Aestuariibacter salexigens gen. nov., sp. nov. and *Aestuariibacter halophilus* sp. nov., isolated from tidal flat sediment, and emended description of *Alteromonas macleodii*

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Two strictly aerobic, halophilic strains of the γ -*Proteobacteria*, designated JC2042^T and JC2043^T, were obtained from a sediment sample of getbol, the Korean tidal flat. Comparative 16S rDNA sequence studies revealed that the test strains were related most closely to the type strains of the genera *Alteromonas* (93.5–95.5%) and *Glaciecola* (91.1–93.3%). Phylogenetic analyses demonstrated that strains JC2042^T and JC2043^T formed a distinct monophyletic clade within the family *Alteromonadaceae* and clustered distantly with the genera *Alteromonas* and *Glaciecola*. Physiological, biochemical and chemotaxonomic data also indicated that the two getbol isolates were significantly different from members of these two genera and others with validly published names. Cells were rod-shaped and motile with a polar flagellum. The major isoprenoid quinone was Q8. The predominant cellular fatty acids were C_{16:0}, C_{18:1 ω 7c} and a mixture of C_{16:1 ω 7c} and iso-C_{15:0} 2-OH. DNA G+C contents were 48–54 mol%. On the basis of this polyphasic study, *Aestuariibacter* gen. nov. is proposed with two novel species, *Aestuariibacter salexigens* sp. nov. (type strain, JC2042^T=IMSNU 14006^T=KCTC 12042^T=DSM 15300^T) and *Aestuariibacter halophilus* sp. nov. (type strain, JC2043^T=IMSNU 14007^T=KCTC 12043^T=DSM 15266^T). *Aestuariibacter salexigens* is the type species of the genus. In addition, an emended description of *Alteromonas macleodii* is proposed.

INTRODUCTION

The family *Alteromonadaceae* Ivanova & Mikhailov 2001 encompasses the genera *Alteromonas*, *Pseudoalteromonas*, *Idiomarina* and *Colwellia*. The genera *Glaciecola* Bowman *et al.* 1998 and *Thalassomonas* Macián *et al.* 2001 are phylogenetically closely related to *Alteromonas macleodii* and also should be regarded as members of this family. All strains of these genera are widespread inhabitants of marine environments and have been isolated from diverse marine sources. In this study, two bacterial strains that were isolated from the sediment of getbol (the Korean tidal flat) were the subject of a taxonomic investigation. On the basis of polyphasic evidence, the test strains formed a novel genus in the family *Alteromonadaceae*, for which the name *Aestuariibacter* gen. nov. is proposed.

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The GenBank/EMBL/DDBJ accession numbers for the 16S rDNA sequences of strains JC2042^T and JC2043^T are AY207502 and AY207503, respectively.

METHODS

Bacterial strains. Test strains were isolated from a sediment sample of the getbol of Ganghwa Island, Korea (37° 35' 31.9'' N, 126° 27' 24.5'' E) on August 31, 2002. The sample was diluted with sterilized artificial sea water (ASW; Lyman & Fleming, 1940), spread onto a plate that contained marine agar 2216 (MA; Difco) and MR2A [R2A (Difco) supplemented with artificial sea salts (Sigma)] and incubated at 25 °C for 3 weeks. Strains JC2042^T and JC2043^T were isolated on MA and MR2A, respectively, and were cultured routinely on MA at 30 °C. *Alteromonas macleodii* LMG 2843^T and *Glaciecola punicea* DSM 14233^T were used as reference strains and were grown on MA at 30 and 10 °C, respectively.

