***Caldimonas manganoxidans* gen. nov., sp. nov., a poly(3-hydroxybutyrate)-degrading, manganese-oxidizing thermophile**

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A poly(3-hydroxybutyrate) (PHB)-degrading, Gram-negative, aerobic bacterium, strain HS^T, was isolated from a hot spring and chemotaxonomically and phylogenetically characterized. The oxidase-positive, weakly catalase-positive, non-pigmented cells (0.6 × 2.6 µm) exhibited a single polar flagellum and accumulated PHB granules. Strain HS^T was capable of manganese oxidation. Highest growth rate was attained at 50 °C. The optimum pH for growth was 7–8. The major respiratory quinone was ubiquinone-8 and major cellular fatty acids were C_{16:0}, C_{16:1} and C_{18:1}. The G+C content of the DNA was 66.2 mol%. Comparative 16S rDNA analysis indicated that strain HS^T is related to the *Rubrivivax* subgroup and the family *Comamonadaceae*. The nearest phylogenetic relatives were *Ideonella dechloratans* (92.1% similarity), *Leptothrix discophora* (93.6%), *Roseateles depolymerans* (92.4%) and *Rubrivivax gelatinosus* (92.2%). On the basis of its phylogenetic and phenotypic properties, it is proposed that this isolate be designated *Caldimonas manganoxidans* gen. nov., sp. nov.; the type strain is HS^T (= JCM 10698^T = IFO 16448^T = ATCC BAA-369^T).

Keywords: thermophile, manganese oxidation, poly(3-hydroxybutyrate) degradation, hot spring, *Caldimonas manganoxidans*

INTRODUCTION

Poly(3-hydroxybutyrate) (PHB) is a carbon and energy storage material of micro-organisms. PHB is physiologically related to polypropylene and polyethylene (Howells, 1982; Byrom, 1987). Biological degradation of PHB and related polymers have been extensively studied in *Alcaligenes*, *Ralstonia*, *Bacillus*, *Pseudomonas* and *Comamonas* strains that grow optimally at moderate temperatures (Blandl *et al.*, 1995; Jendrossek *et al.*, 1996). In a previous study, a moderately thermophilic bacterium capable of PHB degradation was isolated from a hot spring in Matsue, Japan, and its extracellular PHB depolymerase was purified and characterized (Takeda *et al.*, 1998). The gene encoding the polymerase was identified and expressed in *Escherichia coli* (Takeda *et al.*, 2000). The gene exhibited a

similarity to the PHB depolymerase genes of *Comamonas* spp. The isolate (strain HS^T) is a Gram-negative, aerobic, rod-shaped bacterium that grows optimally at 45–50 °C. It has also been suggested that the isolate was oxidized manganese (Takeda *et al.*, 1998), a property shared by a number of other bacteria, including members of the genus *Leptothrix* (Ghiorse, 1984), which are known for their capacity to deposit iron and manganese oxides. In natural ecosystems and under some cultural conditions, *Leptothrix* spp. characteristically form sheaths comprised of protein and polysaccharide fibrils and the sheaths are encrusted with iron or manganese oxides (Emerson & Ghiorse, 1992, 1993a, b). *Leptothrix cholodnii* SP-6 (formerly *Leptothrix discophora* SP-6) (Spring *et al.*, 1996) is a well-studied manganese-oxidizing bacterium whose oxidizing activity is associated with the sheath (Emerson & Ghiorse, 1992). Our preliminary phylogenetic analysis based on 16S rDNA using the FASTDNAML program (Olsen *et al.*, 1994) indicated that strain HS^T is closely related to *Comamonas testosteroni* and *Leptothrix* spp. (Takeda *et al.*, 1998).

Abbreviation: PHB, poly(3-hydroxybutyrate).

The GenBank/EMBL/DBJ accession number for the 16S rRNA gene sequence of strain HS^T is AB008801.

The current paper reports more detailed chemotaxonomic and phylogenetic characteristics of strain HS^T compared with those of several related taxa. It is proposed that strain HS^T represents a new species of a new genus, designated *Caldimonas manganoxidans* gen. nov., sp. nov.

METHODS

Cultivation. Strain HS^T was isolated from a hot spring as a thermophilic PHB-degrading bacterium (Takeda *et al.*, 1998). Unless otherwise described, it was cultivated without shaking in PYG medium (Adams & Ghiorse, 1986a). To maintain the pH of the medium, 2.38 g HEPES l⁻¹ was added and the pH was adjusted to 7.2 with NaOH before autoclaving. After cultivation, cells were harvested by centrifugation.

