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Caenibacterium thermophilum gen. nov., sp. nov., isolated from a thermophilic aerobic digester of municipal sludge

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A bacterial strain, N2-680^T (=DSM 15264^T=LMG 21760^T), isolated from a thermophilic aerobic digester of municipal sludge, was characterized with respect to its morphology, physiology and taxonomy. Phenotypically, the isolate was a Gram-negative rod with a polar flagellum, catalase- and oxidase-positive, containing cytoplasmic inclusions of poly-β-hydroxybutyrate and had an optimal growth temperature of about 47 °C. Strain N2-680^T was unable to reduce nitrate and could use organic acids, amino acids and carbohydrates as single carbon sources. Chemotaxonomic analysis revealed that ubiquinone 8 was the major respiratory quinone of this organism and that phosphatidylethanolamine and phosphatidylglycerol were the major polar lipids. At 50 °C, the major components in fatty acid methyl ester analysis were $C_{16:0}$ and cyclo-$C_{17:0}$. The highest 16S rDNA sequence identity of isolate N2-680^T was to *Leptothrix mobilis* and *Ideonella dechloratans* (95·7 %) and to *Rubrivivax gelatinosus* and *Aquabacterium commune* (95·6 %). 16S rDNA sequence similarities to species of two related thermophilic genera, *Caldimonas manganoxidans* and *Tepidimonas ignava*, were lower (93·6 and 94·7 %). On the basis of phylogenetic analyses and physiological and chemotaxonomic characteristics, it is proposed that isolate N2-680^T represents a new genus and species, for which the name *Caenibacterium thermophilum* gen. nov., sp. nov. is proposed.

INTRODUCTION

The phylogenetic lineage of the β-subclass of *Proteobacteria* that includes the genera *Rubrivivax*, *Roseateles*, *Leptothrix*, *Ideonella*, *Aquabacterium* and *Caldimonas* constitutes a heterogeneous group from physiological and ecological perspectives. Bacteriochlorophyll-containing members of this group are represented by the species *Rubrivivax gelatinosus* (Willems *et al.*, 1991) and *Roseateles depolymerans* (Suyama *et al.*, 1999). *Rubrivivax gelatinosus* comprises phototrophic non-sulphur bacteria that occur frequently in sewage-treatment plants and lagoons (Siefert *et al.*, 1978; Pfennig, 1978; Willems *et al.*, 1991). *Roseateles depolymerans*, isolated from river water in Japan, is an obligately aerobic, heterotrophic organism that produces bacteriochlorophyll *a* and carotenoid pigments only in the presence of low levels of carbon sources. This organism has the ability to degrade biodegradable plastics (Suyama *et al.*, 1998, 1999).

The genus *Leptothrix* includes sheath-forming bacteria capable of manganese oxidation, occurring in natural iron- and manganese-containing systems (Mulder, 1989; Siering & Ghiorse, 1996). Members of the genus *Leptothrix* have been found in both unpolluted natural waters and man-made habitats such as activated sludge (Mulder, 1989; Mulder & Deinema, 1992). *Ideonella dechloratans* was isolated from activated sludge and is characterized by its ability to use chlorate as an electron acceptor (Malmqvist *et al.*, 1994). The genus *Aquabacterium* was defined to accommodate three bacterial strains isolated from biofilm occurring in the Berlin drinking-water distribution system (Kalmbach *et al.*, 1999). These organisms are strict heterotrophs capable of growth on nutrient-rich medium but unable to metabolize carbohydrates.

Among the phylogenetic lineage *Rubrivivax–Roseateles–Leptothrix–Ideonella–Aquabacterium*, thermophily is represented by the genus *Caldimonas* (Takeda *et al.*, 2002).

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Abbreviation: PHB, poly-β-hydroxybutyrate.

The GenBank/EMBL/DDBJ accession number for the 16S rDNA sequence of isolate N2-680^T is AJ512945.

Caldimonas manganoxidans, the single species within this genus with a validly published name, comprises chemo-organotrophic organisms capable of manganese oxidation and able to grow on poly(3-hydroxybutyrate) (Takeda *et al.*, 1998, 2002). The type strain of *Caldimonas manganoxidans* has an optimal temperature for growth around 50 °C and was isolated from a hot spring in Japan, exposed to sun and extensively colonized with cyanobacteria, potential producers of large amounts of poly(3-hydroxybutyrate) (Takeda *et al.*, 1998). More phylogenetically distantly related to this lineage is the thermophile *Tepidimonas ignava* (Moreira *et al.*, 2000), with an optimal temperature for growth around 55 °C. *Tepidimonas ignava*, isolated from a Portuguese hot spring, represents a chemolithoheterotrophic organism, capable of using sulphur compounds as an energy source.

This paper reports the isolation and characterization of a thermophilic bacterium enriched on a poly-ε-caprolactone thermoplastic from a thermophilic aerobic digester of activated sludge. Based on phenotypic, chemotaxonomic and 16S rDNA-based phylogenetic analysis, the definition of a new genus and species is proposed within the β-subclass of *Proteobacteria* with the name *Caenibacterium thermophilum* gen. nov., sp. nov., and the type strain is N2-680^T.

