Desulfovirga adipica gen. nov., sp. nov., an adipate-degrading, Gram-negative, sulfate-reducing bacterium

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INTRODUCTION

Adipic acid is a natural product contained in the lichen Cetraria islandica (Solberg, 1986) and in leaves of the plant Limonia acidissima (Das & Thakur, 1989). This acid is also synthesized industrially as a raw material to synthesize polymers such as ‘nylon 6,6’, polyurethanes, and plasticizers such as di(2-ethylhexyl)-adipate, all of which are important industrial products. This acid is known to be degraded aerobically, for example by Pseudomonas saccharophila (Palleroni, 1984). To the authors’ knowledge, however, its anaerobic degradation by a pure culture of a sulfate reducer has not been documented. Here, we describe the isolation and characterization of a novel Gram-negative, mesophilic and sulfate-reducing strain capable of growing by completely oxidizing adipate.

METHODS

Source of the isolate and micro-organisms. Strain TsuAS1T was isolated from an anaerobic digester of a municipal wastewater treatment centre in Tsuchiura, Ibaraki, Japan, and was deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany, as strain DSM 12016T. Methanospirillum hungatei strain JF1 (DSM 864) was provided by Y. Kamagata (NIBH, Japan).

Chemicals. (E)- and (Z)-2-hexenedioates were synthesized as follows. γ-Butyrolactone was treated with p-toluenesulfonic acid in ethanol to give ethyl 4-hydroxybutyrate. Oxidation of ethyl 4-hydroxybutyrate by DMSO-oxalyl chloride/triethylamine afforded an intermediate aldehyde, which was subjected to the Wittig reaction with (carbethoxymethylene)-triphenylphosphorane in methanol to give a mixture of diethyl (E)- and (Z)-2-hexenedioates (about 2:1). After chromatographic separation over silica gel, each isomer was finally hydrolysed with lithium hydroxide, followed by acid treatment to give the desired (E)- and (Z)-2-hexenedioic acids.

(E)-2-Hexenedioic acid: colourless powder; m.p. 179–180 °C; 1H NMR (500 MHz, DMSO-d6) d 2.37 (m, 4H), 5.76

The GenBank/DBJ accession number for the 16S rDNA gene sequence of strain TsuAS1T is AJ237605.
(Z)-2-Hexenedioic acid: colourless powder; m.p. 78–80 °C; 1H NMR (500 MHz, DMSO-d$_6$) δ 2.33 (t, J = 7.4 Hz, 2H), 2.74 (dq, J = 1.7 and 7.4 Hz, 2H), 5.72 (dt, J = 11.5 and 1.7 Hz, 1H), 6.19 (dt, J = 11.5 and 7.4 Hz, 1H), 12.14 (broad s, 2H); 13C NMR (125 MHz, DMSO-d$_6$) δ 24.2, 33.1, 121.3, 147.8, 167.2, 173.9; IR (KBr), 2939, 1701, 1649, 1604, 1450, 1419, 1389, 1300, 1261, 1226, 1160 cm$^{-1}$. Elemental analysis. Calculated for C$_5$H$_8$O$_2$: C, 50.00; H, 5.59. Found: C, 50.29; H, 5.77. (s, singlet; d, doublet; m, multiplet; t, triplet; dt, double triplet; dq, double quartet)

Hexanoic acid and heptanoic acid were purchased from Wako and Aldrich, respectively.