Quinone analysis. Quinones were extracted from lyophilized cells with chloroform-methanol (2:1, v/v). The extract was passed through a Sep-Pak Plus column (Waters) and analysed by reverse-phase HPLC (Beckman System Gold with a Hewlett Packard Zorbox ODS column) for identification (Tamaoka *et al.*, 1983).

Cellular fatty acid analysis. Cellular fatty acids were converted to methyl esters by treatment with anhydrous methanolic HCl. The methyl esters were analysed using a Hitachi M7200A GC/3DQMS system equipped with a DB-5ms capillary column (30 m × 0.25 mm; J & W Scientific) coated with (5%-phenyl)-methylpolysiloxane (thickness 250 nm). Helium was used as the carrier gas at a flow rate of 1.5 ml min⁻¹. The column temperature profile was as follows: 100 °C for 1 min, then increased to 280 °C at a rate of 18 °C min⁻¹ and held at 280 °C for 12 min.

Electron microscopy. For visualization of the flagella, cells were statically grown for 24 h in the absence of manganese. Cells were harvested by centrifugation and negatively stained with 2% (w/v) uranyl acetate, then subjected to transmission electron microscopy (Phillips EM 300). Cells for thin-section electron microscopy were fixed with 5% (w/v) glutaraldehyde for 6 h and then post-fixed in 2% (w/v) osmium tetroxide at 4 °C for 3 h. The fixed cells were stained with uranyl acetate for 1 h at room temperature, dehydrated and embedded in Spurr low-viscosity resin. Thin sections of the cells were made with an ultramicrotome (Reichert ULTRACUT) and were examined using a transmission electron microscope (Hitachi H7000).

DNA base composition. Total DNA of cells of strain HS^T was extracted according to the procedure of Saito & Miura (1963). Total DNA was digested with P1 nuclease using a Yamasa GC kit (Yamasa Shoyu). The G + C content of the total DNA was measured by HPLC (Kamagata & Mikami, 1991). The reference standard was an equimolar mixture of deoxyribonucleotides included in the kit.

Substrate utilization and growth factor requirement. The capability to utilize various carbon sources was tested on a basal medium containing (l⁻¹): 5 g (carbohydrates and complex organic compounds) or 1 g (organic acids) carbon source; 3 g (NH₄)₂SO₄; 2.6 g K₂HPO₄; 0.1 g NaH₂PO₄; and 0.2 g MgSO₄·7H₂O. All cultures were incubated for 3 d. Substrate utilization was determined by monitoring the increase in OD at 660 nm. Strain HS^T was cultured in a

medium containing 2 g glucose, 3 g (NH₄)₂SO₄, 2.6 g K₂HPO₄, 0.1 g NaH₂PO₄ and 0.2 g MgSO₄·7H₂O for 3 d. After cultivation, a 0.1 vol. of the culture was recovered and inoculated into the same medium. This procedure was repeated three times. Growth factor requirement was determined based on whether or not the final culture became turbid.

Determination of PHB accumulation. Cells grown in a test tube for 24 h were fixed onto a glass microscope slide with poly-L-lysine and stained with the Nile red component of Nile blue A (Ostle & Holt, 1982). Orange fluorescence observed in the highly refractile granules of the cells under short-wavelength epi-illumination using a Zeiss LSM-210 microscope established the presence of PHB in the granules.

Manganese oxidation. The extracellular accumulation of manganic oxide during growth of strain HS^T was examined on PYG agar plates supplemented with various concentrations (0–10 mM) of Mn²⁺ (as MnSO₄·2H₂O). The plates were incubated at 47 °C for 7 d and the presence of brownish manganic oxide deposits in the colonies was confirmed by the addition of Leucoberberlin blue reagent (Ghiorse & Hirsch, 1979).

Catalase and oxidase activities. Catalase activity was assayed with 3% (w/v) hydrogen peroxide solution. Oxidase activity was assayed by applying the cells to a moistened commercial test paper strip (Nissui Pharmaceutical). Cells for these tests were cultured on PYG medium for 24 h without added Mn²⁺, as manganic oxides will oxidize the hydrogen peroxide giving a false-positive result.

Effects of temperature and pH on growth. Strain HS^T was inoculated in nutrient broth and incubated at various temperatures with shaking. At intervals of 1–2 h, the OD at 660 nm was measured and the specific growth rate (h⁻¹) was calculated. The pH of nutrient broth was adjusted to pH 4–10 by the addition of 2 M NaOH or 2 M HCl. The medium was then sterilized by filtration. After inoculation, the cultures were incubated for 24 h. Growth was judged by the increase in OD at 660 nm. Salt tolerance was evaluated in nutrient broth supplemented with 0–4% (w/v) NaCl. After inoculation of strain HS^T, cultures were shaken at 47 °C for 18 h. Cultures that showed visible turbidity were scored as tolerant.