METHODS

Isolation and cultivation conditions. Strain N2-680^T was isolated from a caprolactone polymer enrichment culture obtained from a thermophilic aerobic digester of a domestic wastewater-treatment plant in northern Portugal. In this treatment process, the decanted sludge is submitted to a mesobiotic anaerobic digestion followed by a thermophilic aerobic digestion, which reaches a maximal temperature of about 60 °C. The product obtained through this digestion was used as inoculum for enrichment. The enrichment was carried out at 50 °C, using 1 g inoculum per 10 ml mineral medium (medium A; Manaia & Moore, 2002), supplemented with a pellet of poly-ε-caprolactone thermoplastic (oxepanone homopolymer, with a molecular mass of 80 000; Solvay). Cultures were transferred weekly to fresh medium for 2 months. Isolate N2-680^T was purified from the mixed culture obtained in this enrichment by subculturing on LB broth containing 20 g agar l^{-1} (Carlton & Brown, 1981). This isolate was maintained on LB agar or cryo-preserved in LB broth containing 15 % (v/v) glycerol.

Determination of morphological, growth and biochemical characteristics. Colony and cell morphology of strain N2-680^T were examined using standard protocols (Doetsch, 1981). Cell morphology, Gram-staining reaction, production of spores and the accumulation of poly-β-hydroxybutyrate (PHB) granules were determined by microscopic examination, following procedures described previously (Doetsch, 1981; Smibert & Krieg, 1981). The number and position of flagella were determined by light microscopy, after staining the cells with Ryu stain (Heimbrook *et al.*, 1989).

The growth temperature range was examined by measuring turbidity (at 610 nm) of cultures in 250 ml screw-capped Erlenmeyer flasks containing 50 ml LB medium incubated in an orbital water-bath shaker.

Phenotypic tests on isolate N2-680^T were carried out as described by Smibert & Krieg (1981), using 2-day cultures on LB agar. Unless otherwise stated, all incubations were performed at 50 °C. The pH range for growth was examined in LB medium, using 10 mM MES (Sigma) to adjust the pH between 5·0 and 6·0 or 10 mM CAPS (Sigma) to adjust the pH between 9·0 and 11·0. The enzymic activity was tested using the API ZYM system, following the instructions of the manufacturer (bioMérieux). Hydrogenase activity was determined based on the description of Aragno & Schlegel (1992) and Stöhr *et al.* (2001). A cell suspension was prepared in sterile phosphate buffer (54 mM, pH 7·2) with cells grown on LB agar and washed twice in the same buffer. This suspension was divided into two sets of aliquots, in rubber-sealed vials. In one set, an atmosphere containing about 80 % H_2 was generated, while, in the second set, the atmosphere contained only nitrogen. A solution of triphenyltetrazolium was added in order to obtain a final concentration of 0·25 % (w/v) and incubated at 50 °C, protected from light. Non-inoculated phosphate buffer as well as phosphate buffer inoculated with an organism lacking hydrogenase activity were used as negative controls. The development of a red colour indicated the reduction of triphenyltetrazolium and the presence of hydrogenase activity.

Manganese oxidation was tested on *Sphaerotilus—Leptothrix* medium (l^{-1}: 1 g yeast extract, 1·5 g peptone, 0·2 g $MgSO_4.7H_2O$, 0·5 g ferric ammonium citrate, 50 mg $CaCl_2$, 50 mg $MnSO_4.H_2O$, 10 mg $FeCl_3.6H_2O$, 20 g agar, pH 7·1). *Leptothrix mobilis* DSM 10617^T was used as a positive control and the presence of manganese oxides was evaluated using benzidinium hydrochloride (Nealson, 1992; Spring *et al.*, 1996). Degradation of the polycaprolactone oxydiethylene ester (CAPA 200; Solvay), a polymer derived from ε-caprolactone with a mean molecular mass of 550, was tested using that polymer (2·5 g l^{-1}) dispersed in agar medium with the following composition (l^{-1}): 1 g NH_4NO_3, 0·2 g yeast extract, 0·25 g K_2HPO_4, 0·13 g $MgSO_4$, 0·13 g NaCl, 2·5 mg $Fe_2(SO_4)_3$, 2·5 mg $MnSO_4$, 50 μg K_2MoO_4, 50 μg Na_3BO_3, 50 μg $Co(NO_3)_2$, 50 μg $FeCl_3$, 50 μg $CdSO_4$, 50 μg $CuSO_4$ and 50 μg $ZnSO_4$. Degradation was indicated by the appearance of a clear zone around the colonies.