**Culture conditions.** Strain TusuAS1$^T$ was cultured using the Hungate technique (Bryant, 1972). A basal medium contained 2 mM Na$_2$S, 1 mM resazurin, 30 mM NaHCO$_3$, 1 mM NH$_4$Cl, 1 mM KH$_2$PO$_4$, 1 mM MgCl$_2$, 1 mM CaCl$_2$, 10 µM FeCl$_3$, 1 µM CoCl$_2$, 1 µM MnCl$_2$, 1 µM ZnCl$_2$, 100 mM H$_2$BO$_3$, 100 mM NiCl$_2$, 100 mM Na$_2$MoO$_4$, 100 mM AlCl$_3$, 10 mM Na$_2$SeO$_3$, 10 mM Na$_2$WO$_4$, 10 mM CuCl$_2$, 100 mM biotin, 100 mM 4-aminobenzoic acid, 100 mM pantothenic acid, 100 mM pyridoxine, 100 mM nicotinic acid, 100 mM thiamine, 100 mM lipoic acid, 100 mM folic acid, 100 mM riboflavin and 100 mM vitamin B$_12$, under an atmosphere of N$_2$/CO$_2$ (80:20, v/v). The medium was prepared using a Widdel flask (Widdel & Bak, 1992). The pH of the basal medium was 6.9–7.1 unless otherwise stated. When pH dependence was studied, the N$_2$/CO$_2$ ratio was varied in a range between 100:0 and 50:50. For sulfate reduction, 5 mM sodium sulfate was added unless otherwise stated. A time course study was carried out using half-filled 150 ml serum bottles in duplicate. Substrate tests were carried out using half-filled 20 ml Pyrex tubes in duplicate for water-soluble substrates. Lipophilic substrates such as decanoic acid (5 mM), undecanoic acid (5 mM), dodecanoic acid (5 mM), tridecanoic acid (5 mM), 1-hexanol (10 mM), 1-heptanol (5 mM), 1-octanol (5 mM), 1-nonanol (5 mM), 1-decane (5 mM), 1-undecanol (5 mM), 2-phenylethanol (50 mM), 2-phenylpropanol (50 mM) and indole (10 mM) were prepared in sterile stock solutions in decahydronaphthalene at the concentrations indicated. A test tube for the lipophilic substrate test was prepared to contain 2.0 ml substrate solution and 19.2 ml basal medium in a 25 mm (o.d.)×150 mm order-made screw-capped tube plugged with a Teflon-coated butyl rubber stopper, which was then inoculated with 0.8 ml preculture to give a final aqueous volume of 200 ml. In this case, one-tenth of the substrate concentration in decahydronaphthalene is given in parentheses in Results to make it easy to compare with results with hydrophilic substrates. The inoculum size was 4% (v/v), as with hydrophilic substrates. A pure culture was obtained by application of the agar dilution method (Pfenning, 1978). To test the capacity of strain TusuAS1$^T$ for syntrophic growth on each of adipate, ethanol, 1-propanol, lactate, propionate and butyrate without sulfate in co-culture with *Methanospirillum*, strain TusuAS1$^T$ was pre-cultured on each of the substrates in the presence of sulfate.

**Growth determination.** Cell density was determined with a Shimadzu UV-1600PC spectrophotometer at 600 nm using 1 cm cuvettes.

**Light microscopy.** Cells were observed with an Olympus BH-2 phase-contrast microscope. Micrographs were taken after mounting cultures on glass slides coated with 2% agar (Pfenning & Wagener, 1986).

**Electron microscopy.** Cells on the grid were negatively stained with 1% uranyl acetate. Photographs were taken using a JEM-1010 transmission electron microscope (JEOL) at 100 kV.

**Analysis of adipate and metabolic products.** Determination of adipate, (Z)-2-hexenedioic and (Z)-2-hexenedioic was carried out as follows. Culture fluid (1 ml) was centrifuged in a 1.5 ml polypropylene tube. Then, 0.45 ml supernatant was transferred to a new 1.5 ml polypropylene tube and acidified with 50 µl 1 M H$_2$PO$_4$ followed by analysis of the acidified supernatant by HPLC using a 6×150 mm Shim-pack CLC-ODS column (Shimadzu) at 40 °C, 9 mM H$_2$PO$_4$/2.5% (v/v) methanol as eluant at 1 ml min$^{-1}$, and a UV detector at 210 nm. Benzoate was determined similarly, except 3 mM H$_2$PO$_4$/49% methanol was as eluant (Tanaka et al., 1991). Sulfide was determined spectrophotometrically via a method yielding methylene blue (Cline, 1969).

