Vagococcus acidifermentans sp. nov., isolated from an acidogenic fermentation bioreactor

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A Gram-staining-positive, coccus-shaped, non-spore-forming, facultatively anaerobic bacterium, designated AC-1^T, was isolated from an acidogenic fermentation bioreactor treating food wastewater. On the basis of 16S rRNA gene sequence analysis, strain AC-1^T was shown to belong to the genus *Vagococcus*. The closest phylogenetic relatives were *Vagococcus elongatus* PPC9^T (97.4% 16S rRNA gene sequence similarity), *Vagococcus penaei* CD276^T (96.7%) and *Vagococcus carniphilus* ATCC BAA-640^T (96.6%). The major fatty acids were C_{18:1ω9c} (24.8%) and C_{16:0} (19.5%) and the G + C content of genomic DNA was 44.2 mol%, which supported the affiliation of strain AC-1^T to the genus *Vagococcus*. Strain AC-1^T and *V. elongatus* DSM 21480^T exhibited 11% DNA–DNA relatedness. Physiological and biochemical tests differentiated strain AC-1^T from the type strains of recognized species of the genus *Vagococcus*. Therefore, strain AC-1^T is considered to represent a novel species, for which the name *Vagococcus acidifermentans* sp. nov. is proposed. The type strain is AC-1^T (=KCTC 13418^T =LMG 24798^T).

The genus *Vagococcus* was proposed by Collins *et al.* (1989) and described as a phylogenetically distinct group of Gram-positive cocci that resemble lactococci. The first species to be identified was *Vagococcus fluvialis* (Collins *et al.*, 1989), which was isolated from chicken faeces (Hashimoto *et al.*, 1974). Later, *Vagococcus salmoninarum* was isolated from diseased fish (Wallbanks *et al.*, 1990), *Vagococcus lutrae* was recovered from the common otter (Lawson *et al.*, 1999) and *Vagococcus fessus* (Hoyle *et al.*, 2000), *Vagococcus carniphilus* (Shewmaker *et al.*, 2004), *Vagococcus elongatus* (Lawson *et al.*, 2007) and *Vagococcus penaei* (Jaffrès *et al.*, 2010) were isolated from seal and harbour porpoise, ground beef, a swine-manure storage pit and spoiled cooked shrimp, respectively.

In this study, a *Vagococcus*-like strain, AC-1^T, was isolated from an acidogenic fermentation bioreactor in a food wastewater treatment plant in Naju province, South Korea. A suspension sample was serially diluted with 50 mM phosphate buffer (pH 7.0) and incubated on trypticase soy agar (TSA; Difco) at 30 °C for 1 week. Single colonies on the plates were purified by subcultivation on nutrient agar

(NA) or TSA. Strain AC-1^T was routinely cultured on TSA at 30 °C and maintained as a glycerol suspension (20%, w/v) at –70 °C.

Gram-reaction testing was performed by the non-staining method as described by Buck (1982). Cell morphology was observed at ×1000 magnification with a light microscope (Nikon) using cells grown for 24 h at 30 °C on TSA. Catalase activity was determined by bubble production in 3% (v/v) H₂O₂ and oxidase activity was determined using 1% (w/v) tetramethyl *p*-phenylenediamine. Carbon-source utilization and some enzyme activities were tested at 30 °C with the API 20 NE, API ZYM and API 50 CH systems (bioMérieux), according to the instructions of the manufacturer. Anaerobic growth was tested in serum bottles containing trypticase soy broth (TSB; Difco) and 1 g thioglycolate 1^{–1} with nitrogen gas as the headspace. Tests for the degradation of DNA (DNase agar; Scharlau; tested by flooding plates with 1 M HCl), casein, chitin, starch (Atlas 1993), lipid (Kouker & Jaeger, 1987), xylan and cellulose (Ten *et al.*, 2004) were evaluated after 10 days. The test for haemolysis was performed using TSA supplemented with 5% defibrinated sheep blood. Growth at 4, 15, 25, 30, 37, 42 and 45 °C, at different pH and with 1–10% (w/v) NaCl was assessed in TSB after 5 days. Growth was estimated by monitoring

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain AC-1^T is FJ211190.

A supplementary table is available with the online version of this paper.

OD₆₀₀. Growth on NA and MacConkey agar was also evaluated at 30 °C.

Extraction of the genomic DNA of strain AC-1^T was achieved using a commercial genomic DNA-extraction kit (Solgent). The 16S rRNA gene was amplified from chromosomal DNA using the universal bacterial primer set 9F and 1512R and purified PCR products were sequenced by Solgent (Ten *et al.*, 2008). The full sequence of the 16S rRNA gene was compiled using SeqMan (DNASTAR). 16S rRNA gene sequences of related taxa were obtained from the GenBank database. Multiple alignments were performed using CLUSTAL X (Thompson *et al.*, 1997). Gaps were edited in the BioEdit program (Hall, 1999). Evolutionary distances were calculated using Kimura's two-parameter model (Kimura, 1983). Phylogenetic trees were reconstructed using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods in MEGA4 (Kumar *et al.*, 2008) and bootstrap calculations were based on 1000 replications (Felsenstein, 1985). Sequence similarities were calculated using the EzTaxon server (<http://www.eztaxon.org/>; Chun *et al.*, 2007).