The nutritional pattern was characterized using the API 50CH system and a defined medium (medium B) [l^{-1}: 5 g $(NH_4)_2SO_4$, 0·31 g KH_2PO_4, 0·45 g K_2HPO_4, 1·2 g $Na_2HPO_4.2H_2O$, 0·1 g NaCl, 0·05 g $CaCl_2$, 0·4 g $MgSO_4.7H_2O$, 5 mg histidine, 20 mg tryptophan, 20 mg methionine, 200 μg *p*-aminobenzoic acid, 20 μg biotin, 2 μg folic acid, 10 mg *myo*-inositol, 400 μg nicotinic acid, 2 mg calcium pantothenate, 400 μg pyridoxine hydrochloride, 200 μg riboflavin, 400 μg thiamin hydrochloride, 500 μg H_3BO_3, 200 μg $FeCl_3.6H_2O$, 400 μg $ZnSO_4.7H_2O$, 400 μg $MnSO_4.4H_2O$, 40 μg $CuSO_4.5H_2O$, 200 μg $Na_2MoO_4.2H_2O$, 100 μg KI, 2·5 g agar]. Chemolithoautotrophic growth was tested using medium A supplemented with filter-sterilized 30 mM $NaHCO_3$ and different electron donors. The use of H_2 as energy source was tested according to Suyama *et al.* (1999). The ability to use sulphur or thiosulphate as electron donors was tested by adding 5 g sulphur flowers l^{-1} to medium A or supplementing the same medium with filter-sterilized sodium thiosulphate at final concentrations of 2·5 and 5 g l^{-1}. Positive controls, containing 25 mM acetate or acetate and the inorganic electron donor, were run in parallel. The ability to grow in the absence of a source of combined nitrogen was tested using medium A without ammonium sulphate.

The production of photosynthetic pigments was analysed as described by Suyama *et al.* (1999). Absorption spectra of ultrasonically disrupted cells, pre-grown in medium A supplemented with acetate, were obtained in phosphate buffer.

Determination of genotypic characteristics. For the determination of DNA base composition, genomic DNA was isolated as described by Cashion *et al.* (1977) and the G+C content of DNA was analysed by HPLC (Mesbah *et al.*, 1989).

16S rDNA sequence analysis. The nucleic acid sequence of the 16S rRNA gene was determined after PCR amplification from total

DNA extracts, using procedures described previously (Nogales *et al.*, 2001). The primers described by Lane (1991) were used. The nucleotide sequence was compared with reference 16S rDNA sequences in the EMBL database using the FASTA program (Pearson & Lipman, 1988) and subsequently aligned with reference sequences included in the ARB package (http://www.arb-home.de). Evolutionary distances, derived from sequence-pair dissimilarities (Jukes & Cantor, 1969), were calculated using the PHYLIP package (Felsenstein, 1989). Non-homologous and ambiguous nucleotide positions were excluded from the calculations.

Determination of chemotaxonomic characteristics. Cultures for polar lipid analysis were grown in LB medium until the end of exponential phase of growth. Lipid extractions were performed as described previously (Prado *et al.*, 1988). Individual polar lipids were separated by one-dimensional TLC on silica gel G plates (0·25 mm thickness; Merck), using a solvent system of chloroform/methanol/acetic acid/water (80 : 17 : 10 : 4, by vol.).

For the analysis of methylated fatty acids, isolate N2-680^T was cultivated for 3 days on LB agar at 30 and 50 °C. The harvesting of cells and the preparation of fatty acid methyl esters (FAMEs) were performed as described by Kuykendall *et al.* (1988). FAMEs were separated as described by Moreira *et al.* (2000) and the individual components were identified and quantified by comparison with the retention times of authentic standards, using the MIS Library Generation software (Microbial ID Inc.). FAMEs were extracted and analysed at least twice.

For the analysis of respiratory quinones, cells were cultured on LB agar, harvested, freeze-dried and extracted according to Tindall (1989) and the extracts were analysed as described by Moreira *et al.* (2000).

RESULTS AND DISCUSSION

Cultivation at 50 °C in mineral medium supplemented with a poly-ε-caprolactone thermoplastic was used to enrich a thermophilic population capable of using synthetic polymers as the sole source of carbon and energy. The enrichment procedure resulted in a mixed culture containing isolate N2-680^T, which was purified by successive subculturing on LB agar.

Individual cells of isolate N2-680^T were Gram-negative rods, 1·3 μm long and 0·5 μm wide, containing intracellular PHB granules. A polar flagellum was observed only during the early stages of growth. Endospores, prosthecae or cell sheaths were not observed on isolate N2-680^T. When cultured on LB agar, strain N2-680^T produced non-pigmented colonies, 1–2 mm in diameter after 36–48 h growth.

In pure culture, isolate N2-680^T was unable to grow in mineral medium A supplemented with the caprolactone polymer used for enrichment. The same medium supplemented with acetate supported growth, even after successive transfers, indicating that this organism does not require specific growth factors, such as vitamins or amino acids. Isolate N2-680^T could also grow on nutrient-rich media such as LB. In LB medium, the optimal growth temperature of strain N2-680^T was around 47 °C, with a maximal temperature for growth of 57 °C.

The physiological properties of strain N2-680^T are summarized in Table 1. Strain N2-680^T is composed of oxidase- and catalase-positive, strictly aerobic bacteria, unable to reduce nitrate or nitrite. No photosynthetic pigments or manganese oxidation were observed. This isolate could reduce triphenyltetrazolium in the presence of hydrogen but not in its absence, suggesting that hydrogenase activity is present. However, isolate N2-680^T could not grow autotrophically in the presence of hydrogen gas. Autotrophic growth did not occur in the presence of molecular sulphur or thiosulphate as electron donors.

The nutritional pattern exhibited by strain N2-680^T was very restricted, since only 11 of the 65 carbon sources tested could support growth. Nevertheless, the carbon sources used represent different chemical classes, namely organic acids, amino acids and hydrocarbons (Table 1). Isolate N2-680^T was able to degrade polycaprolactone oxydiethylene ester; growth and polymer degradation were observed after 3 days at 50 °C.

