Pseudonocardia dioxanivorans sp. nov., a novel actinomycete that grows on 1,4-dioxane

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An actinomycete strain (CB1190^T) was previously isolated from industrial sludge contaminated with 1,4-dioxane. The cells of this culture are Gram-positive and exhibit branching aerial and vegetative mycelium. Analysis of the 16S rRNA gene sequence indicates that the strain belongs to the genus *Pseudonocardia*, closely related to *Pseudonocardia hydrocarbonoxydans*, *P. sulfidoxydans* and *P. halophobica*. Physiological and biochemical characteristics of CB1190^T are different from those of other known *Pseudonocardia* species. The novel organism described here is distinguished by its ability to grow on 1,4-dioxane, which is a probable human carcinogen. This culture can also grow on tetrahydrofuran, gasoline aromatics and several other toxic environmental contaminants. Strain CB1190^T is capable of fixing dinitrogen. The predominant fatty acids are 16 : 0 iso, 16 : 1 iso *cis*9 and 17 : 1 iso *cis*9. The major phospholipid fatty acids are 16 : 0 iso, 16 : 0 10-Me and 17 : 0 10-Me. The peptidoglycan belongs to type A1γ, *meso*-diaminopimelic acid. The major menaquinone is MK-8 (H_4). Mycolic acids are absent. The G+C content is 74 mol%. Based on morphological, physiological, chemotaxonomic and phylogenetic evidence, it is proposed that strain CB1190^T (=ATCC 55486^T=DSM 44775^T) be classified as the type strain of a novel species, *Pseudonocardia dioxanivorans* sp. nov. Further studies with this organism will provide insights into metabolic pathways, responsible enzymes, kinetics and the fate of 1,4-dioxane in the environment.

Degradation of toxic pollutants by micro-organisms has emerged as a promising remediation strategy for groundwater contaminants. Actinomycetes have been shown to specifically degrade hydrocarbons (McCarthy & Williams, 1992), chlorinated solvents (Wackett *et al.*, 1989), explosives (Pasti-Grigsby *et al.*, 1996), plasticizers (Klausmeier & Osman, 1976) and azo dyes (Zhou & Zimmermann, 1993). Within the actinomycetes, members of the genus *Pseudonocardia* (Henssen, 1957; Huang *et al.*, 2002) have been reported to use hydrocarbons (Juteau *et al.*, 1999; Lechevalier *et al.*, 1986; Lee *et al.*, 2004), methyl sulfides (Reichert *et al.*, 1998) and tetrahydrofuran (THF) (Kohlweyer *et al.*, 2000) as growth substrates.

In this paper, we describe a novel *Pseudonocardia* species that is the only known pure culture that can grow on 1,4-dioxane, a toxic groundwater pollutant (Parales *et al.*, 1994). This culture can be used for bioaugmentation at 1,4-dioxane- and THF-contaminated sites. 1,4-Dioxane is widely used as a stabilizer for chlorinated compounds and as a solvent in the manufacture of textiles, organic chemicals, fumigants, automotive coolants and hygiene products (Mohr, 2001). 1,4-Dioxane is a probable human carcinogen and a priority pollutant that has been detected in surface and ground waters (Mohr, 2001).

The culture CB1190 was graciously supplied by Dr Rebecca Parales, University of California, Davis. It was originally enriched by Parales and co-workers from 1,4-dioxane-contaminated industrial sludge from Darlington, SC, USA (Parales *et al.*, 1994). The initial enrichments were performed in basal salts medium with 1·5–4·0 mM THF in the presence of 0·1 % (w/v) yeast extract. Enrichments capable of degrading both 1,4-dioxane and THF were selected. The mixed enrichment degraded up to 10 mM 1,4-dioxane per day. Only one prokaryotic isolate, designated strain CB1190^T, was capable of growing on 1,4-dioxane as sole carbon source.

In our laboratory, CB1190^T was grown aerobically in ammonium mineral salts (AMS) liquid medium (Parales *et al.*, 1994) at 30 °C with 5 mM 1,4-dioxane. Cell biomass was quantified as total protein concentration (Lontoh & Semrau, 1998). BSA was used as a standard for the Coomassie Plus protein assay kit (Pierce). Cells of strain

Abbreviations: FAME, fatty acid methyl ester; PLFA, phospholipid fatty acid; THF, tetrahydrofuran.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Pseudonocardia dioxanivorans* CB1190^T is AY340622.