For measurement of the G+C content of chromosomal DNA, genomic DNA of strain AC-1^T was extracted and purified as described by Moore & Dowhan (1995) and degraded enzymically into nucleosides. The G+C content was determined as described by Mesbah *et al.* (1989) using reversed-phase HPLC. Cellular fatty acid profiles were determined for cells grown on TSA for 3 days at 30 °C. Cellular fatty acids were saponified, methylated and extracted according to the protocol of the Sherlock Microbial Identification System (MIDI). Fatty acids were analysed by GC (6890; Hewlett Packard) and identified using the Microbial Identification software package (Sasser, 1990).

Cells of strain AC-1^T were Gram-staining-positive, facultatively anaerobic, non-spore-forming, non-motile cocci that were elongated in the direction of the chain. Colonies grown on TSA for 3 days were smooth, circular, white and convex, 0.2 mm in diameter. The novel strain barely grew on MRS agar and NA. On TSA, strain AC-1^T was able to grow at 15–37 °C, but not at 4 or 42 °C. The physiological characteristics of strain AC-1^T are summarized in the species description and Table 1 lists the characteristics that serve to differentiate strain AC-1^T from its closest phylogenetic relatives.

An almost-complete 16S rRNA gene sequence (1450 bp) of strain AC-1^T (positions 59–1499 with respect to the *Escherichia coli* numbering system) was determined. The closest relatives of strain AC-1^T were *V. elongatus* PPC9^T (97.4% 16S rRNA gene sequence similarity), *V. penaei* CD276^T (96.7%), *V. carniphilus* ATCC BAA-640^T (96.6%), *V. fluvialis* CCUG 32704^T (96.6%), *V. lutrae* CCUG 39187^T (96.2%) and *V. salmoninarum* CCUG 33394^T (94.8%). Lower sequence similarities (<94.7%) were found with all other type strains of recognized species of the family *Enterococcaceae*. This relationship between

Table 1. Differential phenotypic characteristics of strain AC-1^T and type strains of species of the genus *Vagococcus*

Strains: 1, *Vagococcus acidifermentans* sp. nov. AC-1^T; 2, *V. elongatus* DSM 21480^T; 3, *V. carniphilus* ATCC BAA-640^T; 4, *V. fluvialis* CCUG 32704^T; 5, *V. lutrae* CCUG 39187^T; 6, *V. salmoninarum* CCUG 33394^T; 7, *V. fessus* DSM 15697^T. All data were obtained in this study. All strains are positive for acid phosphatase and acid production from D-fructose. All strains are negative for lipase (C14), trypsin, β-galactosidase, α-fucosidase, α-mannosidase, β-glucuronidase and acid production from erythritol, L-xylose, L-sorbose, L-rhamnose, methyl α-D-mannopyranoside, melibiose, inulin, melezitose, raffinose, starch, glycogen, D-lyxose, 2-ketogluconate, 5-ketogluconate and D-fucose. +, Positive; w, weakly positive; –, negative.

Characteristic	1	2	3	4	5	6	7
Acid production from (API 50 CH):							
Glycerol	+	+	+	–	+	–	–
D-Arabinose	–	–	+	–	–	–	–
L-Arabinose	–	+	–	+	–	–	+
D-Ribose	+	–	+	+	+	–	–
D-Xylose	+	–	–	–	–	–	–
Adonitol	+	–	–	–	–	–	–
Methyl β-D-xylose	+	–	–	–	–	–	–
D-Galactose	+	–	–	–	–	–	w
D-Glucose	+	–	+	–	+	+	+
D-Mannose	–	–	–	+	–	–	–
Dulcitol	+	–	–	–	–	–	–
Inositol	w	+	–	+	–	–	–
D-Mannitol	+	+	–	–	+	–	–
D-Sorbitol	–	–	–	+	+	–	–
Methyl α-D-glucopyranoside	+	–	+	–	+	–	–
N-Acetylglucosamine	+	–	+	–	+	–	–
Amygdalin	+	–	–	–	+	–	–
Arbutin	+	–	–	–	+	+	–
Aesculin	+	–	+	–	+	+	–
Salicin	–	–	+	–	+	+	–
Cellobiose	+	+	–	–	+	+	–
Maltose	+	–	+	–	+	–	–
Lactose	+	–	–	–	–	–	–
Sucrose	+	–	–	+	w	+	–
Trehalose	+	–	+	+	w	+	w
Xylitol	w	–	–	–	+	–	–
Gentiobiose	+	–	–	–	+	+	–
Turanose	+	–	–	–	+	–	w
D-Tagatose	+	–	–	–	w	+	+
L-Fucose	–	–	+	–	–	–	–
D- and L-Arabitol	+	–	–	–	–	–	–
Gluconate	+	–	–	–	–	–	–
Enzymes (API ZYM)							
Alkaline phosphatase	+	w	+	w	w	+	–
Esterase lipase (C4)	+	w	+	+	+	+	w
Esterase lipase (C8)	+	w	+	+	+	+	+
Leucine arylamidase	+	–	+	+	+	+	+
Valine arylamidase	–	–	–	+	–	w	–
Cystine arylamidase	+	–	+	–	–	w	–
α-Chymotrypsin	+	w	–	+	+	+	+
Naphthol-AS-BI-phosphohydrolase	+	w	+	+	+	–	+

Table 1. cont.