Isolate N2-680^T presented poor, but visible growth on mineral medium A with acetate, without ammonium sulphate. However, after two successive transfers under the same conditions, no growth occurred, probably indicating that the cell proliferation observed in the initial cultures was due to the use of nitrogen-containing compounds present in reserve materials. Based on these results, is possible to conclude that isolate N2-680^T is unable to use N_2 as a nitrogen source.

Analysis of the polar lipid pattern of strain N2-680^T by TLC revealed the presence of phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) as the major phospholipids. The only respiratory quinone detected was ubiquinone 8. The predominance of the phospholipids PE and PG and the presence of ubiquinone 8 confirm the inclusion of isolate N2-680^T within the β-subclass of the *Proteobacteria* (Wilkinson, 1988; Suzuki *et al.*, 1993).

The fatty acid composition of strain N2-680^T was analysed using LB agar cultures grown at 30 and 50 °C (Table 2). At 30 °C, the predominant components were $C_{16:0}$, $C_{16:1}$ and $C_{18:1}$, in approximately equal proportions. At this temperature, cyclo-$C_{17:0}$ represented only 6·5 % of the total fatty acids. At 50 °C, $C_{16:0}$ and cyclo-$C_{17:0}$ represented about 70 % of the total FAMEs. Since cyclopropane fatty acids are secondary products of fatty acid biosynthesis (Suzuki *et al.*, 1993), the use of cyclo-fatty acids as chemotaxonomic markers should be considered with caution. However, appreciable amounts (more than 20 %) of the fatty acid cyclo-$C_{17:0}$ were reproducibly detected when this isolate was cultivated for 1 and 3 days at 50 °C. The hydroxy fatty acids 3-OH-$C_{10:0}$ and 3-OH-$C_{12:0}$ were detected at 30 and 50 °C. Temperature-induced variations in the fatty acid composition of isolate N2-680^T agree with the tendency observed for other moderately thermophilic *Proteobacteria*, in which higher growth temperatures induce an increase in the content of cyclic fatty acids and a decrease in the degree

Table 1. Phenotypic characteristics of isolate N2-680^T

+, Positive result or growth; −, negative result or no growth. Strain N2-680^T was unable to use these compounds as sole sources of carbon: aesculin, erythritol, D-arabinose, ribose, D-xylose, L-xylose, adonitol, methyl β-xyloside, galactose, D-glucose, D-fructose, D-mannose, L-sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, methyl α-D-mannoside, methyl α-D-glucoside, *N*-acetylglucosamine, amygdalin, arbutin, salicin, maltose, lactose, melibiose, sucrose, trehalose, inulin, melezitose, D-raffinose, starch, glycogen, xylitol, β-gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, 2 ketogluconate, 5-ketogluconate, malate, hydroxyproline, L-glycine, L-histidine, DL-methionine, L-arginine and L-asparagine. The following enzymes were absent from isolate N2-680^T: trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-fucosidase and α-mannosidase.

Characteristic	Response	Characteristic	Response
Presence of:		Growth in presence of/at:	
Polar flagellum	+	Penicillin G (10 U)	+
Catalase	+	Ampicillin (10 μg)	+
Cytochrome-*c* oxidase	+	Nalidixic acid (30 μg)	−
PHB	+	3 % NaCl	+
Photosynthetic pigments	−	4 % NaCl	−
Utilization of carbon sources:		pH 5	−
Acetate	+	pH 6	+
Citrate	+	pH 9	+
Gluconate	+	pH 10	−
Caproate	+	Activity of:	
L-Glutamic acid	+	Cystine arylamidase	+
Cellobiose	+	Alkaline phosphatase	+
L-Arabinose	+	Esterase (C4)	+
Glycerol	+	Esterase lipase (C8)	+
L-Alanine	+	Lipase (C14)	+
Proline	+	Leucine arylamidase	+
Serine	+	Valine arylamidase	+
Autotrophic growth with H_2, S^0 or $S_2O_3^{2-}$	−	Acid phosphatase	+
Reduction of nitrate	−	Naphthol-AS-BI-phosphohydrolase	+
Anaerobic growth with nitrate	−	β-Glucosidase	+
N_2 used as nitrogen source	−	Amylase	−
Mn^{2+} oxidation	−	Tweenase (Tween 80)	+
Requirement for growth factors (vitamins or amino acids)	−	Hydrogenase activity	+
Degradation of polycaprolactone oxydiethylene ester	+		

Table 2. Fatty acid composition of strain N2-680^T

Values are percentages of total fatty acids. Components representing <1 % of total fatty acids are summarized under 'Other' and include iso-$C_{11:0}$ 3-OH, $C_{14:0}$ and $C_{17:0}$.

Fatty acid	50 °C	30 °C
$C_{10:0}$ 3-OH	2·8	3·5
$C_{10:0}$	1·4	1·2
$C_{12:0}$ 3-OH	1·9	1·9
$C_{12:0}$	1·6	1·6
iso-$C_{16:0}$	2·2	0·5
$C_{16:0}$	44·2	23·7
$C_{16:1}$	3·7	29·0
cyclo-$C_{17:0}$	27·6	6·5
$C_{18:0}$	2·7	2·3
$C_{18:1}$	5·7	27·7
cyclo-$C_{19:0}$ (ω8*c*)	3·1	0·1
Other	3·1	2·0
Total	100·0	100·0

of chain unsaturation (Manaia & Moore 2002; Busse *et al.*, 2002).

