Tepidiphilus margaritifer gen. nov., sp. nov., isolated from a thermophilic aerobic digester

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A moderately thermophilic bacterium is described, strain N2-214^T, that was isolated from an enrichment culture, growing on caprolactone, obtained from a sample from a water-treatment sludge aerobic digester operating at temperatures around 60 °C. The organism was aerobic, Gram-negative, oxidase- and catalase-positive, with a polar flagellum, and capable of growth at temperatures as high as 61 °C. The major fatty acids of strain N2-214^T were $C_{16:0}$, $C_{18:1}$ and cyclo-$C_{19:0}$. The phylogenetic relationships of the strain, derived from 16S rRNA gene sequence comparisons, demonstrated it to be a member of the *β*-subclass of the *Proteobacteria*. The highest 16S rDNA sequence similarity of isolate N2-214^T was to *Azoarcus buckelii* (91·9 %), *Thauera aromatica* (92 %) and *Hydrogenophilus thermoluteolus* (92·7 %). On the basis of phylogenetic analyses and physiological and chemotaxonomic characteristics, it is proposed that isolate N2-214^T (=DSM 15129^T=LMG 21637^T) represents a new genus and species, *Tepidiphilus margaritifer* gen. nov., sp. nov.

INTRODUCTION

The vast majority of species belonging to the class *Proteobacteria* of the domain *Bacteria* are mesophilic; nevertheless, some slightly or extremely thermophilic genera and species have been described. Thermophilic organisms are distributed among the different subclasses of the *Proteobacteria*, and most of them are chemolithotrophs that can use H_2 or sulfur/sulfur compounds as electron donors. Within the *β*-subclass of the *Proteobacteria*, eight species of thermophilic organisms, included in five genera, have been described to date. The species *Thiomonas thermosulfata* (Shooner *et al.*, 1996), *Thermothrix azorensis* (Odintsova *et al.*, 1996), *Thermothrix thiopara* (Caldwell *et al.*, 1976) and *Tepidimonas ignava* (Moreira *et al.*, 2000) represent thermophilic organisms, characterized by their ability to use sulfur or sulfur compounds as an energy source. *Tepidimonas ignava*, isolated from a Portuguese hot spring, is the only strictly heterotrophic organism among these sulfur bacteria. The genus *Hydrogenophilus* currently comprises two species, *Hydrogenophilus thermoluteolus* (Hayashi *et al.*, 1999) and *Hydrogenophilus hirschii* (Stöhr *et al.*, 2001), both of which are hydrogen-oxidizing bacteria with the ability to fix CO_2. *Caldimonas manganoxidans*, a chemo-organoheterotrophic, thermophilic member of the *β*-*Proteobacteria*, was described recently (Takeda *et al.*, 2002).

Isolation of these organisms has frequently occurred under conditions favouring the recovery of specific metabolic types, namely hydrogen- and sulfur-oxidizing bacteria; this may explain why the majority of thermophilic members of the *β*-*Proteobacteria* described to date are chemolithotrophic. Indeed, it is intriguing that, despite the abundance of chemo-organoheterotrophic organisms belonging to the *β*-*Proteobacteria*, only a single thermophilic representative of this metabolic group has been described to date.

The present study describes a moderately thermophilic, chemo-organoheterotrophic bacterium, strain N2-214^T, isolated from a thermophilic aerobic digester of water-treatment sludge. On the basis of 16S rDNA-based phylogenetic analyses and physiological and chemotaxonomic characteristics, it is proposed that isolate N2-214^T represents a new genus and species, for which the name *Tepidiphilus margaritifer* gen. nov., sp. nov. is proposed.

Abbreviation: PHB, poly-*β*-hydroxybutyrate.

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METHODS

Isolation, cultivation conditions and bacterial strains. Strain N2-214^T was isolated from a caprolactone 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 subjected to a mesobiotic anaerobic digestion followed by a thermophilic aerobic digestion, which reaches a maximal temperature of about 60 °C. The sample was transported, without temperature control, and used as an inoculum for enrichment on polycaprolactone diol polymer (Solvay) with a molecular mass of 1000 Da. The enrichment was carried out in mineral medium (medium A) (Manaia & Moore, 2002) at 50 °C. Strain N2-214^T was purified from the mixed culture obtained in this enrichment by subculturing on Luria–Bertani (LB) broth containing 20 g agar l^{-1} (LB agar) (Carlton & Brown, 1981). The 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, Gram-staining reaction, production of spores, accumulation of poly-β-hydroxybutyrate (PHB) granules, the number and position of flagella and the temperature range for growth were examined as described by Manaia *et al.* (2003).