Molecular systematics. Bacterial DNA preparation and PCR amplification and sequencing of 16S rDNA were carried out as described previously (Chun & Goodfellow, 1995). The resultant sequences of strains JC2042^T and JC2043^T were aligned manually against sequences obtained from GenBank. Phylogenetic trees were inferred by using the Fitch–Margoliash (Fitch & Margoliash, 1967), maximum-likelihood (Felsenstein, 1993), maximum-parsimony (Fitch, 1971) and neighbour-joining (Saitou & Nei, 1987) methods. Evolutionary distance matrices were generated according to Jukes & Cantor (1969). Resultant tree topologies were evaluated by bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings. Alignment

and phylogenetic analyses were carried out by using the PHYLIT program (available at <http://plaza.snu.ac.kr/~jchun/phydit/>) and PAUP 4.0 (Swofford, 1998), as described previously (Chun *et al.*, 2000).

Morphology and physiological properties. The methods, as well as compositions of media used in this study, have been described previously (Yi *et al.*, 2003). Only additional methods or significant modifications are given below.

Carbon source utilization was tested on basal medium (BM; Baumann *et al.*, 1972) and BM supplemented with 0.01 or 0.1% yeast extract. Oxidation or fermentation of carbohydrates (D-glucose, N-acetylglucosamine and inulin) was examined by using fermentation media (Baumann *et al.*, 1971) and modified oxidation-fermentation medium (MOF; Leifson, 1963). Strips of the API ZYM and 20NE kits (bioMérieux) were inoculated with cell suspensions in half-strength ASW and incubated at 30 °C, except for *G. punicea* ACAM 611^T, for which tests were done at both 10 and 30 °C. Catalase reaction was determined by 3% (v/v) hydrogen peroxide. Specific component requirements for ASW (NaCl, 23.5 g; MgCl₂, 4.9 g; Na₂SO₄, 3.9 g; CaCl₂·2H₂O, 1.1 g; KCl, 0.66 g; NaHCO₃, 0.19 g; KBr, 0.096 g; H₃BO₃, 0.026 g; SrCl₂, 0.024 g; NaF, 0.003 g; distilled water, 1 l) were tested by using synthetic MA (10 g Bacto peptone, 2 g yeast extract, 0.2 g ferric citrate and 15 g Bacto agar in 1 l distilled water) supplemented with various combination of artificial sea salts. Growth in synthetic media was determined after 3 days at 30 °C.

Chemotaxonomy. Chemotaxonomic characteristics were determined from cells grown at 30 °C for 2 days on MA or in marine broth 2216 (MB; Difco). Analysis of fatty acid methyl esters was performed by GLC according to the instructions of the Microbial Identification system (MIDI). Isoprenoid quinones were isolated according to Minnikin *et al.* (1984) and analysed by HPLC (Waters) as described by Collins (1985). DNA G+C content was determined by HPLC of deoxyribonucleosides as described by Mesbah *et al.* (1989), using a reverse-phase column (Supelcosil LC-18-S; Supelco).

RESULTS AND DISCUSSION

Phylogenetic analysis

Almost-complete 16S rDNA sequences of strains JC2042^T (1439 bp) and JC2043^T (1434 bp) were obtained and used for an initial BLAST search against sequences in GenBank.

The result clearly indicated that the getbol isolates belonged to the γ -Proteobacteria. The newly determined sequences were then aligned manually against representatives of the γ -Proteobacteria, based on bacterial 16S rRNA secondary structure. Regions available for all sequences were the domains used to construct the phylogenetic tree (positions 35–1459 of the *Escherichia coli* 16S rDNA sequence), excluding positions that were likely to show ambiguous alignment (positions 75–95, 204–213, 841–846, 1132–1141 and 1448–1454). Sequence similarity between strains JC2042^T and JC2043^T was 96.6%, which indicated that these strains belonged to different species (Stackebrandt & Goebel, 1994). The highest similarity values of our isolates to species with validly published names were as follows: *Alteromonas marina* SW-47^T (93.8%), *Alteromonas macleodii* DSM 6062^T (93.5%), *G. punicea* ACAM 611^T (93.3%) and *Glaciecola pallidula* ACAM 615^T (91.1%) for strain JC2042^T, and *Alteromonas marina* SW-47^T (95.5%), *Alteromonas macleodii* DSM 6062^T (95.3%), *G. pallidula* ACAM 615^T (92.6%) and *G. punicea* ACAM 611^T (92.2%) for strain JC2043^T.