A scanning electron micrograph of cells of strain CB1190^T and details of fatty acid compositions are available as supplementary material in IJSEM Online.

CB1190^T were also maintained on plates containing AMS and 1·5 % Bacto agar. The plates were incubated at 30 °C in a desiccator in which 1,4-dioxane was supplied in the vapour phase by placing a glass microfibre filter (Whatman) in the bottom half of a glass Petri dish placed on the floor of the desiccator. An aliquot of 250 µl 1,4-dioxane that had been filter-sterilized (0·1 µm pore size) was added to the filter. Every 4–5 days, the filters were replaced with new dioxane-laden filters in the fume hood. The colours and shapes of colonies were recorded weekly. A Hitachi model S-5000 microscope was used for scanning electron microscopy of gold-coated, dehydrated cells. Fourteen-day-old cultures on AMS-agar plates were visualized by electron microscopy.

The ability of CB1190^T and reference strains *Pseudonocardia hydrocarbonoxydans* DSM 43281^T and *Pseudonocardia sulfidoxydans* DSM 44248^T to grow aerobically on a variety of carbon sources was investigated using GP2 and SFP2 microplates (Biolog). Cells were grown at 30 °C on Biolog universal growth agar amended with 0·25 % glucose. The OD_{590} of the inoculum was adjusted to 0·2 before loading 150 µl into each well. Similarly, the utilization of various nitrogen sources was tested using the Biolog PM3 microplate. For PM3 plates, the OD_{590} of the inoculum was adjusted to 0·4 before loading 100 µl into each well. The wells were analysed using the Omnilog system after incubating the plates for 7 days at 30 °C. In addition, the capability of *P. sulfidoxydans* and *P. hydrocarbonoxydans* to grow on THF and 1,4-dioxane was tested. The ability of CB1190^T to grow on 1,4-dioxane (99·8 % pure; Aldrich Chemical) and Luria–Bertani (LB) broth (Becton Dickinson) under anaerobic conditions was investigated. The temperature range for growth was examined. Standard phenotypic tests commonly used for describing novel species of actinomycetes were performed (Gordon *et al.*, 1974). The cells were grown in AMS medium with 1,4-dioxane added as the sole carbon source and incubated at 30 °C for up to 28 days. The pH of AMS medium was adjusted to 7·0 for examining acid production from various carbohydrates, which were added to the AMS medium to achieve 1 % (w/v) aqueous concentration.

For monitoring growth on 1,4-dioxane, liquid samples were collected at various intervals and filtered with 0·45 µm syringe filters to eliminate cells. Dioxane disappearance was measured by injecting 5 µl filtered liquid samples into a Varian 3400 gas chromatograph equipped with a flame-ionization detector (FID) and a GraphPac-GB column (Alltech). The injector, oven and detector temperatures were respectively set at 220, 150 and 250 °C. Cell growth was determined by measuring total protein and enzymic activities of soluble monooxgenases were assayed as described by Brusseau *et al.* (1990). Briefly, duplicate 5-ml aliquots of CB1190^T cell suspensions were transferred to 20 ml glass serum vials containing approximately 5 mg crystalline naphthalene. The vials were sealed with Teflon-lined rubber septa and incubated at 30 °C on a platform shaker for 15 min. Fifty microlitres of freshly prepared 5 mg tetrazotized *o*-dianisidine ml^{-1} were added to the samples. Naphthalene was oxidized to a mixture of 1-naphthol and 2-naphthol by the monooxygenase. The naphthols were detected colorimetrically by reaction with tetrazotized *o*-dianisidine to form purple diazo dyes.

The capability of strains to fix dinitrogen was tested by the activity of nitrogenase, which converts acetylene into ethylene in the presence of an external energy source (Chu & Alvarez-Cohen, 1996). Briefly, 1 ml acetylene and 20 mM glucose were added to 5 ml cell culture in nitrogen-free AMS medium. The vials were incubated at 30 °C on a shaker at 150 r.p.m. The headspace concentrations of acetylene and ethylene were analysed by an HP 5890 series II gas chromatograph equipped with an FID. Acetylene-free controls were used to determine background ethylene production. Stoichiometric decrease in acetylene concentration with concomitant production of ethylene was indicative of nitrogenase activity. Ammonia produced by nitrogen-fixing cells was assayed using the Nessler method (Hach method 8038). The colour produced by the reaction of Nessler reagent and ammonium ions was measured at 425 nm using a Hach DR/2500 spectrophotometer. Uninoculated N-free AMS medium was used as a blank for the colorimetric assay.