Phenotypic tests 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 tolerance range was examined in LB medium, using 10 mM MES (Sigma), for pH 5·0–6·0 or 10 mM TAPS (Sigma) for pH 6·0–9·0. Enzymic activities were tested using the API ZYM system by following the instructions of the manufacturer (bioMérieux). Hydrogenase activity was determined on the basis of the descriptions of Aragno & Schlegel (1992) and Stöhr *et al.* (2001) as described by Manaia *et al.* (2003). The nutritional pattern was characterized using the API 50CH system and a defined medium (medium B) as described by Manaia *et al.* (2003). Chemolithoautotrophic growth, the use of H_2 as an energy source and the ability to use sulfur or thiosulfate as electrons donors were tested as described by Manaia *et al.* (2003). The ability to grow in the absence of a source of combined nitrogen was tested using medium A without ammonium sulfate, under aerobic conditions or under an N_2 atmosphere with nitrate as the electron acceptor.

Determination of genotypic characteristics. The DNA G+C content was determined as described by Manaia *et al.* (2003).

16S rDNA sequence analysis. The 16S rRNA gene sequence was determined after PCR amplification as described by Manaia *et al.* (2003). 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). Calculations were done by using a 50 % conservation filter for the *β-Proteobacteria* implemented in the ARB package and by using a second filter that excluded only the most variable regions in the 16S rRNA molecule, which corresponded to inserts of different lengths amongst the reference sequences used in the analysis. The tree shown later is the one obtained using the second type of filter, since the topology of the tree for the most closely related genera did not vary regardless of which filter was used, and the bootstrap values were slightly higher. Ambiguously determined nucleotide positions or positions for which no sequence data were available in any of the reference sequences were excluded from the calculations.

Determination of chemotaxonomic characteristics. Polar lipids, methylated fatty acids and respiratory quinones were extracted and separated as described previously (Manaia *et al.*, 2003).

RESULTS AND DISCUSSION

Cultivation at 50 °C in mineral medium supplemented with a polycaprolactone diol was used to enrich moderate thermophiles capable of using synthetic polymers as the sole source of carbon and energy. The inoculum for this enrichment was taken from a thermophilic aerobic digester of sludge from a domestic wastewater-treatment plant. The enrichment procedure resulted in a mixed culture, predominantly composed by Gram-positive rods, which, on the basis of phenotypic characterization, were identified as members of the genus *Bacillus*. Isolate N2-214^T was obtained through purification of cultivable organisms present in the mixed culture. In pure culture, this isolate was unable to grow in mineral medium supplemented with caprolactones, although good growth was observed when acetate was the only carbon source. At the optimal temperature for growth (50 °C), the doubling time in LB medium was approximately 1 h. When cultured on LB agar, strain N2-214^T produced brilliant non-pigmented colonies, 1–2 mm in diameter, after 36–48 h growth. Under a strong light source, these colonies had a nacre-like appearance.

Cells of strain N2-214^T were Gram-negative, non-spore-forming rods, 2·0 μm long and 0·7 μm wide, with a single polar flagellum. The isolate tested positive for oxidase and catalase and for the presence of intracellular PHB granules. Strain N2-214^T reduced nitrate to a compound more reduced than nitrite, since nitrite was not detected in the supernatant of cultures. The isolate could grow under anaerobic conditions (N_2-saturated atmosphere) using nitrate as an electron acceptor, both in complex media and in mineral medium supplemented with acetate, ethanol or benzoate. Isolate N2-214^T could reduce triphenyltetrazolium in the presence of hydrogen but not in its absence, suggesting the presence of hydrogenase activity. However, since this organism could grow in a N_2-saturated atmosphere in the absence of H_2 gas, it is possible to conclude that this organism is not a strictly hydrogen-oxidizing chemolithoheterotroph. The optimal temperature for growth of strain N2-214^T in LB medium was around 50 °C, with a maximal growth temperature of 61 °C. The nutritional pattern of strain N2-214^T revealed an inability to use the sugars that were tested and a preference for organic acids, e.g. malate, acetate or benzoate, as carbon sources; other substrates, such as ethanol and the amino acids glutamic acid and asparagine, could also be used as single carbon sources (Table 1). The strain could not grow autotrophically in mineral medium supplemented with sodium hydrogen carbonate in the presence of hydrogen, S^0 or thiosulfate; in the presence of acetate as a carbon source, these potential electron donors did not enhance growth.