This relationship between our isolates and other genera was also highlighted in the phylogenetic trees, as shown in Fig. 1. Strains JC2042^T and JC2043^T formed a monophyletic clade with a bootstrap value of 77% that was supported by all tree-making methods employed in this study. The genus *Alteromonas* also formed a strong monophyletic clade (bootstrap value of 100%) and was recovered as a sister group to the monophyletic clade that contained the getbol isolates. Members of the genera *Alteromonas* and *Glaciecola* and the two test strains were recovered in a significant monophyletic clade with 100% bootstrap support; the branch pattern within the clade was identical in all phylogenetic trees. On the basis of sequence similarities and phylogenetic analysis, it is clear that our isolates cannot be assigned to either *Alteromonas* or *Glaciecola*, and should merit a novel genus in the family *Alteromonadaceae*.

Fig. 1. Phylogenetic relationship of strains JC2042^T and JC2043^T and related species within the γ -Proteobacteria, based on 16S rDNA sequences. The tree was created by using the neighbour-joining method; percentage numbers at nodes are levels of bootstrap support from 1000 resampled datasets. Solid circles indicate that the corresponding nodes (groupings) were also recovered in Fitch–Margoliash, maximum-likelihood and maximum-parsimony trees. *Helicobacter pylori* ATCC 43504^T (GenBank accession no. U01330) was used as the outgroup. Bar, 0.1 nucleotide substitution per position.

Culture and growth conditions

Cultural characteristics were studied by using several bacteriological growth media. Abundant growth was observed on MA, CSY-3 (Sawabe *et al.*, 1998) and SMM (Shioi's Marine medium; Shiba, 1981). Both strains grew on MA at pH 6–11; optimal growth occurred at pH 7–8. Strain JC2042^T grew at 20–40 °C and the optimum was 35 °C. Strain JC2043^T grew at 15–40 °C and the optimum was 40 °C. These two strains were common in strict halophilicity, but exhibited slightly different requirements of sea salts. Strain JC2043^T showed growth on 0.5–10 % (w/v) artificial sea salts (Sigma)-based media (the optimum being 2–3 % sea salts) and showed less abundant growth on 1–8 % (w/v) NaCl-containing media (the optimum being 5–6 % NaCl). Similarly, strain JC2042^T required 1–10 % (w/v) artificial sea salts-based media for growth (the optimum being 2–6 % sea salts), but completely failed to grow on modified CSY-3 that contained 0–15 % (w/v) NaCl alone. Strain JC2042^T showed no growth when NaCl was not added to synthetic MA and very weak growth when any of several sea salts components, namely MgCl₂, NaHCO₃, H₃BO₃ and NaF, was omitted. However, growth of strain JC2043^T only decreased a little without NaCl, H₃BO₃ or NaF. No growth was detected under anaerobic conditions, created by a GasPack system (BBL). Strain JC2042^T showed a marked loss of viability within 7 days on MA at 30 °C.

Morphological properties

Cellular and colonial morphologies of cells grown on MA at 30 °C for 2 days were observed. Colonies of both strains did not swarm or luminesce on the agar media tested and spore formation was not observed. Cellular and colonial morphologies are given in the species descriptions.

Physiological and biochemical properties

Physiological and biochemical properties of strains JC2042^T and JC2043^T, as well as some other characteristics, are given in the genus and species descriptions. A summary of the major differential properties between the test strains and phylogenetically related species is given in Table 1.

Several properties of *A. macleodii* LMG 2843^T were discordant with previous results that were reported by others (Baumann *et al.*, 1972, 1984; Gauthier *et al.*, 1995). In contrast to the results described by Gauthier *et al.* (1995), *A. macleodii* LMG 2843^T showed an obvious catalase-positive reaction. In addition, D-ribose, L-arginine and succinate were utilized as sole carbon sources under the same test conditions as in the previous study (Baumann *et al.*, 1972). Finally, *A. macleodii* LMG 2843^T, together with strain JC2043^T, showed slightly fermentative reactions for glucose and N-acetylglucosamine in MOF medium, which may result from residual oxygen in this medium. Based on these differences, an emended description for *A. macleodii* is given later.

