***Janibacter brevis* sp. nov., a new trichloroethylene-degrading bacterium isolated from polluted environments**

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Two strains that were strongly able to degrade trichloroethylene were isolated from contaminated environmental samples. The isolates are non-motile, aerobic, non-spore-forming, Gram-positive coccoid to short rods with cell-wall peptidoglycan containing meso-diaminopimelic acid. The major type of menaquinone is MK-8(H₄). The polar lipids were diphosphatidylglycerol, phosphatidylglycerol and phosphatidylinositol. Mycolic acids were absent. The G+C content of the DNA was 72 mol%. Furthermore, comparison of 16S rDNA sequences shows that the isolates clearly belong to the genus *Janibacter*. The isolates differ from *Janibacter limosus* at the species level, as shown by the value for DNA–DNA hybridization. It is therefore proposed that the strains be assigned to the genus *Janibacter* as *Janibacter brevis* sp. nov.

Keywords: *Janibacter brevis* sp. nov., genus *Janibacter*, trichloroethylene-degrading bacterium, rRNA sequence

INTRODUCTION

Large amounts of halogenated aliphatic compounds such as trichloroethylene (TCE) and tetrachloroethylene have been used as solvents for dry cleaning, washing of metal surfaces and other chemical industrial processes. Emission of the compounds into the environment causes contamination of groundwater, soil and sediments. This pollution poses serious health problems because of the toxic and carcinogenic properties of the compounds (Davidson *et al.*, 1982; Infante & Tsongas, 1982). Gram-positive and -negative bacteria that degrade chlorinated aliphatic hydrocarbons aerobically have been found by many investigators. Among the Gram-positive bacteria identified are *Arthrobacter* sp. (Scholtz *et al.*, 1987), *Corynebacterium* sp. m15-3 (Yokota *et al.*, 1987), *Rhodococcus erythropolis* Y2 (Sallis *et al.*, 1990), *Mycobacterium aurum* L1 (Hartmans & De Bont, 1992), *Rhodococcus rhodochrous* and other *Rhodococcus* strains (Malachowsky *et al.*, 1994). Recently, cometabolic degradation of TCE was evaluated in reactors with distinct phenol-feeding patterns (Shin

et al., 1996). However, the active microbes were not specified in systems characterized by higher concentrations of TCE-degrading organisms and higher rates of TCE transformation.

We isolated two strains that were strongly able to degrade TCE from contaminated environmental samples. The isolates belonged to the genus *Janibacter* established by Martin *et al.* (1997). We describe here the phenotypic and phylogenetic characterization of *Janibacter*-like organisms. On the basis of the taxonomic results shown below, a new species, *Janibacter brevis* sp. nov., is described.

METHODS

Isolation and cultivation. Inorganic salt agar medium (Kimbara *et al.*, 1988) was placed at the bottom of a tightly closed plastic container (about 20 ml) and a groundwater sample was inoculated on the surface of the medium with 0.5% agar and 0.1 M phosphate buffer. The aerial part of the vessels contained 8000 p.p.m. TCE vapour and the vessels were incubated at 25 °C until bacterial colonies appeared on the medium. Among the strains obtained from various colonies, two strains, 10N^T and 16N, which showed higher levels of TCE degradation, were selected as representatives. The isolates were stored on plates of LB agar [10 g tryptone (Difco), 5 g yeast extract (Difco), 10 g NaCl and 15 g agar (Difco) l⁻¹] and in frozen LB liquid medium with 15% (v/v) glycerol.

Abbreviation: TCE, trichloroethylene.

The DDBJ accession numbers for the 16S rRNA gene sequences of strains 10N^T and 16N are AB016438 and AB016439.

Morphological and cultural characteristics. For morphological and cultural observation, bouillon agar (NA) containing 10 g meat extract (Kyokuto, Tokyo, Japan), 5 g bacteriological peptone (Oxoid), 5 g NaCl and 15 g agar (Difco) l^{-1} and LB medium with or without agar (Difco) were used at 30 °C. Gram staining was performed with a FAVOR.EG SET.EF kit (Nissui Pharmaceutical Co.). Cells grown on NA were used for acid-fast and motility tests.

Physiological characteristics. Acid production from carbohydrates was determined with a phenol red broth medium (Difco). Utilization of carbon sources was studied with the medium described by Stanier *et al.* (1966) with a small amount of thiamine. Decomposition of adenine, casein, hypoxanthine, tyrosine and xanthine was examined by the method recommended by Gordon *et al.* (1974).