The inability of isolate N2-214^T to fix nitrogen or assimilate nitrate was evidenced by the fact that this organism could not grow in mineral medium without combined nitrogen under aerobic conditions, or in the same medium supplemented with nitrate under a N_2 atmosphere.

Table 1. Phenotypic characteristics of isolate N2-214^T

Strain N2-214^{T}was unable to use the following compounds as sole sources of carbon: glycerol, erythritol, D-arabinose, L-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, cellobiose, 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, gluconate, 2-ketogluconate, 5-ketogluconate, hydroxyproline, L-alanine, L-serine, L-glycine, L-histidine, DL-methionine, L-arginine, toluene and polycaprolactone (molecular mass 1000 and above). Strain N2-214^T was unable to grow autotrophically with H_2, S^0 or $S_2O_3^{2-}$, did not fix nitrogen and did not grow in the presence of ampicillin (10 μg), penicillin G (10 U) or 3 % NaCl or at pH 5 or 9. Strain N2-214^T did not require growth factors (vitamins or amino acids). The following enzymes were absent from isolate N2-214^T: cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, *N*-acetyl-β-glucosaminidase, α-fucosidase, α-mannosidase, tweenase (Tween 80), amylase and gelatinase.

Characteristic	Response	Characteristic	Response
Presence of:		Anaerobic growth with nitrate	+
Motility/polar flagellum	+	Hydrogenase activity	+
Catalase	+	Growth in presence of/at:	
Cytochrome-*c* oxidase	+	Nalidixic acid (30 μg)	+
PHB	+	3 % NaCl	+
Utilization as carbon source:		pH 6 and 8	+
Acetate	+	Activity of enzymes:	
Malate	+	Alkaline phosphatase	+
Caproate	+	Esterase (C_4)	+
Benzoate	+	Esterase lipase (C_8)	+
Phenylacetate	+	Lipase (C_{14})	+
Ethanol	+	Leucine arylamidase	+
Proline	+	Valine arylamidase	+
L-Asparagine	+	Acid phosphatase	+
L-Glutamic acid	+	Naphthol-AS-BI-phosphohydrolase	+
Reduction of nitrate	+	β-Glucosidase	+

The G+C content of the genomic DNA of strain N2-214^T was 64·8 mol%.

Nearly the complete 16S rDNA sequence of strain N2-214^T was determined (1456 nucleotides). Phylogenetic analysis of the 16S rDNA sequence of strain N2-214^T showed its affiliation to the *β-Proteobacteria*, a result that is in agreement with the polar lipid and respiratory quinone composition (see below). The 16S rDNA sequence was then compared with all species with validly published names of the most closely related genera, *Azoarcus*, *Thauera* and *Hydrogenophilus* (Springer *et al.*, 1998; Hayashi *et al.*, 1999; Song *et al.*, 1998, 1999, 2000, 2001; Reinhold-Hurek & Hurek, 2000; Stöhr *et al.*, 2001; Mechichi *et al.*, 2002), and several other genera in the *β-Proteobacteria*. The 16S rDNA sequence of strain N2-214^T was not closely related to those of other genera within this subclass, the highest similarities (approx. 92–93 %) being to the sequences of *Azoarcus buckelii* (Mechichi *et al.*, 2002), *Thauera aromatica* (Anders *et al.*, 1995) and *H. thermoluteolus* (Hayashi *et al.*, 1999). Comparative evolutionary distance analyses of the 16S rDNA sequence of strain N2-214^T demonstrated that it branched from the lineage leading to the genus *Hydrogenophilus* (Fig. 1), a result that was also supported by parsimony, maximum-likelihood and bootstrapping methods. The polar lipid pattern of strain N2-214^T, obtained by TLC, revealed the presence of phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) as the major phospholipids. A similar polar lipid pattern, characterized by the predominance of PE and PG, is described for other members of the *β-Proteobacteria* (Wilkinson, 1988). The only respiratory quinone detected for isolate N2-214^T was ubiquinone 8, which is also characteristic of micro-organisms belonging to the *β-Proteobacteria* (Suzuki *et al.*, 1993).

