Emendation of the genus *Acidomonas* Urakami, Tamaoka, Suzuki and Komagata 1989

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The genus *Acidomonas* and the species *Acidomonas methanolica* were recharacterized by using the type strain (NRIC 0498^T), three reference strains and 10 methanol-utilizing bacteria that were isolated from activated sludge from three different sewage-treatment plants in Tokyo. Based on 16S rDNA sequences, all strains formed a single cluster within the *Acetobacteraceae* that was clearly different from the genera *Acetobacter*, *Gluconobacter*, *Gluconacetobacter*, *Asaia* and *Kozakia*. The 14 strains were identified as a single species, *Acidomonas methanolica*, by DNA–DNA similarities, showed DNA G + C contents that ranged from 62 to 63 mol% and had Q-10 as the major quinone, accounting for > 87 % of total ubiquinones. Cells of *Acidomonas methanolica* had a single polar flagellum (or occasionally polar tuft flagella); this differs from a previous study that described peritrichous flagella. Oxidation of acetate was positive for all strains, but oxidation of lactate was weakly positive and varied with strains. Dihydroxyacetone was not produced from glycerol. Pantothenic acid was an essential requirement for growth. The strains tested grew at mostly the same extent at pH 3.0–8.0. Therefore, *Acidomonas methanolica* should be regarded as acidotolerant, not acidophilic. The descriptions of the genus *Acidomonas* and the species *Acidomonas methanolica* Urakami, Tamaoka, Suzuki and Komagata 1989 are emended with newly obtained data.

The genus name *Acidomonas* was validly published for acidophilic, methanol-utilizing bacteria (Urakami *et al.*, 1989), incorporating *Acetobacter methanolicus* Uhlig *et al.* 1986. *Acidomonas methanolica* is the type and only species reported so far in this genus. Members of the genus *Acidomonas* are recognized as methanol-utilizing bacteria (Bulygina *et al.*, 1990, 1992; Komagata, 1990; Bulygina *et al.*, 1993; Green, 1993). In fact, strain BNS-25 (an acidophilic, methanol-utilizing bacterium) was once regarded as a promising tool for the production of single-cell proteins (SCPs) from methanol (Urakami *et al.*, 1981). This strain was later identified as a member of *Acidomonas methanolica* (Urakami *et al.*, 1985, 1989). On the other hand, the phylogenetic relationship of the genus *Acidomonas* to acetic acid bacteria (the family *Acetobacteraceae*) was first suggested by 5S rRNA sequencing (Bulygina *et al.*, 1992). This finding was confirmed by 16S rDNA sequences (Yamada *et al.*, 1997; Boesch *et al.*, 1998). The revival of

Acetobacter methanolicus was once proposed (Sievers *et al.*, 1994), but another study justified the description of the genus *Acidomonas* (Yamada *et al.*, 2000). However, since the work of Uhlig *et al.* (1986) and Urakami *et al.* (1989), few *Acidomonas* strains have been isolated until now. In addition, this taxon is problematic, as some characteristics differ in the descriptions by Uhlig *et al.* (1986) and Urakami *et al.* (1989) and only four *Acidomonas methanolica* strains are available from culture collections worldwide. They are the type strain NRIC 0498^T (=DSM 5432^T=JCM 6891^T=LMG 1668^T=MB58^T) (Steudel *et al.*, 1980; Uhlig *et al.*, 1986), LMG 1667, LMG 1669 and LMG 1735. The type strain was isolated from an unsterilized process of SCP production from methanol with *Candida* sp. and LMG 1735 was isolated from *Saccharum officinarum* (Janssens *et al.*, 1998). This low number of strains is not enough to verify the diversity of the species.

The present study aims to isolate novel *Acidomonas* strains from activated sludge samples of sewage-treatment plants and to recharacterize and emend the genus *Acidomonas* by using newly obtained 16S rDNA sequences, DNA–DNA similarities, quinone systems and phenotypic characteristics of the new isolates and strains that were obtained from culture collections.

The enrichment culture approach was employed for the isolation of *Acidomonas* strains by using medium C

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Abbreviation: SCP, single-cell protein.

The GenBank/EMBL/DDBJ accession numbers for the 16S rDNA sequences of *Acidomonas methanolica* NRIC 0498^T, LMG 1667, LMG 1669, LMG 1735, TS 0101, TS 0114, TS 0117, TS 0121, TS 0128, TS 0131, TS 0135, TS 0143, TS 0145 and TS 0156 are AB110702–AB110715, respectively.