The G+C content of genomic DNA of strain N2-680^T was 70·1 mol%. Nearly the complete 16S rDNA sequence of strain N2-680^T was determined (1435 nucleotide positions) and compared with reference sequences in databases. Phylogenetic analysis of the 16S rDNA sequence of strain N2-680^T showed its affiliation to the β-subclass of the *Proteobacteria*, being most closely related to the genera *Ideonella*, *Leptothrix*, *Rubrivivax* and *Aquabacterium* and the species *Alcaligenes latus*, as shown in Fig. 1. The highest sequence similarities were to *Leptothrix mobilis* DSM 10617^T and *Ideonella dechloratans* CCUG 30898^T (95·7 % sequence similarity).

Considerable physiological heterogeneity characterizes the sub-branch *Rubrivivax–Roseateles–Leptothrix–Ideonella–Aquabacterium* of the β-*Proteobacteria*. Among the few common characteristics attributed to members of this phylogenetic lineage are the accumulation of PHB granules,

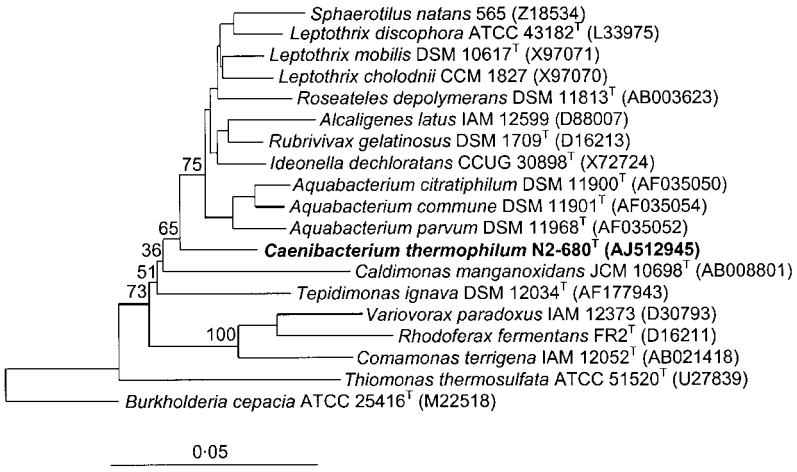

Fig. 1. Phylogenetic relationships of the 16S rDNA sequence of strain N2-680^T with related genera within the β-subclass of the *Proteobacteria*. Evolutionary-distance matrices were calculated using the correction of Jukes & Cantor (1969). The dendrogram was constructed using the FITCH program included in PHYLIP (Felsenstein, 1989). The 16S rDNA sequence of *Burkholderia cepacia* ATCC 25416^T was used as the outgroup. Bootstrap values of relevant branches obtained after 1000 replicates are indicated at the nodes.

the presence of ubiquinone 8 and a DNA base composition ranging from 66 to 72 mol% G+C. The fatty acid composition is not published for all species with validly published names within this phylogenetic lineage; however, based on the data available (Busse & Auling, 1992; Spring *et al.*, 1996; Takeda *et al.*, 2002), the predominance of the fatty acids $C_{16:0}$, $C_{16:1}$ and $C_{18:1}$ seems to represent another feature of this group. As presented in Tables 1–3, strain N2-680^T shares all these characteristics with its closest phylogenetic neighbours.

One important characteristic that distinguishes strain N2-680^T from its closest phylogenetic neighbours, i.e. members of the genera *Rubrivivax*, *Leptothrix*, *Ideonella*

Table 3. Characteristics of strain N2-680^T and related species

Taxa: 1, strain N2-680^T; 2, *Alcaligenes latus*; 3, *Rubrivivax gelatinosus*; 4, *Leptothrix mobilis*; 5, *Ideonella dechloratans*; 6, *Aquabacterium commune*; 7, *Caldimonas manganoxidans*; 8, *Tepidimonas ignava*. Data were obtained from Busse & Auling (1992), Kalmbach *et al.* (1999), Malmqvist *et al.* (1994), Moreira *et al.* (2000), Palleroni & Palleroni (1978), Spring *et al.* (1996), Takeda *et al.* (2002) and Willems *et al.* (1991). NA, No data available; d, variable result within the species.

Characteristic	1	2	3	4	5	6	7	8
Optimal (or range) temperature for growth (°C)	47	35	35	10–37	12–42	6–34	50	55
Photosynthetic pigments	−	−	+	−	−	NA	−	NA
Autotrophic growth with H_2	−	+	+*	NA	NA	NA	NA	NA
Nitrate reduction	−	+	NA	NA	+	+	NA	−
Anaerobic growth with nitrate	−	−	NA	NA	+	+	−	−
N_2 used as nitrogen source	−	+	+	NA	NA	NA	NA	NA
Specific growth requirements	−	NA	+	NA	+	−	−	+
Mn^{2+} oxidation	−	NA	NA	+	NA	−	+	NA
PHB accumulation	+	+	NA	+	NA	+	+	−
Tween 80 hydrolysis	+	+	NA	NA	NA	+	NA	−
Utilization of carbon sources:								
Acetate	+	−	+	−	+	+	−	+
Citrate	+	d	+	−	NA	−	+	−
Glucose	−	+	+	−	+	−	+	−
Arabinose	+	−	NA	NA	NA	−	−	−
Cellobiose	+	−	NA	NA	NA	NA	NA	−
Galactose	−	−	NA	−	NA	−	+	−
Sucrose	−	+	NA	−	NA	−	+	−
Mannitol	−	−	−	−	NA	−	+	−
Sorbitol	−	−	−	NA	NA	NA	+	−
Glycerol	+	+	−	−	NA	−	+	−
DNA G+C content (mol%)	70	69–71	71–72	68	68	66	66	70