Quinone, lipid, fatty acid, mycolic acid and cell wall analyses. These analyses were carried out by using cells grown in trypticase soy broth (BBL) with 0.5% yeast extract (Difco) (TSY medium). Quinones were extracted twice from lyophilized cells with chloroform-methanol (2:1, v/v) for 20 min, partially purified by TLC with benzene as the solvent and analysed by reverse-phase HPLC (Tamaoka *et al.*, 1983), with a model 510 (Waters) and a Lambda-Max model 481 (Waters) and a Wakosil-II5C18 HG column (4.6 × 150 mm; Wako Pure Chemical Industries), with methanol/2-propanol (2:1, v/v) as the eluting solvent. Polar lipids were extracted and identified by the method of Minnikin *et al.* (1984). Fatty acids, mycolic acids and cell wall were analysed by the method described by Komagata & Suzuki (1987). The glycolyl test was performed by the method of Uchida & Aida (1984).

DNA base composition and DNA-DNA hybridization. DNA was isolated and its G+C content determined by HPLC as described by Tamaoka & Komagata (1984). For DNA-DNA hybridization, levels of DNA relatedness were measured by the method of Takahashi *et al.* (1993) using photobiotin and microimmunoplates.

Amplification and sequencing of 16S rRNA genes. Cells were grown in LB broth, harvested by centrifugation in the late-exponential phase of growth, washed with sterile 1% (w/v) saline, resuspended in 100 µl sterile distilled water and stored at -20 °C until used. The preparation of crude lysates for amplification by PCR, the sequence of oligonucleotide primers, their target sites and electrophoretic separation conditions have been described previously (Hiraishi, 1992; Hiraishi *et al.*, 1994; Katayama *et al.*, 1995).

Phylogenetic analysis. Pairs of sequences were aligned with the GENE WORKS software. Multiple alignment of sequences, calculation of nucleotide substitution rates (K_{nuc}) (Kimura, 1980) and construction of neighbour-joining phylogenetic trees (Saitou & Nei, 1987) were performed with the CLUSTAL w program (Thompson *et al.*, 1994). Alignment positions that included gaps or unidentified bases were eliminated from the calculations. After the alignment process, sequences of 16S rDNA were adjusted manually. Confidence intervals of the branch points were determined by the bootstrap technique (Felsenstein, 1985) with 1000 bootstrapped trials.

Nucleotide sequence accession numbers. The strain designations and accession numbers of reference 16S rRNA sequences are as follows: *Intrasporangium calvum* ATCC 23552^T, D85486; *Janibacter limosus* DSM 11140^T, Y08539; *J. limosus* DSM 11141, Y08540; *Sanguibacter inulinus* NCFB 3024^T, X79451; *Sanguibacter keddii* NCFB 3025^T,

X79450; *Sanguibacter suarezii* NCFB 3023^T, X79452; *Terrabacter tumescens* IAM 12345^T, X83812; and *Terracoccus luteus* DSM 44267^T, Y11928.

RESULTS AND DISCUSSION

Morphological and cultural characteristics

Colonies of both strains 10N^T and 16N are white, opaque and convex with a glistening surface and entire margins on NA and LB media. Cells are Gram-positive, non-acid-fast and non-motile, occur singly, in pairs or occasionally in v-shaped clumps at the early stage of growth. Cells are short rods in young culture, 0.9–1.0 µm in diameter and 1.5–1.8 µm long, and

Fig. 1. Micrographs of cells of strain 10N^T grown on NA medium at 30 °C for 6 h (a) and 24 h (b). Bars, 10 µm.

Table 1. Comparison of morphological and biochemical characteristics of isolates 10N^T and 16N with *J. limosus* strains

Data for *J. limosus* were taken from Martin *et al.* (1997) unless stated otherwise. Characteristics are scored as positive (+) or negative (–). All taxa were positive for the following characters: Gram reaction, obligately aerobic growth, utilization of citrate, decomposition of casein and tyrosine, nitrate reduction, catalase reaction, growth in the presence of 7% NaCl. All taxa were negative for the following characters: motility, spore formation, acid production from L-arabinose, D-fructose, D-glucose, lactose, maltose, sucrose, trehalose, D-raffinose, starch and glycerol, utilization of *cis*-aconitate, *trans*-aconitate, benzoate, succinate and L-tartarate, decomposition of adenine, hypoxanthine and xanthine, production of indole, Voges–Proskauer test, methyl red test, oxidase test, growth in the presence of 10% NaCl.