**Isolation of DNA and base composition.** Cells were lysed by treatment with achromopeptidase and SDS (Ezaki et al., 1990). DNA was isolated from the resultant lysate (Marmur, 1961) and base composition of DNA was determined using HPLC (Tamaoka & Komagata, 1984; Kamagata et al., 1992).

**16S rDNA sequence determination.** Genomic DNA extraction, PCR-mediated amplification of the 16S rDNA and sequencing of the PCR products were performed as described by Rainey et al. (1996). The sequence reaction mixtures were electrophoresed using a model 373A automatic DNA sequencer (Applied Biosystems).

**Phylogenetic analysis.** The 16S rDNA sequence of strain TusuAS1$^T$ was aligned to the sequences of a general selection of different bacteria in the DSMZ identification database, and analysed against members and relatives of the δ-subclass of the Proteobacteria using the aa2 editor (Maidak et al., 1997). Evolutionary distances were calculated by the method of Jukes & Cantor (1969). Phylogenetic dendrograms were constructed according to the method of DeSoete (1983) and the neighbour-joining method contained in the PHYLIP package (Felsenstein, 1993).

**RESULTS**

**Enrichment and isolation.** Liquid medium containing 1 mM adipate and 5 mM sulfate in tubes was inoculated with sludge, mud and sand samples from anaerobic digesters, a creek, a pond and a lake, and was incubated at 30 °C. Slight growth was observed after 3 months with a tube inoculated with a sludge sample from an anaerobic digester of the municipal wastewater treatment centre in Tsuchiura, Ibaraki, Japan. The obtained enrichment culture could be subcultured and was subjected to serial liquid dilution with liquid medium for partial
purification. Yeast extract at 0·5 g l\(^{-1}\) enhanced growth of the partially purified enrichment culture. Following agar dilution, lens-shaped white colonies appeared after 4 weeks. A colony in the highest dilution tube was transferred into liquid medium. Purity of the culture (designated strain TsuAS1\(^T\)) was confirmed by cell morphology under the phase-contrast microscope and by the lack of growth on any of glucose (2 mM), peptone (1 g l\(^{-1}\)) and yeast extract (1 g l\(^{-1}\)).

**Morphology**

Cells of strain TsuAS1\(^T\) were Gram-negative, straight or slightly curved short rods, single or in pairs, with rounded ends having the shape of a rice grain (Fig. 1). The majority of the cells were 2·2–40 µm long and 0·8–2·0 µm wide (Fig. 1). Cells were motile and had a single polar flagellum (Fig. 2). Spores were never observed in the culture of strain TsuAS1\(^T\) under the microscope and pasteurized digested sludge from which strain TsuAS1\(^T\) was isolated did not yield microorganisms which could utilize adipate in the presence of sulfate over a 7 month period.

**Physiological characterization**

Growth of the new isolate was observed between pH 6·6 (not tested below pH 6·6) and pH 7·4 with an optimum around pH 7·0. No growth occurred at pH 7·6. Strain TsuAS1\(^T\) grew at 20–36 °C with an optimum around 35 °C. At temperatures of 15 and 37 °C, no growth occurred. Under optimal conditions, strain TsuAS1\(^T\) grew at a rate of 0·06–0·07 d\(^{-1}\) (doubling time = 10–12 d). Growth of strain TsuAS1\(^T\) was enhanced by yeast extract and the growth yield was optimal at 0·5–1 g yeast extract l\(^{-1}\) as estimated from OD\(_{600}\) measurements (data not shown). In the presence of 100 µM EDTA, the yield was optimal at 0·02 g yeast extract l\(^{-1}\) (a comparison between the optimal growth yield with and without EDTA was not carried out). Nitrilotriacetate and citrate were not as effective as EDTA. Addition of 70 mM sodium chloride inhibited growth. In the presence of 2 mM sulfide, growth was inhibited by the addition of 2 mM cysteine or thioglycolic acid.