*Photoautotrophic growth.

and *Aquabacterium* and the species *Alcaligenes latus*, is the temperature range of growth. Strain N2-680^T differs from species of the genus *Leptothrix* in the absence of a cell sheath and the inability to produce manganese oxides, as is typical for these organisms (Siering & Ghiorse, 1996). Characteristics that differentiate strain N2-680^T from *Leptothrix mobilis* include the capacity to use acetate, citrate and glycerol as single carbon sources. The presence of photosynthetic pigments, described for members of *Rubrivivax gelatinosus* (Willems *et al.*, 1991), constitutes another distinction between isolate N2-680^T and this species. Members of *Alcaligenes latus* can grow autotrophically in the presence of hydrogen gas, are able to reduce nitrate and can fix nitrogen (Palleroni & Palleroni, 1978; Busse & Auling, 1992). All these characteristics were absent for isolate N2-680^T. The presence of catalase, the use of carbohydrates as sole carbon sources and the inability to reduce nitrate and to grow anaerobically with nitrate allow distinction between isolate N2-680^T and *Aquabacterium* species (Kalmbach *et al.*, 1999). *Ideonella dechloratans* differs from isolate N2-680^T in the ability to use glucose as a single carbon source and in the capacity to reduce nitrate (Malmqvist *et al.*, 1994).

More distantly related phylogenetic neighbours of strain N2-680^T are the thermophilic species *Caldimonas manganoxidans* and *Tepidimonas ignava*, which share 16S rDNA sequence identity of 93·6 and 94·7 %, respectively, with the novel isolate. Despite the fact that the three organisms are thermophilic, the comparatively low values of 16S rDNA sequence identity and the differences observed for other phenotypic traits are consistent with the definition of distinct genera. *Caldimonas manganoxidans* can be distinguished from strain N2-680^T by its ability to oxidize manganese and to use malate, mannitol, sorbitol, D-glucose, D-galactose, maltose and sucrose as single carbon sources (Takeda *et al.*, 2002). *Tepidimonas ignava* differs from strain N2-680^T in the absence of PHB granules, the inability to grow in the presence of 3 % NaCl and to hydrolyse Tween 80, the requirement for specific growth factors, the inability to use arabinose, cellobiose and glycerol and the ability to use malate and asparagine as single carbon sources. Moreover, the optimum temperature for growth of *Tepidimonas ignava* is 55 °C, slightly higher than that observed for strain N2-680^T (Moreira *et al.*, 2000). The phylogenetic position of strain N2-680^T, along with its phenotypic characteristics, support the description of a new genus. Characteristics that differentiate between strain N2-680^T, its phylogenetic closest relatives and the thermophilic species more phylogenetically closely related to this isolate are summarized in Table 3.

Based on FASTA analysis, isolate N2-680^T showed 99·9 % 16S rDNA identity to a thermophilic organism, strain DhA-71 (EMBL accession no. AF125876), described as capable of degrading dehydroabietic acid (Yu & Mohn, 1999), indicating that the two isolates might belong to the same species. Strain DhA-71 was isolated from municipal compost in Canada, whereas strain N2-680^T was recovered from a thermophilic sludge digester in Portugal, suggesting that this species may have a widespread distribution in such habitats.

The phenotypic and chemotaxonomic characterization of strain N2-680^T and 16S rDNA-based phylogenetic analysis revealed that this bacterium is not affiliated to any validly named genus. The definition of the new genus *Caenibacterium* gen. nov., containing the species *Caenibacterium thermophilum* sp. nov., is proposed, with isolate N2-680^T as the type strain.

Description of *Caenibacterium* gen. nov.

Caenibacterium (Cae′ni.bac.te.ri.um. L. n. *caenum* mud, sludge; N.L. n. *bacterium* from Gr. n. *bakterion* rod; N.L. neut. n. *Caenibacterium* a rod-shaped bacterium isolated from sludge).

Forms rod-shaped cells that stain Gram-negative, with a polar flagellum. Endospores are not formed. PHB granules are accumulated. Oxidase and catalase are positive. Slightly thermophilic. Major phospholipids are phosphatidylethanolamine and phosphatidylglycerol; ubiquinone 8 is the major respiratory quinone. Major fatty acids include $C_{16:0}$, $C_{16:1}$ and $C_{18:1}$ or its secondary products such as cyclo-$C_{17:0}$. The hydroxylated fatty acids 3-OH-$C_{10:0}$ and 3-OH-$C_{12:0}$ are present. Nitrate is not reduced, photosynthetic pigments are not present and Mn^{2+} is not oxidized. No autotrophic growth occurs. Chemo-organotrophic. Organic acids, amino acids and hydrocarbons are used as single carbon sources. The type species is *Caenibacterium thermophilum*.