(Urakami *et al.*, 1985) at pH 4.0 and 30 °C. Medium C contained (l^{-1}): 10 ml methanol, 0.2 g yeast extract, 3.0 g $(NH_4)_2SO_4$, 4.0 g KH_2PO_4 , 0.2 g $MgSO_4 \cdot 7H_2O$, 30 mg $CaCl_2 \cdot 2H_2O$, 5.0 mg $MnCl_2 \cdot 4H_2O$, 5.0 mg $ZnSO_4 \cdot 7H_2O$, 0.5 mg $CuSO_4 \cdot 5H_2O$ and 1.0 ml vitamin solution (pH 4.0). Ferric citrate (present in the original composition) was omitted from the medium used in this study. Vitamin solution contained (l^{-1}): 2 mg biotin, 400 mg calcium pantothenate, 400 mg pyridoxine/HCl, 400 mg thiamin/HCl, 200 mg *p*-aminobenzoic acid, 2 mg folic acid, 2 g inositol, 400 mg nicotinic acid and 200 mg riboflavin. If necessary, 20 g agar l^{-1} was added to solidify the medium. Isolation materials were inoculated into medium C and incubated at 30 °C with shaking. When microbial growth occurred, cultures were subjected to subsequent enrichment. After repeating enrichment cultures, micro-organisms were streaked on PYMe medium agar plates and any colonies that appeared were transferred to medium C broth. Microbial growth in the broth was checked microscopically and methanol-utilizing bacteria were isolated and purified by streaking on medium C agar plates. PYMe medium contained (l^{-1}): 10 ml methanol, 5.0 g peptone and 3.0 g yeast extract (pH 4.0). PYMe medium was also used for isolation of methanol-utilizing bacteria. This medium was enriched with rather high concentrations of yeast extract and peptone and favoured the isolation of a variety of methanol-utilizing bacteria. Pure cultures of methanol-utilizing bacteria were obtained in the same manner as described above. Activated sludge samples were mainly used as isolation sources, followed by vegetables, fruit, decayed woods and leaves, manure, paddy soils etc. Activated sludge samples were obtained at the Ochiai, Morigasaki and Shibaura sewage-treatment plants in Tokyo; 19 activated sludge samples were collected at different sites of the plants. In total, 56 methanol-utilizing bacteria were isolated from the activated sludge samples, but not from other isolation sources. Of the 56 strains, 10 were selected for subsequent study, considering prevention of picking up the same clone, colony appearance, sampling sites and locations of the plants. Strain TS 0101 was isolated from a sample that was collected at the Ochiai plant, strains TS 0114, TS 0117, TS 0121, TS 0128, TS 0131 and TS 0135 were collected at the Morigasaki plant and strains TS 0143, TS 0145 and TS 0156 were collected at the Shibaura plant. They have been deposited in the NODAI Culture Collection, Tokyo University of Agriculture, Tokyo, Japan (NRIC), with the accession numbers NRIC 0548, NRIC 0549, NRIC 0550, NRIC 0551, NRIC 0552, NRIC 0553, NRIC 0554, NRIC 0555, NRIC 0556 and NRIC 0557, respectively. Including *Acidomonas methanolica* NRIC 0498^T, LMG 1667, LMG 1669 and LMG 1735, which were obtained from culture collections, 14 strains were used throughout this study.

Sequencing of 16S rDNA was carried out by using previously reported methods (Lisdiyanti *et al.*, 2000; Yamada *et al.*, 2000). 16S rDNA was amplified by PCR with two primers: 20F (5'-GATTTTGATCCTGGCTCAG-3', positions

9–27) and 1500R (5'-GTTACCTTGTTACGACTT-3', positions 1509–1429). Numbering of positions was based on the *Escherichia coli* numbering system (GenBank accession no. V00348; Brosius *et al.*, 1981). Purified PCR products were sequenced directly by using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit and an ABI PRISM model 310 genetic analyser. The following six primers were used: 20F, 1500R, 520F (5'-CAGCAGCCG-CGGTAATAC-3', positions 519–536), 520R (5'-GTATTA-CCGCGGCTGCTG-3', positions 536–519), 920F (5'-AACTAAATGAATTGACGG-3', positions 907–926) and 920R (5'-CCGTCAATTCATTTGAGTTT-3', positions 926–907).