Characteristic	10N ^T	16N	<i>J. limosus</i> DSM 11140 ^T	<i>J. limosus</i> DSM 11141
Colour of colonies	White	White	White	Yellow
Morphology	Short rod to coccoid	Short rod to coccoid	Coccoid, rod	Coccoid
Utilization of:				
D-Mannose	–	–	+	+
Lactose	+	+	–†	–†
Acetate	+	+	+	–
Formate	–	–	+	+
Hippurate	+	+	–	–
Malate	–	–	+	+
Requirement of thiamine for growth	+	+	–	–
Production of H ₂ S	–	–	+	+
Growth in the presence of:*				
8% NaCl	+	+	+	–
9% NaCl	–	+	–	–

* Cultured for 2 weeks; all data taken from this study.

† Determined in this study.

coccoid cells in the stationary phase of growth that are 1.0–1.2 µm in diameter and 1.2–1.3 µm long (Fig. 1). Spore formation and a marked rod–coccus cycle were not observed. Both strains are obligately aerobic. They grow within a week at 4 and 39 °C and optimal growth was observed at 25–28 °C.

Physiological characteristics

A comparison of physiological characteristics of 10N^T and 16N with two strains of *J. limosus* is shown in Table 1. The characteristics of both strains are similar to those of *J. limosus*, but thiamine is required for growth of the isolates. Acid was not produced from any of the substrates tested (L-arabinose, D-fructose, D-glucose, lactose, maltose, sucrose, trehalose, D-raffinose, starch and glycerol). Lactose and hippurate were utilized only by strains 10N^T and 16N. On the other hand, D-mannose, formate and malate were utilized only by *J. limosus*.

Chemotaxonomic characteristics

Cell wall murein analysis of the two isolates revealed that *meso*-diaminopimelic acid was the dibasic amino acid. The acyl type was acetyl. The organisms did not contain arabinose or galactose, but ribose and glucose were present in whole-cell hydrolysates. Strains 10N^T and 16N resemble each other in their cellular fatty acid

profiles (Table 2). They are characterized by branched fatty acids such as i-C_{15:0}, i-C_{16:0}, i-C_{17:0}, i-C_{17:1}, ai-C_{17:0} and C_{18:1} (Δ¹¹) as well as minor amounts of straight chain unsaturated (C_{16:0}, C_{17:0} and C_{18:0}) and saturated (C_{17:1}) acids. These profiles differ from those of *J. limosus*, which consist of C_{17:1}, C_{17:0} and i-C_{16:0} as major components as well as minor amounts of straight chain saturated and unsaturated fatty acids. The isoprenoid quinone was represented by MK-8(H₄) as the major component. Mycolic acids were absent.

DNA base composition

The DNA base composition of strains 10N^T and 16N was 72 mol % G + C.

DNA–DNA relatedness

The level of DNA–DNA relatedness between strains 10N^T and 16N was 97%. The DNA–DNA relatedness to other species was therefore determined using strain 10N^T. DNA of strain 10N^T exhibited homology values of 51–47% to DNA from two strains of *J. limosus*, DSM 11140^T and DSM 11141. As shown in Table 3, strains 10N^T and 16N both belonged to different species from *J. limosus*.

Table 2. Cellular fatty acid profiles for isolates 10N^T and 16N and *J. limosus* strains

Fatty acid	10N ^T	16N	<i>J. limosus</i> DSM 11140 ^T	<i>J. limosus</i> DSM 11141
i-C _{14:0}	—	—	0.5	0.7
C _{14:0}	—	—	0.4	0.2
i-C _{15:0}	13.8	12.2	1.7	0.7
ai-C _{15:0}	1.2	1.1	0.3	0.1
C _{15:0}	0.3	0.3	3.8	0.9
C _{15:1}	—	—	0.3	0.3
i-C _{16:0}	21.4	22.6	17.5	40.8
i-C _{16:1}	—	—	—	0.3
ai-C _{16:0}	0.2	0.2	—	—
C _{16:0}	2.9	3.0	3.9	1.3
C _{16:1}	—	—	2.4	2.6
i-C _{17:0}	16.1	16.4	1.3	1.0
i-C _{17:1}	10.2	10.1	—	0.2
ai-C _{17:0}	10.1	10.0	—	—
ai-C _{17:1}	—	—	0.2	0.1
C _{17:0}	2.1	2.1	20.5	5.0
C _{17:1}	2.0	2.4	29.7	27.6
i-C _{18:0}	—	—	1.4	0.3
i-C _{18:1}	—	—	0.7	2.9
C _{18:0}	2.5	3.5	2.4	0.7
C _{18:1} (Δ^9)	1.1	1.1	—	—
C _{18:1} (Δ^{11})	16.1	15.0	8.1	8.5