Whole-cell fatty acid methyl ester (FAME) analysis was performed by Microchek, Inc. (Northfield, VT, USA) using gas chromatography. The cells were grown at 30 °C on BBL trypticase soy broth (BD Diagnostic Systems) for 7 days prior to FAME analysis. Phospholipid fatty acid (PLFA) analysis was performed on 14-day-old cultures grown in AMS and 5 mM 1,4-dioxane at 30 °C. The cells were saponified, methylated, solvent-extracted and analysed by GC-MS by using the modified Bligh/Dyer method (White & Ringelberg, 1998). Analysis of quinones and mycolic acids was carried out by the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Braunschweig, Germany. The diagnostic diamino acid was determined by analysis of a whole-cell hydrolysate by TLC on cellulose (Hasegawa *et al.*, 1983; Rhuland *et al.*, 1955). Gram staining was performed according to the standard method (Gerhardt *et al.*, 1994).

Genomic DNA was extracted from CB1190^T by a two-step enzymic treatment followed by bead-beating lysis and organic extraction (Dojka *et al.*, 1998). PCR amplification of the 16S rRNA gene using bacterial primers 8F (5′-AGAGTTTGATCCTGCCTCAG-3′), 338F (5′-CCTACG-GGAGGCAGCAG-3′) and 1492R (5′GGYTACCTTGTTA-CGACTT-3′) was carried out as described by Richardson *et al.* (2002). Purified PCR products were directly sequenced using an Applied Biosystems 3100 Genetic Analyzer at the University of California Berkeley Sequencing Facility. The partial sequences were aligned using the Autoassembler program version 2.1 (PE Biosystems). CHECK_CHIMERA software (http://rdp.cme.msu.edu/) was used to confirm that the sequences were not of chimeric origin. The 16S

rRNA gene sequences were compared with those in the GenBank database using the BLAST program (http://www.ncbi.nlm.nih.gov/blast). Detailed phylogenetic analyses were carried out using the ARB program (http://www.arb-home.de). Evolutionary distance dendrograms were constructed using the Felsenstein substitution model and neighbour-joining tree-building algorithm. Since all input sequences were longer than 1250 bp, the parsimony tool was not used. Statistical support for branch points in the dendrograms was estimated by bootstrap resampling analysis of 1000 independent trees (Felsenstein, 1985) using PAUP 4.0 program.

DNA base composition (G+C mol%) was determined by the DSMZ by HPLC analysis using DNA isolated from CB1190^T as described above.

Strain CB1190^T grows slowly on AMS medium containing 1,4-dioxane. It also grows slowly on rich media such as LB, TSA and R2A in the absence of 1,4-dioxane. When grown in liquid medium, the cells appear as powdery white colonies floating on the liquid surface and form biofilms on the insides and the bottom of the flask. The cells produce white aerial mycelium and yellowish substrate mycelium, which branch and fragment into rod-shaped, non-motile units. No diffusible pigments or sporangia are produced. Hyphal swelling and budding are sometimes observed with this culture. The cells are 1–3 μm in size (a scanning electron micrograph is available as supplementary material in IJSEM Online).

CB1190^T is an aerobic micro-organism capable of utilizing 1,4-dioxane as its sole source of carbon and energy for growth. In batch experiments, cell yield was $0{\cdot}09 \pm 0{\cdot}002$ g protein (g dioxane)$^{-1}$. The observed maximum growth rate was 90 μg l^{-1} h^{-1} and the maximum dioxane degradation rate was 0·1 μg dioxane (μg protein)$^{-1}$ h^{-1}, and is described by Monod kinetics. The addition of 10 mg vitamins stock l^{-1} (Tanner, 1997) to the AMS medium did not significantly affect the growth rate. CB1190^T was capable of growing aerobically on benzene, toluene, formate, Casamino acids, LB broth and trypticase soy broth. No growth was observed on methyl *tert*-butyl ether or *tert*-butyl alcohol. While *P. sulfidoxydans* was able to use THF as a sole growth substrate but not 1,4-dioxane, *P. hydrocarbonoxydans* was unable to grow on either compound. The substrates in Biolog GP2 and SFP2 microplates metabolized by CB1190^T, *P. hydrocarbonoxydans* and *P. sulfidoxydans* are listed in Table 1.