Chemotaxonomic characteristics

Cellular fatty acid profiles of the test strains are given in Table 2. These fatty acid compositions are not only similar to each other, but also to those of the genus *Alteromonas*. As reported for other members of the family *Alteromonadaceae* (Ivanova & Mikhailov, 2001), Q8 was the predominant isoprenoid quinone in both strains. The DNA G + C contents of strains JC2042^T and JC2043^T were 48 and 54 mol%, respectively.

Taxonomic conclusions

Polyphasic analyses demonstrate that the two getbol isolates belong to a coherent taxon and represent a novel genus within the family *Alteromonadaceae*. Phenotypic data also indicate that strains JC2042^T and JC2043^T are not affiliated closely with any previously described genus and are sufficiently different from each other to be recognized as separate species (Table 1). Strain JC2043^T shows *Alteromonas*-like phenotypic properties in colonial morphology, lecithinase, acid phosphatase, utilization of D-glucose as sole carbon source and relatively high 16S rDNA sequence similarity to members of the genus *Alteromonas* (95.1–95.5 %). However, monophyly of the two getbol isolates is not only strongly supported by all tree-inferring methods, but also by several phenotypic characteristics, namely sea salts requirement, nitrate reduction, alginase, β -galactosidase, valine arylamidase and utilization of D-cellobiose, D-fructose, D-raffinose, D-salicin, D-xylose and lactose (Table 1). It is therefore proposed that the two getbol isolates should be assigned to a novel genus in the family *Alteromonadaceae*, *Aestuariibacter* gen. nov., as *Aestuariibacter salexigens* sp. nov. for strain JC2042^T and *Aestuariibacter halophilus* sp. nov. for strain JC2043^T.

Description of *Aestuariibacter* gen. nov.

Aestuariibacter (Aes.tu.ar.i.i.bac'ter. L. neut. n. *aestuarium* -i tidal flat; N.L. masc. n. *bacter* rod; N.L. masc. n. *Aestuariibacter* rod-shaped bacterium from tidal flat).

Gram-negative and oxidase- and catalase-positive. Strictly aerobic, chemoheterotrophic, halophilic, mesophilic and neutrophilic. Cells are rod-shaped and motile with a polar flagellum. Abundant growth occurs on MA, CSY-3 and SMM media. Spores are not formed. Major isoprenoid quinone is Q8. Predominant cellular fatty acids are C_{16:0}, C_{18:1}ω7c and a mixture of C_{16:1}ω7c and iso-C_{15:0} 2-OH. DNA G + C content is 48–54 mol%. Phylogenetically, the genus belongs to the family *Alteromonadaceae* and currently contains two species. The type species is *Aestuariibacter salexigens*.

Description of *Aestuariibacter salexigens* sp. nov.

Aestuariibacter salexigens (sa.lex'i.gens. L. n. *sal salis* salt, sea water; L. v. *exigo* to demand; N.L. part. adj. *salessigens* sea water-demanding).

Table 1. Characteristics that differentiate strains JC2042^T and JC2043^T from phylogenetically related species

Strains: 1, JC2042^T; 2, JC2043^T; 3, *Alteromonas macleodii* LMG 2843^T; 4, *Alteromonas marina* SW-47^T; 5, *G. punicea* ACAM 611^T; 6, *G. pallidula* ACAM 615^T. Data from this and earlier studies (Baumann *et al.*, 1972, 1984; Bowman *et al.*, 1998; Gauthier *et al.*, 1995; Mikhailov *et al.*, 2002; Yoon *et al.*, 2003). +, Positive reaction; –, negative reaction; W, weakly positive; ND, no data available; NG, no growth.