The fatty acid composition of strain N2-214^T was analysed using LB agar cultures grown at 50 °C. Under these conditions, the methyl esters of fatty acids $C_{16:0}$ and cyclo-$C_{19:0}$ were predominant, followed by the unsaturated fatty acid $C_{18:1}$. These components constituted more than 75 % of the total fatty acids (Table 2). A lower growth temperature (30 °C) promoted a dramatic shift between the content of the unsaturated fatty acids $C_{18:1}$ and $C_{16:1}$, which doubled, and that of cyclo-$C_{19:0}$, which, under these conditions, represented only 3 % of total fatty acids (results not shown). The hydroxy fatty acid 3-OH $C_{10:0}$ was also detected, and its

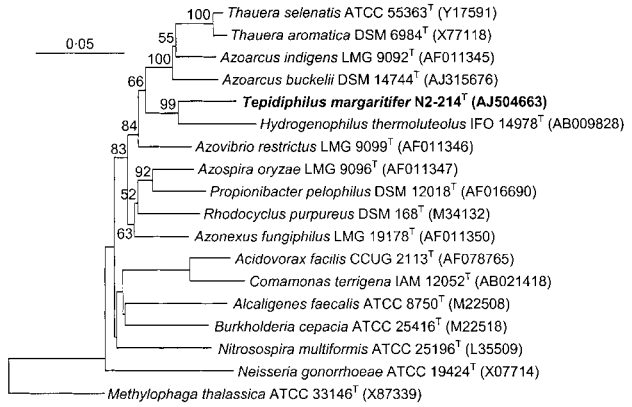

Fig. 1. Phylogenetic relationships based on 16S rDNA sequences between strain N2-214^T and representatives of related genera within the *β-Proteobacteria*. The dendrogram was generated from evolutionary distances, calculated from pairwise dissimilarities, using the FITCH program from the PHYLIP package. Accession numbers are given in parentheses. Bootstrap values for branches leading to the sequence of N2-214^T and its closest relatives, obtained after 1000 resamplings, are shown in the tree. The sequence of *Methylophaga thalassica* ATCC 33146^T was used as outgroup.

level was not temperature-dependent. Characteristics that distinguish isolate N2-214^T from the type strains of the type species of genera with the closest 16S rRNA similarity to the proposed new genus are shown in Table 3.

To date, few thermophilic organisms have been described in the *β-Proteobacteria*. However, this may be due to the difficulty in cultivating these organisms under laboratory conditions, since bacteria belonging to the *β-Proteobacteria* have been detected in studies employing culture-independent molecular methods to describe the biological diversity of high-temperature natural (e.g. Ward *et al.*, 1998; Hugenholtz *et al.*, 1998) and man-made environments (e.g. LaPara *et al.*, 2000). The work of LaPara *et al.* (2000), describing the phylogenetic diversity of bacterial communities in thermophilic and mesophilic bioreactors used to

Table 2. Mean fatty acid composition of strain N2-214^T

Values are percentages of total fatty acids. Cells were grown on LB agar at 50 °C. Components that made up less than 1 % of the total are summed as 'Other' and included iso-$C_{11:0}$ 3-OH, $C_{12:0}$, $C_{15:0}$, $C_{17:0}$, $C_{19:1}\omega12t$ and $C_{20:1}\omega9t$.

Fatty acid	Content (%)
$C_{10:0}$ 3-OH	1·4
$C_{14:0}$	2·7
Summed feature 4*	3·7
$C_{16:0}$	36·8
cyclo-$C_{17:0}$	6·2
$C_{16:0}$ 3-OH	2·9
Summed feature 7*	15·6
$C_{18:0}$	1·6
cyclo-$C_{19:0}$ ($\omega8c$)	25·9
Other	3·2

*Summed features contain one or more of the following: feature 4, $C_{16:1}$ $\omega7c$ and/or iso-C_{15} 2-OH; feature 7, $C_{18:1}\omega7c$, $\omega9t$ and/or $\omega12t$.