Strain CB1190^T is capable of utilizing a variety of nitrogen sources, including NH_4^+, NO_3^- and N_2. The capability of CB1190^T to fix atmospheric nitrogen was indicated by (i) slow but positive growth in ammonium-free AMS medium, as well as in the negative control well of the Biolog PM3 plate, (ii) production of ammonium ions in cell cultures grown in ammonium-free AMS medium and (iii) the presence of nitrogenase activity as measured by stoichiometric reduction of acetylene to ethene. The measured rate of ethylene production was 18 nmol h^{-1} (mg protein)$^{-1}$. Abiotic loss of acetylene was negligible. No cell growth or ammonia production was observed in control vials containing N-free medium with headspace containing argon and 5% oxygen. Neither *P. hydrocarbonoxydans* nor *P. sulfidoxydans* was able to grow in nitrogen-free medium in the presence of glucose or lactate as carbon source. Neither strain was capable of using N_2 as a nitrogen source in the Biolog PM3 microplate test. Both strains were unable to reduce acetylene to ethylene, indicating the absence of nitrogenase activity. No other *Pseudonocardia* species has been reported to fix dinitrogen.

Growth on 1,4-dioxane in AMS was tested at temperatures ranging from 10 to 45 °C, and the optimum growth temperature was found to be 30 °C, with no observed growth at temperatures higher than 37 °C. CB1190^T grown on 1,4-dioxane was able to oxidize naphthalene to a mixture of 1-naphthol and 2-naphthol, indicating soluble monooxygenase activity. A comparison of physiological properties of CB1190^T with *P. hydrocarbonoxydans* and *P. sulfidoxydans* is presented in Table 1.

The whole-cell fatty acid pattern of CB1190^T was of the iso-/anteiso-branched type. The dominant fatty acid was hexadecanoate (details available as supplementary material in IJSEM Online). Saturated, unsaturated and 10-methyl-branched fatty acids were also present. These signatures are diagnostic for members of the family *Pseudonocardiaceae* (Embley *et al.*, 1988; Kroppenstedt, 1992). Ninety-nine per cent of the total peaks were named. The major PLFAs are hexadecanoate (27·5%), 10-methylhexadecanoate (9·3%) and 10-methylheptadecanoate (23·6%). Methyl branching on the tenth carbon atom in a fatty acid molecule is specific for many mycolateless nocardioform actinomycetes. Like all other *Pseudonocardia* species, CB1190^T does not synthesize mycolic acids. The dominant menaquinone was MK-8(H_4). In addition, a small amount of MK-7(H_4) was also present. The cell-wall peptidoglycan of strain CB1190^T contained *meso*-diaminopimelic acid (*m*-Dpm). This indicates A1γ peptidoglycan type, which is commonly found in representatives of *Pseudonocardia*.

The almost complete sequence of the 16S rRNA gene (1422 bp) was used for phylogenetic analyses. Preliminary phylogenetic placements based on the results of BLAST analyses revealed that strain CB1190^T is closely related to the members of the family *Pseudonocardiaceae*. The closest phylogenetic neighbours with validly published names are *P. sulfidoxydans* (1401/1423 bp) and *P. hydrocarbonoxydans* (1400/1423 bp). Rigorous analysis of the phylogenetic position was carried out by constructing phylogenetic trees using 23 species containing 1353 nucleotides (Fig. 1). Kimura two-parameter neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) methods were employed. Both methods consistently placed CB1190^T in the cluster encompassing members of *Pseudonocardia* with high bootstrap support. This phylogenetic placement was not affected by the choice of different outgroups. The

Table 1. Physiological characteristics of strain CB1190^T and reference *Pseudonocardia* strains