Other physiological characterizations. Anaerobic growth was tested by the formation of colonies on PYG agar plates within 5 d in the BBL GasPac Pouch (Becton Dickinson Microbiology Systems). Gram staining was performed using 24 h old cells grown on PYG and nutrient broth at 47 °C by a commercially available Gram definition kit (Favour-G set-S 'Nissui'; Nissui Pharmaceutical). The motility of individual cells (24 h old) was determined by light microscopic observation.

16S rRNA gene sequence and phylogenetic analysis. Almost the full length of the 16S rDNA sequence was previously determined (Takeda *et al.*, 1998). It was aligned with reference sequences by CLUSTAL W (Thompson *et al.*, 1994). Alignment gaps and ambiguous bases were not taken into consideration and 1210 bases were compared. A phylogenetic tree was constructed from the evolutionary distance matrix calculated by the neighbour-joining method (Saitou & Nei, 1987). The neighbour-joining analysis was performed with a MEGA program (Kumar *et al.*, 1993). Bootstrap resampling analysis (Felsenstein, 1985) for 100 replicates was performed in order to estimate the confidence of topologies.

RESULTS AND DISCUSSION

Manganese oxidation

Strain HS^T formed colonies on PYG plates containing 0–4 mM Mn²⁺ within 4 d. Only a few small colonies appeared after incubation for 7 d on plates containing 5 mM Mn²⁺. No colonies formed even after 2 weeks on plates containing 10 mM Mn²⁺. Creamy-grey colonies formed on plates containing 0–1 mM Mn²⁺ and slightly brownish colonies formed on those containing 2–5 mM Mn²⁺. Reaction with Leucoberbelin blue reagent revealed formation of manganese oxide in colonies appearing on the plates containing 0.5–5.0 mM Mn²⁺, whereas no comparable reaction occurred on plates supplied with lower concentrations (0.0–0.1 mM) of Mn²⁺. Colonies grown with 2 mM Mn²⁺ very quickly turned blue upon the addition of Leucoberbelin blue reagent. Additionally, slightly brownish coloured zones, presumably attributable to the forma-

tion of manganese oxides, formed around the colonies in plates containing 2 and 3 mM Mn²⁺.

Morphology

Colonies of strain HS^T on PYG agar or nutrient agar plates were creamy-grey. No diffusible pigment was produced. Granular cell clumps were often observed in liquid culture. The number and size of the clumps increased in the presence of Mn²⁺. Neither filamentous cellular clumps nor sheath formation were observed. Motile cells were observed under the microscope especially in the younger stage of culture without shaking. As shown in Fig. 1(top), strain HS^T had a single polar flagellum. A number of electron-transparent granules were detected by transmission electron microscopy (Fig. 1, bottom). These granules were probably composed of PHB because PHB granules were recognized in Nile red-stained cells by epifluorescence microscopy. The presence of PHB in cells of strain HS^T was previously revealed by GLC (Takeda *et al.*, 1998). Cells of strain HS^T were straight rods 0.5–0.7 µm wide and 2.2–3.5 µm long (Fig. 1). Spores were not observed. Gram-staining was negative. The cells possessed a typical Gram-negative cell wall structure (Fig. 1, bottom).

Substrate utilization and growth properties

Strain HS^T was a chemo-organotrophic bacterium and only grew aerobically. Its optimum growth temperature was 50 °C and growth was not detected at 65 °C. The pH for optimum growth was between 8 and 9. It was oxidase-positive and weakly catalase-positive. Growth factors were not required. Strain HS^T was able to grow at NaCl concentrations ranging from 0 to 2 % (w/v), but not at 3 or 4 %.

Growth of strain HS^T was not observed on the following substrates: D-glucosamine, *myo*-inositol, D-arabinose, L-fucose, D-xylose, D-salicin, trehalose, D-mannose, D-fructose, D-ribose, lactose, L-rhamnose, raffinose, propionate, 4-hydroxybutyrate, oxalate, quinate, acetate and glucuronate. Strain HS^T did grow on D-sorbitol, maltose, sucrose, D-glucose, mannitol, D-galactose, starch, glycerol, L-malate, lactate, citrate, tartrate, succinate, pyruvate, gluconate, malonate, 3-hydroxybutyrate, beef extract, yeast extract, malt extract, tryptone, Casamino acids and peptone. Vigorous growth was observed on beef extract, yeast extract, tryptone, peptone and gluconate.