**Metabolism**

With adipate provided as electron donor, strain TsuAS1\(^T\) utilized sulfate, sulfite, thiosulfate and elemental sulfur (weak growth) as electron acceptors. Nitrate was not utilized as electron acceptor. Consumption of 1 mM adipate and 5 mM sulfate (excess), lead to the production of 2·90 ± 0·06 mM H\(_2\)S. Electron recovery was 89±2 % as calculated based on complete oxidation of adipate. Trace amounts (about 6 µM) of (E)-2-hexenedioate accumulated transiently during the oxidation of adipate, while (Z)-2-hexenedioate was not detected (see Fig. 3).

Electron donors utilized by strain TsuAS1\(^T\) include adipate (1 mM), (E)-2-hexenedioate (1 mM), (E)-3-hexenedioate (1 mM), pyruvate (2 mM), lactate (2 mM), formate (10 mM), acetate (2 mM), propionate (2 mM), butyrate (1 mM), valerate (1 mM), caproate (1 mM), heptanoate (1 mM), octanoate (0·5 mM), nonanoate (0·5 mM), decanoate (0·5 mM), undecanoate (0·5 mM), dodecanoate (0·5 mM), isobutyrate (1 mM), isovalerate (1 mM), isocaproate (1 mM), 3-phenylpropionate (0·5–2 mM), ethanol (2 mM), 1-propanol (2 mM), 1-butanol (1 mM), 1-pentanol (1 mM), 1-hexanol (1 mM), 1-heptanol (0·5 mM), 1-octanol (0·5 mM), 1-nonanol (0·5 mM) and 1-decanol (0·5 mM). The amount of H\(_2\)S produced was close to the amount expected for complete oxidation of substrates, except with decanoate, undecanoate, dodecanoate and 1-decanol (stoichiometry not studied with these substrates). 3-Phenylpropionate was almost stoichiometrically oxidized to benzoate (98±3 mol%).

Electron donors not utilized for growth by strain TsuAS1\(^T\) include H\(_2\)/CO\(_2\) (80:20, v/v), (Z)-2-hexenedioate (1 mM), methylmalonate (2 mM), malonate (2 mM), succinate (2 mM), glutarate (1 mM), pimelate (1 mM), suberate (1 mM), muconate (1 mM), fumarate (2 mM), maleate (2 mM), L-malate (2 mM), δ-malate (2 mM), tricarboxysalicylate (0·5 mM), benzoate (1 mM), cinnamate (0·5 mM), phenylacetate (1 mM), 2-phenylpropionate (0·5–5 mM), 4-phenylbutyrate (0·5–5 mM), 6-phenylhexanoate (0·5 mM), cyclohexane-carboxylate (1 mM), methanol (2–5 mM), 2-methoxy-ethanol (2 mM), ethylene glycol (2 mM), 1,3-propandiol (2 mM), 1,4-butanediol (2 mM), 1,5-pentanediol (2 mM), 1-undecanol (0·5–5 mM), 2-phenylethanol (5 mM), 2-phenylpropanol (5 mM), phenol (1 mM), catechol (1 mM), glucose (1 mM), fructose (1 mM), xylose (1 mM), mannitol (1 mM), acetone (2 mM) and indole (1 mM).

**DNA base composition**

The G + C content of the DNA was 59·9 ± 0·5 mol %.
Fig. 2. Electron micrograph of a single, flagellated cell of strain TsuAS1T negatively stained with 1% uranyl acetate. Bar, 1 µm.

Fig. 3. Time course of anaerobic oxidation of adipate by strain TsuAS1T. Two bottles showed similar profiles; data from one bottle are shown. ▼, pH; ● (dotted line), OD600; ▲, (E)-2-hexenedioate; ■ (solid line), H2S; ■, adipate.

Pigments

Desulfoviridin was not detected in cell-free extract of strain TsuAS1T. A redox difference spectrum of dithionite-reduced minus air-oxidized cell-free extract had peaks at 421, 523 and 552 nm, indicating the presence of cytochrome c.