Strains: 1, CB1190^T; 2, *P. hydrocarbonoxydans* DSM 43281^T; 3, *P. sulfidoxydans* DSM 44248^T. +, Positive or present; W, weakly positive; −, negative or absent. All strains were able to metabolize the following substrates in the Biolog GP2 and SFP2 microplates: dextrin, α-D-glucose, maltose, maltotriose, D-mannose, D-trehalose, turanose, acetic acid, L-lactic acid, pyruvic acid methyl ester, pyruvic acid, glycerol, adenosine, 2′-deoxyadenosine, inosine, uridine and DL-α-glycerol phosphate. The following compounds were not utilized in the Biolog plate: α-cyclodextrin, inulin, Tween 80, *N*-acetyl-D-glucosamine, *N*-acetyl-β-D-mannosamine, L-arabinose, D-arabitol, D-galacturonic acid, methyl β-D-galactoside, sedoheptulosan, D-sorbitol, stachyose, D-tagatose, α-, β- and γ-hydroxybutyric acid, D-lactic acid methyl ester and L-pyroglutamic acid. All strains were able to utilize the following nitrogen sources: ammonia, nitrite, nitrate, urea, L-alanine, L-aspartic acid, L-phenylalanine, D-aspartic acid, methylamine, ethylamine and uric acid. None of the strains could utilize the following compounds as nitrogen sources: biuret, *N*-phthaloyl-L-glutamic acid, histamine, β-phenylethylamine, tyramine, D-mannosamine, *N*-acetyl-D-mannosamine, DL-α-amino-*N*-butyric acid, γ-amino-*N*-butyric acid, ε-amino-*N*-caproic acid, DL-α-aminocaprylic acid, D-amino-*N*-valeric acid and 2-amino-*N*-valeric acid.

Characteristic	1	2	3	Characteristic	1	2	3
Carbon sources:				Nitrogen sources:			
1,4-Dioxane	+	−	−	Dinitrogen	+	−	−
Tetrahydrofuran	+	−	+	L-Arginine	−	−	+
Glycogen	−	−	+	L-Asparagine	−	−	+
Amygdalin	W	−	+	L-Cysteine	W	+	−
Arbutin	+	−	+	L-Glutamic acid	+	−	+
D-Cellobiose	+	−	−	L-Glutamine	+	−	−
D-Fructose	+	+	W	Glycine	+	+	−
D-Galactose	+	−	−	L-Histidine	−	+	+
Gentiobiose	+	−	−	L-Isoleucine	−	−	W
D-Gluconic acid	+	−	−	L-Leucine	−	−	+
m-Inositol	−	−	+	L-Lysine	−	+	+
α-D-Lactose	−	+	+	L-Methionine	+	−	+
Lactulose	−	+	−	L-Proline	−	+	−
D-Mannitol	+	W	−	L-Serine	−	+	+
D-Melezitose	+	+	−	L-Threonine	+	+	−
D-Melibiose	−	−	+	L-Tryptophan	−	+	+
3-Methyl glucose	−	+	W	L-Tyrosine	W	+	−
Methyl α-D-glucoside	+	−	+	L-Valine	+	−	−
Methyl β-D-glucoside	+	−	+	L-Citrulline	+	−	−
Methyl α-D-mannoside	−	−	+	L-Homoserine	−	+	−
Palatinose	+	−	−	L-Ornithine	−	+	−
D-Psicose	+	+	−	Hydroxylamine	+	+	−
D-Raffinose	−	W	−	Ethanolamine	−	+	+
L-Rhamnose	+	−	−	Ethylenediamine	+	−	+
D-Ribose	+	+	−	Putrescine	+	+	−
Salicin	W	−	+	Acetamide	−	+	+
Sucrose	−	+	+	Formamide	−	+	+
Xylitol	+	−	−	Xanthine	+	−	W
D-Xylose	+	W	+	Xanthosine	+	−	+
α-Ketoglutaric acid	−	+	−	Uridine	−	W	+
α-Ketovaleric acid	−	+	+	Alloxan	+	+	−
Lactamide	W	−	+	Allantoin	−	+	+
D-Malic acid	+	−	−	Parabanic acid	−	−	+
L-Malic acid	+	−	−	Decomposition of:			
Succinamic acid	−	+	+	Adenine	−	W	−
Succinic acid	+	−	+	L-Tyrosine	−	+	+
L-Alaninamide	+	−	W	Xanthine	+	−	+
D-Alanine	−	+	+	Hypoxanthine	+	−	+
L-Alanine	−	+	+	Growth on:			
L-Asparagine	−	+	+	3 % NaCl	+	−	+
L-Glutamic acid	−	−	+	4 % NaCl	+	−	−
L-Serine	−	+	−	5 % NaCl	W	−	−
Putrescine	+	+	−				
Thymidine	−	+	+				