Table 3. Characteristics of isolate N2-214^T and related type strains

Strains: 1, *Thauera selenatis* AX^T (data from Macy *et al.*, 1993; Song *et al.*, 2000); 2, *Azoarcus indigens* VB32^T (Reinhold-Hurek *et al.*, 1993); 3, *Hydrogenophilus thermoluteolus* TH-1^T (Goto *et al.*, 1978; Hayashi *et al.*, 1999; Stöhr *et al.*, 2001); 4, N2-214^T. NA, Data not available. All strains utilize acetate.

Characteristic	1	2	3	4
Optimal temperature for growth (°C)	25–30	28	50–52	50
Hydrogenase activity	NA	NA	+	+
Autotrophic growth with H_2	NA	NA	+	−
Anaerobic growth with nitrate	−	−	−	+
N_2 used as nitrogen source	NA	+	−	−
Motility/flagella	+/1 polar	+/1 polar	−	+/1 polar
Specific growth requirements	+	+	−	−
Utilization of carbon source:				
Glucose	+	−	−	−
Glutamate	+	+	NA	+
Asparagine	+	−	NA	+
Benzoate	+	−	−	+
Major fatty acids	$C_{16:0}$, $C_{16:1}$, $C_{18:1}$	$C_{16:0}$, $C_{16:1}$, $C_{18:1}$	$C_{16:0}$, $C_{18:0}$	$C_{16:0}$, $C_{18:1}$, cyclo-$C_{19:0}$
DNA G+C content (mol%)	66	67	63–65	65
Isolated from	Se-contaminated water	Kallar grass	Hot spring soil	Thermophilic digester

treat pharmaceutical wastewater, refers to the presence of clones that have the closest phylogenetic affiliation to the β-proteobacterium HMD 444 (accession number AB015328) in both meso- and thermophilic bioreactors. In this respect, is interesting to note that strains HMD 444 and N2-214^T share 16S rRNA sequence identity above 99 % (according to FASTA analysis), suggesting that they might eventually represent the same species. However, such a conclusion would only be possible on the basis of a DNA–DNA hybridization study (Stackebrandt & Goebel, 1994).

Description of *Tepidiphilus* gen. nov.

Tepidiphilus (Te.pi.di′phi.lus. L. adj. *tepidus* lukewarm; Gr. adj. *philos* friendly to; N.L. masc. n. *Tepidiphilus* liker of lukewarm conditions).

Forms rod-shaped cells that stain Gram-negative. Endospores are not formed. Slightly thermophilic. PHB granules are accumulated. Grows anaerobically in the presence of nitrate. Oxidase- and catalase-positive. Major phospholipids are PE and PG; ubiquinone 8 is the major respiratory quinone. Major fatty acids are $C_{16:0}$ and cyclo-$C_{19:0}$. Chemo-organotrophic. Organic acids and amino acids, but no sugars, are used as single carbon sources. The type species is *Tepidiphilus margaritifer*.

Description of *Tepidiphilus margaritifer* sp. nov.

Tepidiphilus margaritifer (mar.ga.ri′ti.fer. L. n. *margarita* pearl; L. masc. suffix *-fer* carrying; N.L. masc. adj. *margaritifer* pearl-carrying, referring to the nacre-like appearance of the colonies).

Shows the following properties in addition to those in the genus description. Forms rod-shaped cells, 2·0 μm long and 0·7 μm wide. Cells are motile by a single polar flagellum. Colonies grown on LB agar are nacre-like and 1–2 mm in diameter after 36–48 h growth. Growth occurs above 25 °C and below 61 °C; the optimal growth temperature is approximately 50 °C. Growth occurs between pH 6 and 8. Hydrogenase-positive. The major fatty acids at the optimal temperature for growth are $C_{16:0}$, $C_{18:1}$ and cyclo-$C_{19:0}$; $C_{10:0}$ 3-OH is also present. The DNA G+C content of the type strain is 64·8 mol%.

The type strain, strain N2-214^T (=DSM 15129^T=LMG 21637^T), was isolated from a thermophilic digester of wastewater-treatment sludge.

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