Characteristic	1	2	3	4	5	6
Pigment	None	None	None	None	Pink	Pink
Sea salts requirement	+	+	–	–	+	+
Nitrate reduction	+	+	–	–	–	–
Alginase	–	–	+	ND	ND	ND
Amylase	+	+	+	+	–	+
β -Galactosidase	–	–	+	ND	+	+
Caseinase	–	+	–	+	–	–
DNase	+	+	NG	ND	–	–
Aesculinase	+	+	+	+	+	–
Gelatinase	+	+	+	+	–	–
Lecithinase	–	+	+	ND	–	–
Tweenase	+	+	+	+	–	+
Utilization of:						
Acetate	+	+	+	+	–	+
D-Cellobiose	–	–	+	ND	–	–
D-Fructose	–	–	+	+	–	–
D-Galactose	W	–	+	+	–	–
D-Glucose	–	+	+	ND	–	–
D-Raffinose	–	–	+	ND	–	–
D-Salicin	–	–	+	ND	ND	ND
D-Sorbitol	W	–	–	–	–	–
D-Xylose	–	–	+	ND	–	–
Ethanol	W	–	+	ND	ND	ND
Glycine	+	–	+	ND	ND	ND
Inulin	+	–	–	ND	ND	ND
Lactose	–	–	+	+	–	–
L-Lysine	W	–	–	ND	ND	ND
L-Ornithine	+	–	–	ND	–	–
N-Acetylglucosamine	+	+	+	ND	–	–
Succinate	W	–	+	–	+	–
Sucrose	–	+	+	+	–	–
DNA G + C content (mol%)	48	54	45–46	45	44	40
Major fatty acid composition (%):						
C _{16:1} ω 7 <i>cl</i> -iso-C _{15:0} 2-OH	27.2	32.5	34.6	28.5	60.7	54.2
C _{16:0}	15.8	23.9	25.0	21.2	10.0	9.4
C _{18:1} ω 7 <i>c</i>	14.0	11.1	9.4	11.8	16.3	12.6
API ZYM:						
Esterase lipase (C8)	+	+	+	+	–	ND
Valine arylamidase	–	–	+	–	–	ND
Trypsin	+	+	–	–	–	ND
Acid phosphatase	–	+	+	+	–	ND

*Difference between the results of this study and previously reported data (Baumann *et al.*, 1984; Gauthier *et al.*, 1995).

Cells are approximately 1.0–1.8 μ m long and 0.4–0.6 μ m wide. Optimal growth is observed at 35 °C, pH 7–8 and 2–6 % artificial sea salts. Colonies are circular, raised,

entirely margined, brittle, rough, opaque and white on MA and hard to emulsify. Viability is lost rapidly after 7 days on MA at 30 °C. Reduces nitrate to nitrite. Decomposes DNA,

Table 2. Cellular fatty acid composition (%) of strains JC2042^T and JC2043^T

Only fatty acids that represent $\geq 1\%$ of the total fatty acids of at least one of the strains are shown.

Fatty acid	JC2042 ^T	JC2043 ^T
C _{10:0} 3-OH	0.6	4.6
C _{12:0}	1.8	3.0
C _{11:0} 3-OH	1.0	0.3
C _{12:1} 3-OH	2.2	1.1
C _{12:0} 3-OH	1.0	0.6
C _{14:0}	3.9	3.1
C _{15:1} ω 8c	1.0	0.5
C _{15:0}	3.8	2.4
C _{16:1} ω 7c alcohol	2.1	0.7
Sum 2* (C _{12:0} alde/iso-C _{16:1} I/C _{14:0} 3-OH)	2.0	1.6
iso-C _{16:0}	1.1	0.1
Sum 3* (C _{16:1} ω 7c/iso-C _{15:0} 2-OH)	27.2	32.5
C _{16:0}	15.8	23.9
iso-C _{17:1} ω 5c	0.0	1.6
C _{17:1} ω 8c	6.8	3.1
C _{17:0}	4.5	2.8
C _{18:1} ω 7c	14.0	11.1
C _{18:0}	2.9	0.9
Sum 7* (C _{19:1} ω 6c/C _{19:0} ω 10c cyclo/C _{19:0} ω 6c)	1.1	0.3