Chemotaxonomic characteristics

The major respiratory quinone of strain HS^T was ubiquinone-8. The main cellular fatty acids were C_{16:0} (42 % of the total cellular fatty acid methyl ester detected), C_{16:1} (19 %), and C_{18:1} (16 %). Small amounts (2–5 %) of 3-OH C_{10:0}, C_{12:0}, C_{14:0}, C_{14:0}, C_{15:0}, C_{17:1} (or cyclo C_{17:0} and C_{18:0}) were also detected.

Fig. 1. Negatively stained electron micrograph (top; bar, 1 µm) and thin section micrograph (bottom; bar, 0.5 µm) of strain HS^T. The cell bears single polar flagellum (top). Note the electron-transparent intracellular granules (indicated by arrows) and absence of extracellular sheath structure (bottom).

Fig. 2. Phylogenetic position of strain HS^T among the related strains of the β -Proteobacteria. The tree was constructed from the evolutionary distance matrix based on the neighbour-joining method (Saitou & Nei, 1987). Bar, 1 nt substitution per 100 nt in the 16S rDNA sequence. The sequence of *Burkholderia pseudomallei* was used to root the tree. Bootstrap probabilities (Felsenstein, 1985) are indicated at the branch points. Sequence accession numbers are shown next to the strain names.

The G + C content of the total DNA of strain HS^T was 66.2 mol %. The fatty acid composition and the lipoprotein are typical of the β -Proteobacteria.

Phylogenetic analysis and phenotypic comparison

Strain HS^T was closely related to the strains of the *Rubrivivax* subgroup and the family *Comamonadaceae* (Fig. 2). The nearest phylogenetic relatives of strain HS^T were *Ideonella dechloratans* (92.1 % similarity), *L. discophora* (93.6 %), *Roseateles depolymerans* (92.4 %) and *Rubrivivax gelatinosus* (92.2 %).

Table 1 summarizes the properties of strain HS^T that can differentiate it from phylogenetically related strains of the β -Proteobacteria. One of the interesting features of strain HS^T was its ability to oxidize Mn²⁺. The ability has been found in *L. discophora* (Adams & Ghiorse, 1986b; Emerson & Ghiorse, 1992; Spring *et al.*, 1996), which is the closest relative (93.6 % 16S rDNA sequence similarity) to strain HS^T. However, strain HS^T differed from *L. discophora* in that it had a higher growth temperature, was incapable of forming a sheath, had a lower G + C content and in its carbon source utilization. *Sphaerotilus natans* is a close relative of *L. discophora* and forms sheaths, but cannot oxidize Mn²⁺ (Corstjens & Muyzer, 1993; Siering & Ghiorse, 1996). *L. discophora* grows at moderate temperatures, utilizes a wide variety of organic compounds and has a much larger cell size than strain HS^T, thus allowing an easy distinction between the two. *Roseateles depolymerans* has the ability to produce bacteriochlorophyll and carotenoids under certain conditions and forms several polar flagella; these two properties were not observed in strain HS^T. *Rubrivivax gelatinosus* is also a pigmented bacterium due to its bacteriochlorophyll and carotenoid production and can grow photosynthetically; strain HS^T cannot grow photosynthetically. *I. dechloratans* can grow anaerobically by assimilating acetate which is different from strain HS^T. *Alcaligenes*

Table 1. Characteristics of strain HS^T and related species

Taxa: 1, strain HS^T; 2, *Leptothrix discophora*; 3, *Sphaerotilus natans*; 4, *Roseateles depolymerans*; 5, *Rubrivivax gelatinosus*; 6, *Ideonella dechloratans*; 7, *Alcaligenes latus*; and 8, *Tepidimonas ignava*. Data was obtained from the following references: Imhoff & Trüper (1989); Kersters & De Ley (1984); Malmqvist *et al.* (1994); Moreira *et al.* (2000); Palleroni & Palleroni (1978); Suyama *et al.* (1998, 1999); Spring *et al.* (1996); and Willems *et al.* (1991a, b). +, 90 % or more positive; -, 90 % or more absent; d, 11–89 % present; NT, not tested.