Characteristic	1	2	3	4	5	6	7
α -Galactosidase	—	+	—	—	+	—	—
α -Glucosidase	+	—	+	—	+	—	—
β -Glucosidase	—	—	—	w	+	+	—
N-Acetyl- β -glucosaminidase	—	—	—	—	+	—	—

strain AC-1^T and other members of the genus *Vagococcus* was also evident in the phylogenetic analysis based on 1400 nt. Strain AC-1^T and the seven members of the genus *Vagococcus* formed a monophyletic lineage supported by a bootstrap value of 100 % that was recovered by both of the tree-making methods employed in this study (Fig. 1).

DNA–DNA hybridization was performed between strain AC-1^T and *V. elongatus* DSM 21480^T with the method described by Ezaki *et al.* (1989) using photobiotin-labelled DNA probes and microdilution wells. Hybridization was performed with five replications for each sample. The highest and lowest values obtained for each sample were excluded and the means of the remaining three values were converted to percentage values. DNA–DNA relatedness between strain AC-1^T and *V. elongatus* DSM 21480^T was 11 %, which was low enough to assign strain AC-1^T to a novel species of genus *Vagococcus* (Wayne *et al.*, 1987).

The G + C content of genomic DNA of strain AC-1^T was 44.2 mol%. The fatty acid profiles of strain AC-1^T and the seven members of the genus *Vagococcus* are shown in Supplementary Table S1 (available in IJSEM Online). The fatty acid profile of strain AC-1^T was composed mainly of C_{18:1} ω 9c (24.8 %), C_{16:0} (19.5 %), summed feature 4 (C_{16:1} ω 7c and/or iso-C_{15:0} 2-OH; 7.7 %), C_{18:0} (7.4 %), C_{16:1} ω 9c (6.8 %) and C_{14:0} (6.4 %), which is typical of members of the genus *Vagococcus*. However, some minor qualitative and quantitative differences in fatty acid content

could be observed between strain AC-1^T and its closest phylogenetic relatives.

The results obtained from phenotypic and phylogenetic characterization indicated that strain AC-1^T belongs to the genus *Vagococcus*. Phylogenetic distinctiveness and DNA–DNA relatedness, in addition to some phenotypic differences (Table 1), confirmed that strain AC-1^T represented a species that is distinct from recognized *Vagococcus* species. Therefore, on the basis of the data presented, strain AC-1^T should be classified in a novel species of the genus *Vagococcus*, for which the name *Vagococcus acidifermentans* sp. nov. is proposed.

Description of *Vagococcus acidifermentans* sp. nov.

Vagococcus acidifermentans (a.ci.di.fer.men'tans. N.L. neut. n. *acidum* acid; N.L. part. adj. *fermentans* fermenting; N.L. part. adj. *acidifermentans* fermenting to an acid).

Cells are Gram-staining-positive, facultatively anaerobic, non-spore-forming, non-motile cocci after 3 days on TSA. Colonies after 3 days on TSA are smooth, white and convex, 0.2 mm in diameter. Grows well at 15–37 °C (but not at 4 or 42 °C) and at pH 4.0–9.0. Growth occurs with 0–1.0 % (w/v) NaCl, but not with 2.0 % (w/v) NaCl. Grows weakly on MRS agar and NA. Catalase- and oxidase-negative. Does not reduce nitrate to nitrite. Indole and H₂S is not produced. Negative for haemolysis and production of arginine dihydrolase, urease and protease (gelatin hydrolysis). Acid production from sole carbon sources (API 50 CH) and enzyme activities (API ZYM) are listed in Table 1. C_{18:1} ω 9c, C_{16:0}, summed feature 4 (C_{16:1} ω 7c and/or iso-C_{15:0} 2-OH), C_{18:0}, C_{16:1} ω 9c and C_{14:0} are the major cellular fatty acids (>6 %). The DNA G + C content of the type strain is 44.2 mol% (HPLC).

The type strain, AC-1^T (=KCTC 13418^T =LMG 24798^T), was isolated from an acidogenic fermentation bioreactor in a food wastewater treatment plant in Naju province, South Korea.

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Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships between strain AC-1^T and related type strains. Filled circles indicate that the corresponding nodes were also recovered in a tree generated with the maximum-parsimony algorithm. Bootstrap values (>70 %) based on 1000 replications are shown at branch nodes. Bar, 0.01 substitutions per nucleotide position.

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