*Sum, summed features (groups of two or three fatty acids that could not be separated by GLC with the MIDI system).

aesculin, gelatin, starch and Tween 80, but not agar, alginate, casein, cellulose, chitin or egg yolk. Does not produce arginine dihydrolase, β -galactosidase, fluorescein, H₂S, indole, polyhydroxybutyrate or urease. Does not ferment carbohydrates. Produces alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, trypsin and naphthol-AS-BI-phosphohydrolase, but not lipase (C14), valine arylamidase, cystine arylamidase, α -chymotrypsin, acid phosphatase, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase or α -fucosidase. Utilizes acetate, glycine, inulin, L-arginine, L-ornithine and *N*-acetylglucosamine as sole carbon sources. D-Galactose, D-ribose, D-sorbitol, ethanol, L-lysine and succinate are utilized weakly. Does not utilize acetamide, adenine, benzoate, citrate, D-cellobiose, D-fructose, D-glucose, D-mannose, D-raffinose, D-salicin, D-xylose, inositol, 2-propanol, lactose, L-cysteine, L-rhamnose, sucrose, salicylate or thiamin. Cellular fatty acid composition is given in Table 2. DNA G + C content is 48 mol%.

The type strain is JC2042^T (=IMSNU 14006^T=KCTC 12042^T=DSM 15300^T). Isolated from the sediment of getbol, the Korean tidal flat.

Description of *Aestuariibacter halophilus* sp. nov.

Aestuariibacter halophilus (ha.lo'phi.us. Gr. n. *hals halos* salt; Gr. adj. *philos* loving; N.L. masc. adj. *halophilus* salt-loving).

Cells are approximately 1.2–1.8 μ m long and 0.5–0.6 μ m wide. Optimal growth is observed at 40 °C, pH 7–8 and 2–3 % artificial sea salts. Colonies are circular, convex, entirely margined, butyraceous, glistening, opaque and white on MA. Reduces nitrate to nitrite. Decomposes casein, DNA, egg yolk, aesculin, gelatin, starch and Tween 80, but not agar, alginate, cellulose or chitin. Does not produce arginine dihydrolase, β -galactosidase, fluorescein, H₂S, indole, polyhydroxybutyrate or urease. Does not ferment carbohydrates. Produces alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase and β -glucosidase, but not lipase (C14), valine arylamidase, cystine arylamidase, α -chymotrypsin, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase or α -fucosidase. Utilizes acetate, D-glucose, D-ribose, L-arginine, *N*-acetylglucosamine and sucrose as sole carbon sources. Does not utilize acetamide, adenine, benzoate, citrate, D-cellobiose, D-fructose, D-galactose, D-mannose, D-raffinose, D-salicin, D-sorbitol, D-xylose, ethanol, glycine, inositol, inulin, 2-propanol, lactose, L-cysteine, L-lysine, L-ornithine, L-rhamnose, salicylate, succinate or thiamin. Cellular fatty acid composition is given in Table 2. DNA G + C content is 54 mol%.

The type strain is JC2043^T (=IMSNU 14007^T=KCTC 12043^T=DSM 15266^T). Isolated from the sediment of getbol, the Korean tidal flat.

Emended description of *Alteromonas macleodii*

The description remains as those given by Baumann *et al.* (1972, 1984) and Gauthier *et al.* (1995), with the following modification: positive for catalase reaction. The following is added to the descriptions of Baumann *et al.* (1972, 1984) and Gauthier *et al.* (1995). Major isoprenoid quinone is ubiquinone-8. Positive for β -galactosidase, aesculinase and lecithinase. Negative for urease, caseinase and cellulase. Does not produce indole. Utilizes D-raffinose, D-ribose, L-arginine and succinate as sole carbon sources, but not D-sorbitol or thiamin. Produces alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase, but not lipase (C14), cystine arylamidase, trypsin, α -chymotrypsin, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase or α -fucosidase.

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