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

Célia M. Manaia, 1 Olga C. Nunes 2 and Balbina Nogales 3

1Escola Superior de Biotecnologia, Universidade Católica Portuguesa, 4200-072 Porto, Portugal
2LEPAE-Departamento de Engenharia Química, Faculdade de Engenharia, Universidade do Porto, 4200-465 Porto, Portugal
3Area de Microbiologia, Universitat de les Illes Balears, 07071 Palma de Mallorca, Spain

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 C16 : 0 and cyclo-C17 : 0. The highest 16S rDNA sequence identity of isolate N2-680 T was to Leptothrix mobilis and Ideonella dechloratans (95 %) and to Rubrivivax gelatinosus and Aquabacterium commune (95 %). 16S rDNA sequence similarities to species of two related thermophilic genera, Caldimonas manganoxidans and Tepidimonas ignava, were lower (93 % and 94 %). 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, thermophilic is represented by the genus Caldimonas (Takeda et al., 2002).
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(3-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 N2-680T.

**METHODS**

**Isolation and cultivation conditions.** Strain N2-680T 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(3-caprolactone) thermoplastic (oxygenated homopolymer, with a molecular mass of 80 000; Solvay). Cultures were transferred weekly to fresh medium for 2 months. Isolate N2-680T 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 N2-680T 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 Ruy 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 N2-680T 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% H2 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 (1 g yeast extract, 1 g peptone, 0.2 g MgSO4·7H2O, 0.5 g ferric ammonium citrate, 50 mg CaCl2, 50 mg MnSO4·H2O, 10 mg FeC12·6H2O, 20 g agar, pH 7.1). *Leptothrix mobilis* DSM 10617T 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 e-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 NH4NO3, 0.2 g yeast extract, 0.25 g K2HPO4, 0.13 g MgSO4·7H2O, 2.5 mg FeC2(SO4)3, 2.5 mg MnSO4, 50 μg K2MnO4, 50 μg Na2BO3, 50 μg Co(NO3)2·6H2O, 50 μg FeCl3, 50 μg CdSO4·50 μg CuSO4 and 50 μg ZnSO4. 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 (NH4)2SO4, 0.31 g KH2PO4, 0.45 g K2HPO4, 1.2 g Na2HPO4·2H2O, 0.1 g NaCl, 0.05 g CaCl2, 0.4 g MgSO4·7H2O, 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 H3BO3, 200 μg FeCl3·6H2O, 400 μg ZnSO4·7H2O, 400 μg MnSO4·H2O, 40 μg CuSO4·5H2O, 200 μg Na2MoO4·2H2O, 100 μg KI, 2.5 g agar]. Chemolithoautotrophic growth was tested using medium A supplemented with filter-sterilized 30 mM NaHCO3 and different electron donors. The use of H2 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 assayed 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.

**RESULTS AND DISCUSSION**

**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\textsuperscript{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).

Isolate N2-680\textsuperscript{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, it is possible to conclude that isolate N2-680\textsuperscript{T} is unable to use N\textsubscript{2} as a nitrogen source.

Analysis of the polar lipid pattern of strain N2-680\textsuperscript{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\textsuperscript{T} within the \( \beta \)-subclass of the *Proteobacteria* (Wilkinson, 1988; Suzuki et al., 1993).

The fatty acid composition of strain N2-680\textsuperscript{T} was analysed using LB agar cultures grown at 30 and 50 °C (Table 2). At 30 °C, the predominant components were C\textsubscript{16:0}, C\textsubscript{16:1} and C\textsubscript{18:1\textsuperscript{a}} in approximately equal proportions. At this temperature, cyclo-C\textsubscript{17:0} represented only 6-5 % of the total fatty acids. At 50 °C, C\textsubscript{16:0} and cyclo-C\textsubscript{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\textsubscript{17:0} were reproducibly detected when this isolate was cultivated for 1 and 3 days at 30 °C. The hydroxy fatty acids 3-OH-C\textsubscript{10:0} and 3-OH-C\textsubscript{12:0} were detected at 30 and 50 °C. Temperature-induced variations in the fatty acid composition of isolate N2-680\textsuperscript{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...
The G + C content of genomic DNA of strain N2-680T was 70.1 mol%. Nearly the complete 16S rDNA sequence of strain N2-680T was determined (1435 nucleotide positions) and compared with reference sequences in databases. Phylogenetic analysis of the 16S rDNA sequence of strain N2-680T showed its affiliation to the beta 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 10617T and Ideonella dechloratans CCUG 30898T (95-7% sequence similarity).

Considerable physiological heterogeneity characterizes the sub-branch Rubrivivax–Roseateles–Leptothrix–Ideonella–Aquabacterium of the beta-Proteobacteria. Among the few common characteristics attributed to members of this phylogenetic lineage are the accumulation of PHB granules,
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 C16:0, C16:1 and C18:1 seems to represent another feature of this group. As presented in Tables 1–3, strain N2-680T shares all these characteristics with its closest phylogenetic neighbours.

One important characteristic that distinguishes strain N2-680T from its closest phylogenetic neighbours, i.e. members of the genera Rubrivivax, Leptothrix, Ideonella...
and Aquabacterium and the species Alcaligenes latus, is the temperature range of growth. Strain N2-680<sup>T</sup> 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<sup>T</sup> 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<sup>T</sup> 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<sup>T</sup>. 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<sup>T</sup> and Aquabacterium species (Kalmbach et al., 1999). Ideonella dechloratans differs from isolate N2-680<sup>T</sup> 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<sup>T</sup> are the thermophilic species Caldimonas manganoxidan 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 manganoxidan can be distinguished from strain N2-680<sup>T</sup> 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<sup>T</sup> 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 glyceral 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<sup>T</sup> (Moreira et al., 2000). The phylogenetic position of strain N2-680<sup>T</sup>, along with its phenotypic characteristics, support the description of a new genus. Characteristics that differentiate between strain N2-680<sup>T</sup>, 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<sup>T</sup> showed 99·9 % 16S rDNA identity to a thermophilic organism, strain DhaA-71 (EMBL accession no. AF125876), described as capable of degrading dehydroabiatic acid (Yu & Mohn, 1999), indicating that the two isolates might belong to the same species. Strain DhaA-71 was isolated from municipal compost in Canada, whereas strain N2-680<sup>T</sup> 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<sup>T</sup> 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<sup>T</sup> 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 phosphatidyglycerol; ubiquinone 8 is the major respiratory quinone. Major fatty acids include C<sub>16:0</sub>, C<sub>16:1</sub> and C<sub>18:1</sub> or its secondary products such as cyclo-C<sub>17:0</sub>. The hydroxylated fatty acids 3-OH-C<sub>10:0</sub> and 3-OH-C<sub>12:0</sub> are present. Nitrate is not reduced, photosynthetic pigments are not present and Mn<sup>2+</sup> 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. thermoe 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<sub>16:0</sub> and cyclo-C<sub>17:0</sub>. The DNA G + C content of the type strain is 70·1 mol%.

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

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