Characteristic	1	2	3	4	5	6	7	8
Cell size (μ m)	0.5–0.7 $\times$ 2.2–3.5	0.6–1.4 $\times$ 1.0–12.0	1.2–2.5 $\times$ 2.0–10.0	0.5 $\times$ 2.0	0.4–0.7 $\times$ 1.0–3.0	0.7–1.0 $\times$ 2.5–5.0	1.1–1.4 $\times$ 1.6–2.4	0.5–1.0 $\times$ 1.2–2.0
Flagella	Single, polar	Single, polar	Polar bunch	Several, polar	Single, polar	Several, polar	5–10, peritrichous	Single, polar
Sheath formation	—	+	+	—	—	—	—	—
Photosynthesis	—	—	—	—	+	—	—	—
Pigments	—	—	—	+	+	—	—	—
Mn ²⁺ oxidation	+	+	—	NT	NT	NT	NT	NT
Optimum temp. or range (°C)	50	15–33	10–37	35	35	12–42	35	50–55
Utilization of:								
Acetate	—	—	+	d	+	+	—	+
Lactate	+	—	+	+	+	+	+	+
Pyruvate	+	+	+	+	+	+	—	+
Citrate	+	—	+	+	+	NT	d	—
Glycerol	+	+	+	—	—	NT	+	—
Mannitol	+	NT	+	+	—	NT	—	—
Sorbitol	+	NT	+	—	—	NT	—	—
D-Fructose	—	—	+	+	+	+	+	—
D-Glucose	+	+	+	+	+	+	+	—
D-Galactose	+	NT	+	+	NT	NT	—	—
Sucrose	+	+	+	—	NT	NT	+	—
G + C content (mol %)	66.2	71	69.9	66.2–66.3	70.0–72.5	68.1	69.1–71.1	69

latus is distinguishable from strain HS^T by its short coccoid cell shape and the formation of peritrichous flagella. All these close relatives of strain HS^T mentioned above are mesophilic. *Tepidimonas ignava* is the only thermophilic bacterium ever recognized in the related strains. Its optimum growth temperature of 50–55 °C is similar to that of strain HS^T. However, the following properties of *T. ignava* (Moreira *et al.*, 2000) are different from those of strain HS^T: no carbohydrate is assimilated; short rod cell shape; and one of the major cellular fatty acids is C_{17:0}.

On the basis of its phenotypic characteristics and 16S rRNA gene analysis, it is proposed that strain HS^T represents a new species of a new genus, for which the name *Caldimonas manganoxidans* gen. nov., sp. nov. is proposed.

Description of the genus *Caldimonas* gen. nov.

Caldimonas [Cal.di.mo'nas. L. adj. *calidus* hot; Gr. fem. n. *monas* unit, monad; N.L. fem. n. *Caldimonas* hot (heat-loving) monad].

Cells are motile by means of a single polar flagellum, especially during the early stages of culture without shaking. Gram-negative, aerobic rods (0.5–0.7 × 2.2–3.5 µm). Neither sheath formation nor filamentous growth is observed. Oxidase-positive and weakly catalase-positive. Optimum growth temperature and pH are 50 °C and 8–9, respectively. Many organic acids and carbohydrates are utilized. PHB granules are accumulated as storage material. Chemo-organotroph. The G+C content of the DNA is around 66 mol % and ubiquinone-8 is the major quinone. The type species of the genus is *Caldimonas manganoxidans*.

Description of *Caldimonas manganoxidans* sp. nov.

Caldimonas manganoxidans (man.gan.ox'i.dans. N.L. neut. n. *manganum* manganese; M.L. part. adj. *oxidans* oxidizing; N.L. part. adj. *manganoxidans* manganese-oxidizing).

Cells are rods (average of 0.6 × 2.6 µm), form granular clumps in media supplemented with manganese. Cells slowly and weakly oxidize manganese on agar plates supplemented with 0.5–5.0 mM Mn²⁺. Colonies on PYG plates are circular, convex, opaque and creamy-grey. Growth factors are not required. Growth is not observed in the presence of 3 % (w/v) NaCl. Cultures are not maintained at 65 °C. The following compounds are utilized as energy and carbon sources: D-sorbitol, maltose, sucrose, D-glucose, mannitol, D-galactose, starch, glycerol, L-malate, lactate, citrate, tartrate, succinate, pyruvate, gluconate, malonate, 3-hydroxybutyrate, beef extract, yeast extract, malt extract, tryptone, Casamino acids and peptone. In addition to these substrates, PHB is rapidly degraded and utilized. No growth observed on D-glucosamine, *myo*-inositol, D-arabinose, L-fucose, D-xylose, D-salicin, trehalose, D-mannose, D-fructose, D-ribose, lactose, L-rhamnose, raffinose, propionate, 4-hydroxybutyrate, oxalate,

qui-nate, acetate or glucuronate. The major cellular fatty acids are C_{16:0}, C_{16:1} and C_{18:1}. The type strain is HS^T (= JCM 10698^T = IFO 16448^T = ATCC BAA-369^T). Isolated from a hot spring in Matsue, Japan.

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