Description of *Caenibacterium thermophilum* sp. nov.

Caenibacterium thermophilum (ther.mo′phi.lum. Gr. n. *therme* warm; Gr. adj. *philos* friendly to; N.L. neut. adj. *thermophilum* loving warmth, thermophilic).

Forms rod-shaped cells, 1·3 μm long and 0·5 μm wide. A single polar flagellum is observed at the early stages of growth. Colonies grown on LB agar are non-pigmented, slightly brilliant and 1–2 mm in diameter after 36–48 h growth. Growth occurs above 25 °C and below 57 °C; the optimal growth temperature is approximately 47 °C. Growth occurs between pH 6 and 9. Hydrogenase- and tweenase-positive. Acetate, citrate, gluconate, caproate, glutamic acid, cellobiose, arabinose, glycerol, alanine, proline and serine are used as single carbon sources. Capable of degradation of polycaprolactone oxydiethylene ester. The major fatty acids at 50 °C are $C_{16:0}$ and cyclo-$C_{17:0}$. The DNA G+C content of the type strain is 70·1 mol%.

The type strain, strain N2-680^T (=DSM 15264^T=LMG 21760^T), was isolated from a thermophilic aerobic digester of wastewater-treatment sludge.

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REFERENCES

Aragno, M. & Schlegel, H. G. (1992). The mesophilic hydrogen oxidizing (Knallgas) bacteria. In *The Prokaryotes*, 2nd edn, pp. 3917–3933. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K.-H. Schleifer. New York: Springer.

Busse, H. J. & Auling, G. (1992). The genera *Alcaligenes* and '*Achromobacter*'. In *The Prokaryotes*, 2nd edn, pp. 2544–2555. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K.-H. Schleifer. New York: Springer.

Busse, H.-J., Kämpfer, P., Moore, E. R. B. & 7 other authors (2002). *Thermomonas haemolytica* gen. nov., sp. nov., a γ-proteobacterium from kaolin slurry. *Int J Syst Evol Microbiol* **52**, 473–483.

Carlton, B. C. & Brown, B. J. (1981). Gene mutation. In *Manual of Methods for General Bacteriology*, pp. 409–443. Edited by P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg & G. H. Phillips. Washington, DC: American Society for Microbiology.

Cashion, P., Holder-Franklin, M. A., McCully, J. & Franklin, M. (1977). A rapid method for the base ratio determination of bacterial DNA. *Anal Biochem* **81**, 461–466.

Doetsch, R. N. (1981). Determinative methods of light microscopy, In *Manual of Methods for General Bacteriology*, pp. 21–33. Edited by P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg & G. H. Phillips. Washington, DC: American Society for Microbiology.

Felsenstein, J. (1989). PHYLIP – phylogeny inference package. *Cladistics* **5**, 164–166.

Heimbrook, M. E., Wang, W. L. L. & Campbell, G. (1989). Staining bacterial flagella easily. *J Clin Microbiol* **27**, 2612–2615.

Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.

Kalmbach, S., Manz, W., Wecke, J. & Szewzyk, U. (1999). *Aquabacterium* gen. nov., with description of *Aquabacterium citratiphilum* sp. nov., *Aquabacterium parvum* sp. nov. and *Aquabacterium commune* sp. nov., three *in situ* dominant bacterial species from the Berlin drinking water system. *Int J Syst Bacteriol* **49**, 769–777.

Kuykendall, L. D., Roy, M. A., O'Neill, J. J. & Devine, T. E. (1988). Fatty acids, antibiotic resistance, and deoxyribonucleic acid homology groups of *Bradyrhizobium japonicum*. *Int J Syst Bacteriol* **38**, 358–361.

Lane, D. J. (1991). 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*, pp. 115–175. Edited by E. Stackebrandt & M. Goodfellow. Chichester: Wiley.

Malmqvist, A., Welander, T., Moore, E., Ternström, A., Molin, G. & Sternström, I. (1994). *Ideonella dechloratans*, gen. nov., sp. nov., a new bacterium capable of growing anaerobically with chlorate as an electron acceptor. *Syst Appl Microbiol* **17**, 58–64.

Manaia, C. M. & Moore, E. R. B. (2002). *Pseudomonas thermotolerans* sp. nov., a thermotolerant species of the genus *Pseudomonas sensu stricto*. *Int J Syst Evol Microbiol* **52**, 2203–2209.

Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.

Moreira, C., Rainey, F. A., Nobre, M. F., da Silva, M. T. & da Costa, M. S. (2000). *Tepidimonas ignava* gen. nov., sp. nov., a new chemolithoherotrophic and slightly thermophilic member of the β-*Proteobacteria*. *Int J Syst Evol Microbiol* **50**, 735–742.

Mulder, E. G. (1989). Genus *Leptothrix* Kützing 1843, 198^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. 3, pp. 1998–2003. Edited by J. T. Staley, M. P. Bryant, N. Pfennig & J. G. Holt. Baltimore: Williams &Wilkins.

Mulder, E. G. & Deinema, M. H. (1992). The sheathed bacteria. In *The Prokaryotes*, 2nd edn, pp. 2612–2624. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K.-H. Schleifer. New York: Springer.