Multiple alignment was performed by using the program CLUSTAL X (version 1.18) (Thompson *et al.*, 1997). Distance matrices for the aligned sequences were calculated by using the two-parameter method (K_{nuc}) (Kimura, 1980). The neighbour-joining method was used for constructing a phylogenetic tree (Saitou & Nei, 1987). Robustness of individual branches was estimated by bootstrapping with 1000 replicates (Felsenstein, 1985). Species, type strains, strain numbers and GenBank accession numbers are presented in Fig. 1.

Acidomonas methanolica NRIC 0498^T, 10 isolates and three reference strains were subjected to phylogenetic analysis based on 16S rDNA sequences. Similarity values of 16S rDNA sequences (on the basis of 1408 bases) of *Acidomonas methanolica* NRIC 0498^T and the new isolates and reference strains were calculated in pairs; similarities among the strains tested were 99.9–100 %. The above strains formed a single cluster within the *Acetobacteraceae* (Fig. 1) that was clearly distant from the genera *Acetobacter*, *Gluconobacter*, *Gluconacetobacter*, *Asaia* and *Kozakia*.

DNA was extracted and purified by the method of Saito & Miura (1963). Levels of DNA–DNA similarity were determined by the fluorometric DNA–DNA hybridization method in microdilution wells (Ezaki *et al.*, 1989). A probe was prepared with DNA from *Acidomonas methanolica* NRIC 0498^T and hybridization was carried out at 50 °C. DNA G+C contents were determined by reverse-phase HPLC (Tamaoka & Komagata, 1984).

Levels of DNA–DNA similarity between the type strain of *Acidomonas methanolica* and the 10 isolates and three reference strains were 76–100 %. Thus, all strains tested were identified as *Acidomonas methanolica*. DNA G+C contents ranged from 62 to 63 mol%.

Isoprenoid quinone compositions were determined by HPLC (Komagata & Suzuki, 1987). *Acidomonas methanolica* NRIC 0498^T, the 10 isolates and the three reference strains contained ubiquinones, with Q-10 accounting for >87 % and Q-9 for <11 % of the total ubiquinones.

Phenotypic characteristics were examined by using previously described methods (Asai *et al.*, 1964; Lisdiyanti *et al.*, 2000) unless otherwise stated. PYM medium

Fig. 1. Phylogenetic relationships of *Acidomonas methanolica* and other acetic acid bacteria, deduced from 16S rDNA sequences. *Acidiphilium cryptum* ATCC 33463^T was used as the outgroup. Numbers at nodes represent bootstrap values (1000 replicates). Bar, 0.005 K_{nuc} .

(Urakami *et al.*, 1989), which contained (l^{-1}): 10 g glucose, 5 g peptone, 3 g yeast extract and 3 g malt extract (pH 6.0), was used as a basal medium and strains were cultivated at 30 °C. Motility was examined by the hanging-drop method. PYM medium and modified PYM medium, in which glucose was replaced by methanol, were used for examining motility and were incubated at 25 and 30 °C. Oxidation of acetate to CO₂ and H₂O was examined by the method of Leifson (1954) and oxidation of lactate was determined by the methods of Leifson (1954) and Shimwell *et al.* (1960). Urease was examined by using the medium of Christensen (1946). Requirement of pantothenic acid was determined by using medium C from which yeast extract was omitted. Several passages through this medium were needed to prevent carry-over of growth factors from inocula. Assimilation of carbon compounds was also investigated by using yeast extract-omitted medium C. Growth in the presence of 30 % glucose was detected by using a medium that contained (l^{-1}): 300 g glucose, 5 g peptone, 3 g yeast extract and 3 g malt extract. Production of gluconic and keto-gluconic acids was examined by using a medium that contained (l^{-1}): 30 g glucose and 3 g yeast extract (pH 6.8); products were determined by HPLC. Growth in the presence of 0.35 % acetic acid was examined by using a medium that contained (l^{-1}): 10 g glucose, 5 g peptone, 3 g yeast extract, 3 g malt extract and 3.5 g acetic acid.