Phylogenetic analysis

The phylogenetic analysis of the 1524 nt 16S rDNA sequence revealed that strain TsuAS1T clusters with Syntrophobacter sp. strain HP1.1 (Zellner et al., 1996), Syntrophobacter fumaroxidans DSM 10017T (Harmsen et al., 1998), Syntrophobacter pfnennii DSM 10092T (Wallrabenstein et al., 1995), Desulforhabdus amnigenus DSM 10338T (Oude Elferink et al., 1995) and Syntrophobacter wolinii DSM 2805T (Boone & Bryant, 1980), only strains TsuAS1T and Desulforhabdus amnigenus DSM 10338T do not grow in co-culture with Methanospirillum hungatei on propionate and oxidized acetate. In spite of the physiological similarity and a moderate phylogenetic relationship these two strains do not form a phylogenetically consistent ‘non-syntrophic subcluster’ within the genus Syntrophobacter. However, the position of Syntrophobacter wolinii DSM 2805T as the deepest branching strain of the genus Syntrophobacter is not supported by a high bootstrap value, indicating a need for future extension of the database so that the non-syntrophic members may fall outside the radiation of the genus Syntrophobacter. It seems reasonable to consider that strain TsuAS1T represents a new species in a new genus, Desulfovirga adipica gen. nov., sp. nov., in the δ-subclass of the Proteobacteria.

At the moment it is unknown whether the transient accumulation of (E)-2-hexenedioate during batch culture growth of strain TsuAS1T on adipate and sulfate, and the ability of the strain to utilize (E)-2-hexenedioate as electron donor, indicates that adipate is degraded via (E)-2-hexenedioate, i.e. by β-oxidation.

Concerning growth of strain TsuAS1T, colonies appeared after 4 weeks in spite of the doubling time of 10–12 d. It seems that the strain grows faster in agar medium than in the liquid medium. In addition, it is unknown why the yeast extract concentration for optimal growth was lowered in the presence of 100 µM EDTA.
Description of Desulfovirga gen. nov.

Desulfovirga (de.sul.fo.vir'ga. M.L. pref. desulfo desulfuricating; L. fem. n. virga twig, rod; Desulfovirga sulfate-reducing rod).

Rod-shaped cells. No spore formation. Gram-negative. Strictly anaerobic. Chemo-organotrophic. Sulfate, sulfite, thiosulfate and sulfur are utilized as electron acceptors. Nitrate is not utilized. Desulfoviridin has not been detected. Cytochrome c is present. Propionate is not utilized in the absence of sulfate in co-culture with Methanospirillum hungatei. Growth occurs at 20–36 °C. G+C content of DNA is 60 mol%. According to 16S rDNA analysis, Desulfovirga represents a new genus, belonging to the δ-subclass of the Proteobacteria. Type species is Desulfovirga adipica.

Description of Desulfovirga adipica sp. nov.

Desulfovirga adipica (a.di'pi.ca. M.L. n. acidum adipic acid (adipate); M.L. fem. adj. adipica pertaining to adipic acid and the organism’s ability to degrade it).

Cells are 0.8–2.0 × 2.2–4.0 µm with round ends, singly or in pairs. Motile. In the presence of excess sulfate, adipate, (E)-2-hexenedioate, (E)-3-hexenedioate, pyruvate and lactate, C₁₅₋C₉ straight-chain fatty acids, C₁₀₋C₂₀ iso-fatty acids and C₆₋C₈ straight-chain primary alcohols are completely oxidized and 3-phenylpropionate is stoichiometrically oxidized to benzoate. Straight-chain saturated C₀₋C₂ω-dicarboxylates are not utilized except adipate. G+C content of DNA is 60 mol%. Habitat: anaerobic digestor of the municipal wastewater treatment centre, Tsuchiura, Ibaraki, Japan. Type strain is TsuAS1T (= DSM 12016T).

ACKNOWLEDGEMENTS

We thank Professor K. Kakinuma (Tokyo Institute of Technology, Japan) for encouragement to this work and helpful discussions, and Professor H. G. Truper (University of Bonn, Germany) for advice on the naming of strain TsuAS1T.

REFERENCES


