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

Célia M. Manaia, Olga C. Nunes and Balbina Nogales

**INTRODUCTION**

The phylogenetic lineage of the \( \beta \)-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 chloride 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).
Caldimonas manganoxidans, the single species within this genus with a validly published name, comprises chemoorganotrophic 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 chemolithoautotrophic 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 N2-680T.

**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-ε-caprolactone thermoplastic (oxygenane 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 morphology, Gram-staining reaction, production of spores and the characteristics were examined using standard protocols (Doetsch, 1981). Cell characteristics.

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Manganese oxidation was tested on Sphaerotilus—Leptothrix medium (1−1: 1 g yeast extract, 1·5 g peptone, 0·2 g MgSO4·7H2O, 0·5 g ferric ammonium citrate, 50 g CaCl2, 50 g MnSO4·H2O, 10 mg FeCl3·6H2O, 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 benzidin 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 (1−1): 1 g NH4NO3, 0·2 g yeast extract, 0·25 g K3PO4, 0·13 g NaCl, 2·5 mg Fe(NO3)3, 2·5 mg MnSO4, 50 µg K2MoO4, 50 µg Na2MoO4, 50 µg Co(NO3)2, 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) [1−1: 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 MnSO4·4H2O, 40 µg CuSO4·5H2O, 200 µg Na2MoO4·2H2O, 100 µg KCl, 2·5 g gel] 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\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).

**RESULTS AND DISCUSSION**

Cultivation at 50 °C in mineral medium supplemented with a poly-e-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\textsuperscript{T}, which was purified by successive subculturing on LB agar.

Individual cells of isolate N2-680\textsuperscript{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\textsuperscript{T}. When cultured on LB agar, strain N2-680\textsuperscript{T} produced non-pigmented colonies, 1-2 mm in diameter after 36-48 h growth.

In pure culture, isolate N2-680\textsuperscript{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\textsuperscript{T} could also grow on nutrient-rich media such as LB. In LB medium, the optimal growth temperature of strain N2-680\textsuperscript{T} was around 47 °C, with a maximal temperature for growth of 57 °C.

The physiological properties of strain N2-680\textsuperscript{T} are summarized in Table 1. Strain N2-680\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{T} was able to degrade polyacaprolactone oxycarboxylic ester; growth and polymer degradation were observed after 3 days at 50 °C.

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, 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} 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 50 °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
of chain unsaturation (Manaia & Moore 2002; Busse et al., 2002).

The G+C content of genomic DNA of strain N2-680\textsuperscript{T} was 70.1 mol%. Nearly the complete 16S rDNA sequence of strain N2-680\textsuperscript{T} was determined (1435 nucleotide positions) and compared with reference sequences in databases. Phylogenetic analysis of the 16S rDNA sequence of strain N2-680\textsuperscript{T} 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 1061\textsuperscript{7} and Ideonella dechloratans CCUG 30898\textsuperscript{T} (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, chain unsaturation (Manaia & Moore 2002; Busse et al., 2002).

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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

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

<table>
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<th>Characteristic</th>
<th>1</th>
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<th>6</th>
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<th>8</th>
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<td>35</td>
<td>35</td>
<td>10–37</td>
<td>12–42</td>
<td>6–34</td>
<td>50</td>
<td>55</td>
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<td>Autotrophic growth with H₂</td>
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<td>+</td>
<td>*</td>
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<td>Nitrate reduction</td>
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<td>NA</td>
<td>+</td>
<td>+</td>
<td>NA</td>
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<td>Anaerobic growth with nitrate</td>
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<td>N₂ used as nitrogen source</td>
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<td>NA</td>
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<td>Specific growth requirements</td>
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<td>+</td>
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<td>69–71</td>
<td>71–72</td>
<td>68</td>
<td>68</td>
<td>66</td>
<td>66</td>
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*Photoautotrophic growth.

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**Fig. 1.** Phylogenetic relationships of the 16S rDNA sequence of strain N2-680ᵀ 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 25416ᵀ was used as the outgroup. Bootstrap values of relevant branches obtained after 1000 replicates are indicated at the nodes.
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 & Ghirose, 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 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<sup>T</sup> by its ability to oxidize manganese and to use malate, mannnitol, 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 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<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 DhA-71 (EMBL accession no. AF125876), described as capable of degrading dehydroabiotic 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<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.rum. 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:</sub>0, 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. 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, glyceral, 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> 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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REFERENCES