Cells were Gram-negative and rod-shaped, measuring 0.5–0.8 × 1.5–2.0 μm. Of the 14 strains tested, only five showed motility. Cells that were cultivated on glucose at 25 °C were more actively motile than those that were cultivated on methanol and incubated at 30 °C. Strains LMG 1667, LMG 1669, TS 0101, TS 0131 and TS 0156 showed motility when cultivated on glucose at 25 °C. Cells that were incubated at lower temperatures were more actively motile than those incubated at the optimal temperature. This finding was reported previously for acetic acid bacteria (Leifson, 1954; Asai *et al.*, 1964). Motile cells showed a single polar flagellum or, occasionally, polar tuft flagella (Fig. 2). Uhlig *et al.*

(1986) described peritrichous flagella of *Acetobacter methanolicus* but, in contrast, Urakami *et al.* (1989) reported no motility of *Acidomonas methanolica*. All *Acidomonas*

Fig. 2. Flagellation of *Acidomonas methanolica* LMG 1667 grown on YPM agar at 25 °C. A single polar flagellum (top) and polar tuft flagella (bottom) (found very rarely).

methanolica strains were unable to grow without pantothenic acid in yeast extract-omitted medium C. This indicated the requirement of pantothenic acid for growth of *Acidomonas methanolica*, as reported previously (Uhlir *et al.*, 1986; Urakami *et al.*, 1989). In addition, this pointed to the utilization of ammoniacal nitrogen as the sole source of nitrogen by *Acidomonas methanolica* strains when pantothenic acid was supplied. All strains tested grew on methanol, but glucose was preferred over methanol for growth. The strains produced catalase but not oxidase, as did acetic acid bacteria. Urakami *et al.* (1985) reported the production of oxidase by *Acidomonas methanolica*; oxidation of acetate to CO₂ and H₂O by *Acidomonas* strains was detected readily by the method of Leifson (1954). Oxidation of lactate was not detected clearly by the method of Leifson (1954), but was detected in some strains by the method of Shimwell *et al.* (1960). Of the 14 strains tested, seven strains oxidized lactate weakly. The strains tested did not produce dihydroxyacetone from glycerol. Uhlir *et al.* (1986) reported weak ketogenesis from glycerol, but Urakami *et al.* (1989) did not. No strains tested produced urease. Production of acetic acid from ethanol was determined by GC (Urakami *et al.*, 1989). Acid was produced from D-glucose and eight other sugars and sugar alcohols, but not from D-fructose or 13 other sugars, sugar alcohols or alcohols. Production of acid from D-arabinose and four other sugars and alcohols varied with strains. Of the 14 strains tested, four strains produced acid from methanol and 10 did weakly. Production of acid from sugars and sugar alcohols did not agree with the data of Uhlir *et al.* (1986) and Urakami *et al.* (1989). This may be due to medium compositions and procedures. Assimilation of carbon compounds was tested under nutritionally restricted conditions. D-Glucose, D-mannose, glycerol, succinate, ethanol and methanol were assimilated by all strains tested, but D-fructose, L-arabinose, trehalose, maltose, mannitol and inositol were not. Assimilation of D-galactose, D-ribose, D-xylose, citrate, malate, lactate and acetate varied with strains. There were differences in assimilation patterns between the data of Uhlir *et al.* (1986), Urakami *et al.* (1989) and the present study. This is due to the methods employed: Uhlir *et al.* (1986) employed auxanography by using a medium that contained yeast extract and Urakami *et al.* (1989) employed a liquid medium that contained yeast extract. pH range for growth was a decisive character to delineate the genus *Acidomonas*. The 14 strains grew at mostly the same extent at pH 3.0–8.0. Exceptionally, strains LMG 1735, TS 0101, TS 0128, TS 0143 and TS 0156 grew at pH 2.5 and TS 0128 grew at pH 8.5. Strain TS 0135 did not grow at pH 7.5 or 8.0. Uhlir *et al.* (1986) reported growth of *Acetobacter methanolicus* below pH 6.5 and optimally at pH 4.0 and 4.5. Urakami *et al.* (1989) described growth of *Acidomonas methanolica* between pH 2.0 and 5.5 and no growth at pH 1.5 or 6.0. The strains tested grew in the presence of 30% glucose and 0.35% acetic acid and produced gluconic acid from glucose, but not ketogluconic acids. This finding was reported by Uhlir *et al.* (1986). This feature will be due to high

oxidative activity and complete oxidation of glucose at low concentrations. When high concentrations of glucose are supplied, products other than gluconic acid may accumulate. Major cellular fatty acids were reported to be C_{18:1} and C_{16:0} and hydroxy acids were 2-hydroxy C_{16:0} and 3-hydroxy C_{16:0} (Urakami & Komagata, 1987). 2-Hydroxy C_{16:0} was found only in *Acidomonas* strains, compared to other methanol-utilizing bacteria (Urakami & Komagata, 1987). Other phenotypic characteristics are given in the description of the genus *Acidomonas*.