16S rDNA phylogenetic analysis

Two almost complete 16S rRNA sequences were obtained from isolates 10N^T (1487 nucleotides) and 16N (1470 nucleotides). A phylogenetic tree is depicted in Fig. 2. A total of 1371 nucleotides present in all strains between positions 22 and 1524 (*E. coli* numbering) were used for this analysis. The phylogenetic positions of both strains were found to be close to *J. limosus*. The level of 16S rDNA sequence similarity between strains 10N^T and 16N was 98% when all 1470

Table 3. DNA–DNA hybridization between isolate 10N^T and related strains

Strain	DNA–DNA hybridization with 10N ^T (%)
10N ^T	100
<i>J. limosus</i> DSM 11140 ^T	51
<i>J. limosus</i> DSM 11141	47
<i>Terrabacter tumescens</i> IAM 12345 ^T	22
<i>Sanguibacter keddiei</i> ATCC 51767 ^T	24
<i>Nocardioides simplex</i> IAM 1660 ^T	11
<i>Corynebacterium ammoniagenes</i> IAM 1641 ^T	6
<i>Escherichia coli</i> IAM 12119 ^T	2

aligned nucleotide positions were compared. The level of 16S rDNA sequence similarity to *J. limosus* ranged from 96 to 98%.

On the bases of morphological and cultural characteristics, physiological characteristics, chemotaxonomic characters and the phylogenetic position of 16S rRNA sequences, the TCE-degrading isolates clearly belong to the genus *Janibacter*, but differ from *J. limosus* at the species level, as shown by the value for DNA–DNA hybridization. It is therefore proposed that the strains be assigned to the genus *Janibacter* as *Janibacter brevis* sp. nov. An examination of the ability of strains 10N^T and 16N to degrade TCE will be published elsewhere.

Description of *Janibacter brevis* sp. nov.

Janibacter brevis (bre'vis. L. adj. *brevis* short).

Colonies are white, opaque and convex with a glistening surface and entire margins on good growth media. Cells are Gram-positive and non-motile, occur singly, in pairs or occasionally in v-shaped clumps at the early stage of growth. Cells are short rods in young culture, 0.9–1.0 µm in diameter and 1.5–1.8 µm long, and coccoid cells in the stationary phase of growth,

Fig. 2. Phylogenetic tree based on results of a 16S rDNA sequence comparison. Bootstrap values over 50% are shown at branch points.

1.0–1.2 µm in diameter and 1.2–1.3 µm long. A marked rod–coccus cycle is not observed. The optimal growth temperature is 25–28 °C. Obligately aerobic. Chemo-organotrophic. Oxidase-negative. Catalase-positive. H₂S and indole are not produced. Nitrate is reduced to nitrite. Thiamine is required for growth. Formation of acids from sugars and alcohols is not observed. Cells utilize D-fructose, D-glucose, lactose, maltose, sucrose, trehalose, inositol, acetate, butyrate, citrate, gluconate, hippurate, DL-lactate, pyruvate, valeric acid and various amino acids as sole carbon sources. L-Arabinose, D-xylose, D-mannose, L-sorbose, D-cellobiose, galactose, mannitol, D-sorbitol, methanol, ethanol, propanol, *cis*-aconitate, *trans*-aconitate, benzoate, formate, malate, malonate, succinate and L-tartarate are not utilized. *meso*-Diaminopimelic acid is contained in peptidoglycan. The acyl type is acetyl. In whole-cell hydrolysates, ribose and glucose are present, but arabinose, galactose and mannose are absent. The major menaquinone is MK-8(H₄). The major fatty acids are i-C_{15:0}, i-C_{16:0}, i-C_{17:0}, i-C_{17:1} and ai-C_{17:0} as the branched acids and C_{18:1}(Δ¹¹) as the straight chain acid. The polar lipids are diphosphatidylglycerol, phosphatidylglycerol and phosphatidylinositol. Mycolic acids are absent. The DNA base composition is 72 mol% G + C.

The type strain is strain 10N^T. Strains 10N^T and 16N have been deposited in the IAM (Institute of Cellular and Molecular Sciences, University of Tokyo) Culture Collection as IAM 14781^T and IAM 14782, respectively.

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