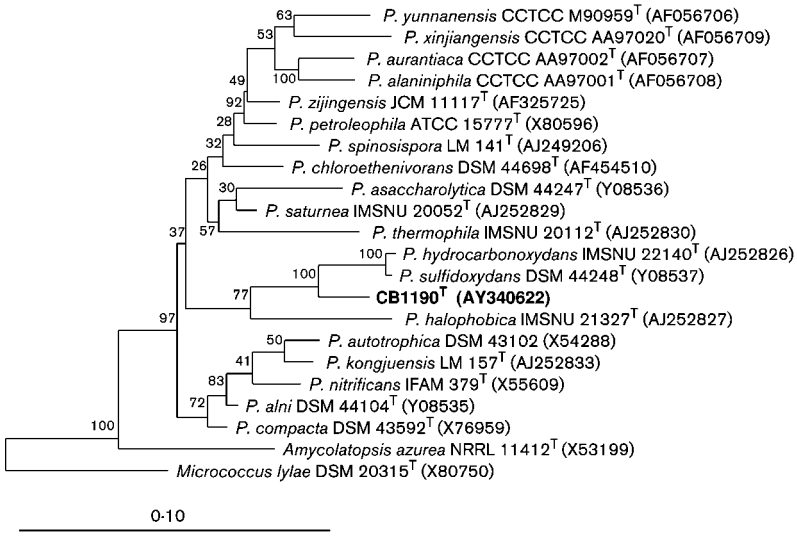

Fig. 1. Evolutionary distance dendrogram displaying the phylogenetic position of *Pseudonocardia dioxanivorans* sp. nov. CB1190^T obtained by comparative analysis of 16S rRNA gene sequence data. The tree is based on 1353 unambiguous nucleotide positions present in all sequences. Bootstrap percentages based on 1000 resamplings are listed at nodes. The scale bar represents 0·1 changes per nucleotide.

bootstrap confidence for the clade containing CB1190^T, *P. sulfidoxydans* and *P. hydrocarbonoxydans* was 100 %.

The analysis of genotypic and phenotypic data suggests that CB1190^T merits recognition as a novel species in the genus *Pseudonocardia*. CB1190^T exhibits 91 % 16S rRNA gene sequence similarity to the type species *Pseudonocardia thermophila* (Embley *et al.*, 1988) and 98·4 % sequence similarity to its closest neighbours, *P. sulfidoxydans* (Reichert *et al.*, 1998) and *P. hydrocarbonoxydans* (Lechevalier *et al.*, 1986; Warwick *et al.*, 1994). Moreover, strain CB1190^T is unique among *Pseudonocardia* species in its ability to metabolize 1,4-dioxane and to fix dinitrogen. It is proposed that the organism be classified as *Pseudonocardia dioxanivorans* sp. nov.

Note added in proof

Since this article was submitted for publication, a novel species, *Pseudonocardia benzenivorans*, has been described (Kämpfer & Kroppenstedt, 2004).

Description of *Pseudonocardia dioxanivorans* sp. nov.

Pseudonocardia dioxanivorans (di.ox.a.ni.vo′rans. N.L. n. *dioxanum* 1,4-dioxane; L. part. adj. *vorans* devouring; N.L. part. adj. *dioxanivorans* dioxane-devouring).

The species description is based on the results of this study and Parales *et al.* (1994). Gram-positive, strictly aerobic, mycolateless actinomycete. Both aerial and vegetative mycelium fragment into rod-shaped elements. Capable of growing on 1,4-dioxane, THF, tetrahydropyran, 1,3-dioxane, 2-methyl-1,3-dioxolane, diethyl ether and butyl methyl ether. A variety of other organic compounds, such as ethers, alcohols, carbohydrates, carboxylic acids and amino acids, are also utilized (Table 1). Aerobic autotrophic growth occurs on H_2 and CO_2. This organism is able to fix dinitrogen. Acid is produced from (+)-D-glucose, (−)-L-rhamnose, (+)-D-xylose, (−)-D-fructose, (−)-D-trehalose, cellobiose, (−)-D-mannitol, maltose and salicin. Xanthine and hypoxanthine are decomposed. (−)-L-Tyrosine and adenine are not degraded. Optimal growth temperature is 30 °C. Nitrogenase-positive and oxygenase-positive. Predominant fatty acids are 16 : 0 iso, 16 : 1 iso *cis*9 and 17 : 1 iso *cis*9. The major menaquinone [MK-8(H_4)] has eight isoprene units, two of which are hydrogenated. Cell wall contains *meso*-Dpm. The G+C content is 74 mol%.

The type strain, CB1190^T (=ATCC 55486^T=DSM 44775^T), was isolated from a 1,4-dioxane-contaminated sludge sample collected at Darlington, SC, USA (Parales *et al.*, 1994).

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