The genus *Acidomonas* is phylogenetically located within the *Acetobacteraceae* in the α -*Proteobacteria* and shares some characteristics with acetic acid bacteria, such as being catalase-positive and oxidase-negative, production of gluconic acid from glucose and growth in the presence of 0.35% acetic acid. However, methanol utilization and a unique ecological niche characterize the genus *Acidomonas*. In addition, the genus *Acidomonas* should be regarded as acidotolerant but not acidophilic, as strains of *Acidomonas methanolica* are able to grow at mostly the same extent at pH 3.0–8.0. Some characteristics of *Acidomonas methanolica* (*Acetobacter methanolicus*) differed in the descriptions by Uhlir *et al.* (1986) and Urakami *et al.* (1989). These differences were made clear in the present study. Therefore, emendation of the genus *Acidomonas* and the species *Acidomonas methanolica* is proposed.

Emended description of *Acidomonas* Urakami *et al.* emend. Yamashita, Uchimura and Komagata

Acidomonas (A.ci.do.mo' nas. Gr. adj. *acid* acid; Gr. n. *monas* unit, monad; N.L. fem. n. *Acidomonas* acidophilic monad).

Cells are Gram-negative, short rods with rounded ends, measuring 0.5–0.8 × 1.5–2.0 µm, and are occasionally up to 4 µm in length. Cells occur singly, in pairs or (rarely) in short chains and are either motile by a single polar flagellum or non-motile. Cells with polar tuft flagella are found very rarely. Non-spore-forming. Aerobic. Catalase-positive. Oxidase-negative. Colonies on PYM agar (pH 4.5) are shiny, smooth, circular, convex, entire, beige to pink and 1–3 mm in diameter after 5 days at 30 °C. Pellicles are produced in PYM medium, but they are not real cellulose. Acetic acid is produced from ethanol. Dihydroxyacetone is not produced from glycerol. Acid is produced from L-arabinose, D-xylose, D-ribose, D-galactose, D-mannose, D-glucose, glycerol, n-propanol, n-butanol, 2-methyl propan-1-ol, ethanol and methanol, but not from L-rhamnose, D-fructose, sucrose, maltose, lactose, raffinose, trehalose, D-mannitol, inositol, D-sorbitol, dulcitol or soluble starch. Production of acid from D-arabinose, D-ribose, melibiose and 2-methyl propan-1-ol varies with strains. Methanol, ethanol, D-glucose, D-mannose, glycerol and succinic acid are utilized as sole sources of carbon, but D-fructose, L-arabinose, maltose, trehalose, inositol and D-mannitol are not utilized. Acetate is oxidized, but lactate is not or is only weakly oxidized. Urease-negative.

Pantothenic acid is an essential requirement for growth. Growth occurs at the same extent between pH 3.0 and 8.0; acidotolerant. Growth occurs at 30 °C, but not at 45 °C. Growth occurs in the presence of 30 % glucose and 0.35 % acetic acid. Gluconic acid is produced from glucose, but 2-keto-, 5-keto- and 2,5-diketogluconic acids do not accumulate in culture media. DNA G+C content ranges from 62 to 63 mol%. Major cellular fatty acids are C_{18:1} and C_{16:0} and major hydroxy acids are 2-OH C_{16:0} and 3-OH C_{16:0}. Major ubiquinone is Q-10. Distributed in activated sludge. The type species is *Acidomonas methanolica* (Uhligh *et al.*) Urakami *et al.* emend. Yamashita, Uchimura and Komagata.

Emended description of *Acidomonas methanolica* Urakami *et al.* emend. Yamashita, Uchimura and Komagata

Acidomonas methanolica (me.tha.no'li.ca. N.L. adj. *methanolica* name of a chemical compound, of methanol).

Basonym: *Acetobacter methanolicus* Uhligh *et al.* 1986.

The type strain is IMET 10945^T=DSM 5432^T=JCM 6891^T=LMG 1668^T=NRIC 0498^T=MB58^T. Isolated from a septic methanol/yeast process (Steudel *et al.*, 1980; Uhligh *et al.*, 1986). Characteristics are the same as those given in the description of the genus. Cells are non-motile. DNA G+C content of the type strain is 62 mol%.

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