis* subspecies formed separate branches (see Supplementary Fig. S2 available in IJSEM Online).

Small-scale DNA extracts were prepared using the method of Pitcher *et al.* (1989) and a 1082 nt fragment of the 16S rRNA gene of strain LMG 22996^T was amplified by a PCR using conserved primers (Coenye *et al.*, 1999). PCR products were purified using a QIAquick PCR purification kit (Qiagen) according to the instructions of the manufacturer. Sequence analysis was performed as described previously (Van Trappen *et al.*, 2004). Evolutionary distances were calculated using the algorithm of Jukes & Cantor (1969) and a phylogenetic tree (Fig. 1) was

constructed using the neighbour-joining method with the TREECON program (Van de Peer & De Wachter, 1994).

The 16S rRNA gene sequence of strain LMG 22996^T was compared with available 16S rRNA gene sequences of other betaproteobacteria and was found to show 100 % similarity to strain ATCC 700596, 99.6 % similarity to *A. faecalis* subsp. *parafaecalis*, 98.3 % similarity to *A. faecalis* subsp. *faecalis*, 96.4 % similarity to '*A. faecalis* subsp. *phenolicus*' and 94.0 % similarity to *A. defragrans*. The phylogenetic tree in Fig. 1 illustrates the phylogenetic relationships within the genus *Alcaligenes*. Bootstrap analysis indicated that strains LMG 22996^T and ATCC 700596 form a stable phylogenetic group (with a bootstrap value of 93 %).

The genomic relatedness between strains LMG 22996^T and ATCC 700596 and the most closely related strains, *A. faecalis* subsp. *faecalis* LMG 1229^T, *A. faecalis* subsp. *parafaecalis* LMG 22680^T and *A. defragrans* LMG 18538^T, was determined by DNA–DNA hybridization. DNA was prepared according to the method of Pitcher *et al.* (1989) and DNA–DNA hybridizations were carried out with photo-biotin-labelled probes in microplate wells as described by Ezaki *et al.* (1989), using a HTS7000 Bio Assay Reader (Perkin Elmer) for the fluorescence measurements. The hybridization temperature was 42 °C and reciprocal experiments were performed for every pair of strains. The level of hybridization between strains LMG 22996^T and ATCC 700596 was 84 %, indicating that they belong to a single species (Wayne *et al.*, 1987), whereas the DNA–DNA binding values with the other *Alcaligenes* strains were low (60 % with LMG 1229^T, 59 % with LMG 22680^T and 10 % with LMG 18538^T). Differences between reciprocal experiments were less than 10 %. These results demonstrate that the five isolates are genotypically distinct from *A. faecalis* subsp. *faecalis* and *A. faecalis* subsp. *parafaecalis*, their nearest neighbours in phylogenetic terms, and constitute a novel species within the genus *Alcaligenes*.

The DNA G+C contents of the isolates were determined using an HPLC method as described by Van Trappen *et al.* (2003): the values for strains LMG 22996^T, LMG 22997,

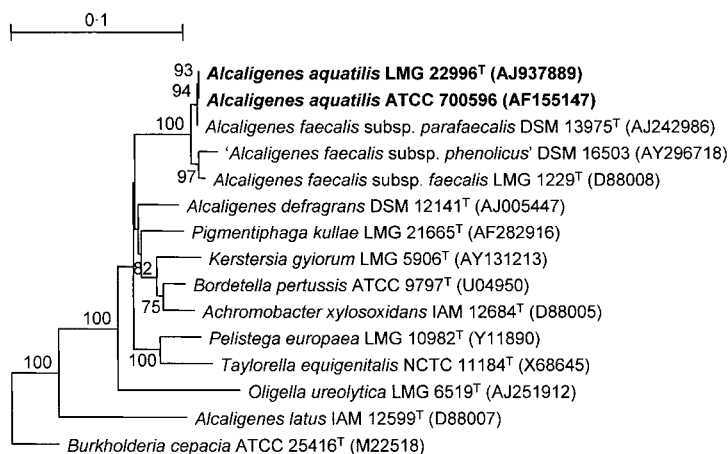


Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the position of *Alcaligenes aquatilis* sp. nov. Bootstrap values are shown as percentages of 500 replicates, if greater than 70 %. Bar, 1 nucleotide substitution per 10 nt. GenBank accession numbers are shown in parentheses. Names that have not been validly published are in quotes.