Nealson, K. H. (1992). The manganese-oxidizing bacteria. In *The Prokaryotes*, 2nd edn, pp. 2310–2320. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K.-H. Schleifer. New York: Springer.

Nogales, B., Moore, E. R. B., Llobet-Brossa, E., Rossello-Mora, R., Amann, R. & Timmis, K. N. (2001). Combined use of 16S ribosomal DNA and 16S rRNA to study the bacterial community of polychlorinated biphenyl-polluted soil. *Appl Environ Microbiol* **67**, 1874–1884.

Palleroni, N. J. & Palleroni, A. V. (1978). *Alcaligenes latus*, a new species of hydrogen-utilizing bacteria. *Int J Syst Bacteriol* **28**, 416–424.

Pearson, W. R. & Lipman, D. J. (1988). Improved tools for biological sequence comparison. *Proc Natl Acad Sci U S A* **85**, 2444–2448.

Pfennig, N. (1978). *Rhodocyclus purpureus* gen. nov. and sp. nov., a ring-shaped, vitamin B_{12}-requiring member of the family *Rhodospirillaceae*. *Int J Syst Bacteriol* **28**, 283–288.

Prado, A., da Costa, M. S. & Madeira, V. M. C. (1988). Effect of growth temperature on the lipid composition of two strains of *Thermus* sp. *J Gen Microbiol* **134**, 1653–1660.

Siefert, E., Irgens, R. L. & Pfennig, N. (1978). Phototrophic purple and green bacteria in a sewage treatment plant. *Appl Environ Microbiol* **35**, 38–44.

Siering, P. L. & Ghiorse, W. C. (1996). Phylogeny of the *Sphaerotilus-Leptothrix* group inferred from morphological comparisons, genomic fingerprinting, and 16S ribosomal DNA sequence analyses. *Int J Syst Bacteriol* **46**, 173–182.

Smibert, R. M. & Krieg, N. R. (1981). General characterization. In *Manual of Methods for General Bacteriology*, pp. 409–443. Edited by P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg & G. H. Phillips. Washington, DC: American Society for Microbiology.

Spring, S., Kämpfer, P., Ludwig, W. & Schleifer, K. H. (1996). Polyphasic characterization of the genus *Leptothrix*: new descriptions of *Leptothrix mobilis* sp. nov. and *Leptothrix discophora* sp. nov. nom. rev. and emended description of *Leptothrix cholodnii* emend. *Syst Appl Microbiol* **19**, 634–643.

Stöhr, R., Waberski, A., Liesack, W., Völker, H., Wehmeyer, U. & Thomm, M. (2001). *Hydrogenophilus hirschii* sp. nov., a novel thermophilic hydrogen-oxidizing β-proteobacterium isolated from Yellowstone National Park. *Int J Syst Evol Microbiol* **51**, 481–488.

Suyama, T., Hosoya, H. & Tokiwa, Y. (1998). Bacterial isolates degrading aliphatic polycarbonates. *FEMS Microbiol Lett* **161**, 255–261.

Suyama, T., Shigematsu, T., Takaichi, S., Nodasaka, Y., Fujikawa, S., Hosoya, H., Tokiwa, Y., Kanagawa, T. & Hanada, S. (1999). *Roseateles depolymerans* gen. nov., sp. nov., a new bacteriochlorophyll *a*-containing obligate aerobe belonging to the β-subclass of the *Proteobacteria*. *Int J Syst Bacteriol* **49**, 449–457.

Suzuki, K., Goodfellow, M. & O'Donnell, A. G. (1993). Cell envelopes and classification. In *Handbook of New Bacterial Systematics*,

pp. 195–250. Edited by M. Goodfellow & A. G. O'Donnell. London: Academic Press.

Takeda, M., Koizumi, J., Yabe, K. & Adachi, K. (1998). Thermostable poly(3-hydroxybutyrate) depolymerase of a thermophilic strain of *Leptothrix* sp. isolated from a hot spring. *J Ferment Bioeng* **85**, 375–380.

Takeda, M., Kamagata, Y., Ghiorse, W. C., Hanada, S. & Koizumi, J. (2002). *Caldimonas manganoxidans* gen. nov., sp nov., a poly(3-hydroxybutyrate)-degrading, manganese-oxidizing thermophile. *Int J Syst Evol Microbiol* **52**, 895–900.

Tindall, B. J. (1989). Fully saturated menaquinones in the archaebacteriun *Pyrobaculum islandicum*. *FEMS Microbiol Lett* **60**, 251–254.

Wilkinson, S. G. (1988). Gram-negative bacteria. In *Microbial Lipids*, vol. 1, pp. 299–488. Edited by C. Ratledge & S. G. Wilkinson. London: Academic Press.

Willems, A., Gillis, M. & De Ley, J. (1991). Transfer of *Rhodocyclus gelatinosus* to *Rubrivivax gelatinosus* gen. nov., comb. nov., and phylogenetic relationships with *Leptothrix*, *Sphaerotilus natans*, *Pseudomonas saccharophila*, and *Alcaligenes latus*. *Int J Syst Bacteriol* **41**, 65–73.

Yu, Z. & Mohn, W. W. (1999). Isolation and characterization of thermophilic bacteria capable of degrading dehydroabietic acid. *Can J Microbiol* **45**, 513–519.