LMG 22998, LMG 22999 and ATCC 700596 are respectively 56, 57, 56, 56 and 55 mol%. These values are consistent with the G + C contents of the genera *Alcaligenes*, *Achromobacter* and *Pigmentiphaga*, which range from 54 to 69 mol% (Stolz *et al.*, 2005).

The cellular fatty acid patterns of the strains were determined as described by Mergaert *et al.* (2001). The five strains showed very similar fatty acid profiles and the mean composition was 2.3% C_{10:0}, 2.6% C_{12:0} 2-OH, 32.7% C_{16:0}, 13.3% C_{17:0} cyclo, 1.0% C_{18:0}, 9.1% C_{18:1}ω7c, 11.3% summed feature 2 (which comprises any combination of C_{12:0} aldehyde, an unknown fatty acid of equivalent chain length 10.928, C_{16:1} iso I and C_{14:0} 3-OH) and 24.6% summed feature 3 (C_{15:0} iso 2-OH and/or C_{16:1}ω7c). In Table 1, the fatty acid compositions of several *Alcaligenes* species are compared: the profiles of the novel strains resemble those determined for the other members of the genus *Alcaligenes*. The dominant fatty acids are C_{16:0}, C_{17:0} cyclo, C_{18:1}ω7c, summed feature 2 and summed feature 3; the novel strains differ from the other *Alcaligenes* species in their relative amounts of C_{14:0} 3-OH, C_{17:0} cyclo and summed feature 3.

The morphological, physiological and biochemical properties of the five novel strains were determined previously

Table 1. Fatty acid compositions of *Alcaligenes* species

Mean percentages of total fatty acids (with the corresponding standard deviations) are given for *A. aquatilis* (from five strains); data for *A. faecalis* subspecies represent single strains. Data are from Schroll *et al.* (2001), Rehfuss & Urban (2005) and this study. Other fatty acids accounted for less than 1% each. Summed feature 2 comprises any combination of C_{12:0} aldehyde, an unknown fatty acid of equivalent chain length 10.928, C_{16:1} iso I and C_{14:0} 3-OH. Summed feature 3 comprises C_{15:0} iso 2-OH and/or C_{16:1}ω7c. Symbols: –, not detected; tr, trace amounts (<1% of total).

Fatty acid	<i>A. aquatilis</i>	<i>A. faecalis</i> subspecies		
		subsp. <i>faecalis</i>	subsp. <i>parafaecalis</i>	'subsp. <i>phenolicus</i> '
C _{10:0}	2.3 ± 0.2	1.8	–	–
C _{12:0}	tr	1.5	tr	3.2
C _{12:0} 2-OH	2.6 ± 0.4	2.6	1.9	2.2
C _{14:0}	tr	1.1	2.2	tr
C _{14:0} 3-OH	–	–	8.6	8.1
C _{16:0}	32.7 ± 3.9	30.6	31.4	35.8
C _{16:1}	–	–	30.4	–
C _{17:0} cyclo	13.3 ± 5.5	27.7	16.3	9.5
C _{18:0}	1.0 ± 0.1	1.1	tr	1.1
C _{18:1} ω7c	9.1 ± 2.3	11.2	7.5	1.1
Summed feature 2	11.3 ± 1.2	11.0	–	8.4
Summed feature 3	24.6 ± 2.5	10.2	–	29.3

(Rüger *et al.*, 1983; de Souza & Yoch, 1995) and are listed below. Tests in the commercial API 20NE and API ID 32 GN systems (bioMérieux) were performed according to the instructions of the manufacturer. Anaerobic growth of the strains in the presence of nitrate and nitrite has been investigated and the reduction of nitrate and nitrite under anaerobic conditions has been tested according to the protocol of Smibert & Krieg (1994). Alkali production from some organic salts and amides has been tested using the medium of Hugh and Leifson (see species description).

On the basis of this polyphasic taxonomic analysis, the five strains can be clearly differentiated from the other species within the genus *Alcaligenes* (Table 2) and should be assigned to a novel species, for which the name *Alcaligenes aquatilis* sp. nov. is proposed.

The status of *A. defragrans* merits further consideration. The large differences in DNA G + C content (67 mol% versus 56–59 mol% for the other *Alcaligenes* species) and the absence of a significant phylogenetic bond (Fig. 1) indicate that it would be more appropriate to allocate this taxon to a distinct genus in the family *Alcaligenaceae*.

Description of *Alcaligenes aquatilis* sp. nov.

Alcaligenes aquatilis (a.qua'ti.lis. L. masc. adj. *aquatilis* living, growing or found in, or near, water, aquatic).

Cells are Gram-negative short rods (0.7–1.1 µm × 1.0–2.5 µm) and are motile via peritrichous flagella. They form non-pigmented or yellow-pigmented (strains LMG

Table 2. Characteristics useful for differentiating *A. aquatilis* from related species

Taxa: 1, *A. aquatilis*; 2, *A. faecalis* subsp. *parafaecalis*; 3, *A. faecalis* subsp. *faecalis*; 4, '*A. faecalis* subsp. *phenolicus*'. +, Positive; –, negative; W, weak reaction; ND, not determined.

Characteristic	1	2	3	4
DNA G + C content (mol%)	56	56	56–59	54.8
Growth at 42 °C	–	–	+	+
Nitrite reduction	+	–	+	+
Assimilation of:				
Glycogen	+	W	–	–
L-Histidine	+	–	+	+
L-Proline	–	+	+	+
L-Tryptophan	–	–	+	+
L-Serine	+	–	+	–
Malonate	–	+	+	+
Propionate	–	+	+	+
Suberate	+	ND	–	ND
Valerate	+	+	–	ND
α-Hydroxybutyric acid	–	+	–	–
α-Ketoglutaric acid	–	–	–	+
Degradation of gelatin	–	+	–	ND

22999 and ATCC 700596), circular, low-convex and smooth colonies with spreading, irregular edges and a diameter of 2·0–5·0 mm on trypticase soy agar plates after 2 days incubation at 28 °C. The growth temperature is in the range 4–35 °C, while the optimal growth temperature ranges from 18 to 24 °C. Tests for catalase and cytochrome oxidase are positive (except for strain ATCC 700596, which is catalase-negative). Nitrate and nitrite are not reduced under anaerobic conditions, but slight growth is observed under these conditions after 5 days incubation at 28 °C. Positive for the alkalization of asparagine, citrate and glutamine. Positive for the utilization of sodium acetate, sodium citrate and malonate for most of the strains (with the exception of strain ATCC 700596, which is negative for utilization of sodium citrate and malonate). β -Galactosidase activity is not detected. Indole and hydrogen sulfide are not produced, and starch, gelatin, aesculin and urea are not degraded. Strains LMG 22996^T, LMG 22997, LMG 22998 and LMG 22999 are able to reduce nitrite but not nitrate. No acids are produced from carbohydrates (except for strain ATCC 700596, which produces acid from maltose, mannitol, xylose and L-arabinose). No activity detected for arginine dihydrolyase (variable for strain ATCC 700596), lysine decarboxylase or ornithine decarboxylase. Growth is not observed on glucose, L-arabinose, mannose, mannitol, N-acetylglucosamine, maltose, gluconate, adipate, salicin, D-melibiose, L-fucose, D-sorbitol, propionate, 2-ketogluconate, 3-hydroxybutyrate, 4-hydroxybenzoate, L-proline, rhamnose, D-ribose, inositol, D-sucrose, itaconate, malonate, L-alanine or 5-ketogluconate. Growth is observed on caprate (except for strain ATCC 700596), malate, phenylacetate, valerate, citrate, histidine, suberate, acetate, DL-lactate, glycogen, 3-hydroxybenzoate and L-serine. Cells contain the fatty acids C_{16:0}, C_{17:0} cyclo, C_{18:1} ω 7c, summed feature 2 (comprising any combination of C_{12:0} aldehyde, an unknown fatty acid of equivalent chain length 10·928, C_{16:1} iso I and C_{14:0} 3-OH) and summed feature 3 (C_{15:0} iso 2-OH and/or C_{16:1} ω 7c) as the main constituents. The DNA G+C content of the strains is 56 mol%.

The type strain is LMG 22996^T (=CCUG 50924^T). Reference strains are LMG 22997, LMG 22998 and LMG 22999, isolated from sediments of the Weser Estuary, Germany, as was the type strain, and M3A (=ATCC 700596), isolated from sediments of Shem Creek, Charleston Harbor, USA.

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