NOTE

Pseudomonas thermotolerans* sp. nov., a thermotolerant species of the genus *Pseudomonas sensu strictoCélia M. Manaia¹ and Edward R. B. Moore^{2,3}¹ Escola Superior de Biotecnologia, Universidade Católica Portuguesa, 4200-072 Porto, Portugal² Bereich Mikrobiologie, Gesellschaft für Biotechnologische Forschung, D-38124 Braunschweig, Germany³ The Soil Health Initiative, Soil Quality and Protection Programme, The Macaulay Land Use Research Institute, Aberdeen AB15 8QH, UK

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A bacterium, strain CM3^T, which has an optimal growth temperature of approximately 47 °C and a maximal growth temperature of 55 °C, was isolated from a hexadecane enrichment culture, obtained from a sample of the industrial cooking water of a cork-processing plant. 16S rRNA gene sequence analysis demonstrated that this Gram-negative, aerobic, rod-shaped bacterium is related to species of the genus *Pseudomonas*. Phosphatidylethanolamine and phosphatidylglycerol were the major polar lipids of this isolate and the total fatty acid methyl ester profile revealed predominantly C16:1 and C18:1 at a growth temperature of 25 °C, whereas, at a growth temperature of 50 °C, C16:0 was the dominant fatty acid. This is the first report of a species of *Pseudomonas sensu stricto* that may be considered to be truly thermotolerant. Isolate CM3^T (= DSM 14292^T = LMG 21284^T) represents the type strain of a novel species of the genus *Pseudomonas*, named *Pseudomonas thermotolerans* sp. nov.

Keywords: *Pseudomonas*, *Pseudomonas thermotolerans* sp. nov., thermotolerant, polyphasic taxonomy, genotypic and chemotaxonomic characterization

Descriptions of thermophilic bacteria have been based, predominantly, upon strains isolated from pristine sites using selective media and cultivation temperatures above 60 °C. These procedures have contributed to the discovery of a considerable diversity of microorganisms that, in most cases, have been integrated within taxonomic groups comprised exclusively of thermophiles. Within the *Proteobacteria* (Stackebrandt *et al.*, 1988), the vast majority of described species are mesophilic, although the β -subclass is known to include a limited number of thermophilic organisms, as reviewed recently by Moreira *et al.* (2000). Although no thermophilic organisms of the γ -*Proteobacteria* have been described to date, a novel thermotolerant organism, *Thermomonas haemolytica*, related phylogenetically to species of the *Xanthomonas*–*Pseudoxanthomonas*–*Stenotrophomonas*–*Luteimonas*–*Xylella*–*Rhodobacter* lineage, with optimal

growth temperatures between 37 and 50 °C and a maximal growth temperature of 50 °C, has recently been described (Busse *et al.*, 2002).

Pseudomonas species are bacteria of considerable scientific and technological importance. These organisms are ubiquitous, found in a diverse range of ecosystems, and represent one of the most divergent bacterial groups in terms of their metabolic versatility (Palleroni, 1984). A maximal growth temperature of 46 °C, reported for *Pseudomonas balearica* (Bennasar *et al.*, 1996), is described for species affiliated to this genus.

The present study describes a moderately thermotolerant bacterium, strain CM3^T, isolated from a sample of the cooking water of a cork-processing factory at a temperature of 50 °C in an enrichment culture grown on hexadecane. Strain CM3^T is proposed to represent a novel species of the genus *Pseudomonas*, *Pseudomonas thermotolerans* sp. nov.

Strain CM3^T was isolated from the cooking water of a cork-processing factory in northern Portugal. The water is used repeatedly, being heated to 100 °C and allowed to cool until the next use. The sample was transported, without controlling the temperature, and

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A table comparing the fatty acid composition of various *Pseudomonas* species and strain CM3^T at different growth temperatures is available as supplementary material in IJSEM Online (<http://ijs.sgmjournals.org/>).

The EMBL accession number for the nearly complete 16S rDNA sequence of strain CM3^T is AJ311980.

used as an inoculum for enrichment cultivation. The enrichment was carried out in mineral medium containing 10 g hexadecane l⁻¹, at 50 °C. The composition of 1 l mineral medium was: 2.7 g Na₂HPO₄ · 2H₂O, 1.4 g KH₂PO₄, 0.2 g MgSO₄ · 7H₂O, 0.5 g (NH₄)₂SO₄, 0.02 g yeast extract, 20 mg NaOH, 120 mg disodium EDTA dihydrate, 20 mg FeSO₄ · 7H₂O, 10 mg CaCl₂, 100 mg Na₂SO₄, 4 mg ZnSO₄ · 7H₂O, 4 mg MnSO₄ · 4H₂O, 1 mg CuSO₄ · 5H₂O, and 1 mg Na₂MoO₄ · 2H₂O. Strain CM3^T was purified from the mixed culture by subsequent subculturing on Luria–Bertani medium containing 20 g agar l⁻¹ (LA) (Carlton & Brown, 1981). The composition of 1 l Luria–Bertani broth medium (LB) was: 10 g tryptone, 5 g yeast extract and 10 g NaCl.

Reference strains used for comparative analyses [obtained from the Division of Microbiology at the German National Research Centre for Biotechnology (GBF) and from the IAM Culture Collection, Institute of Molecular and Cellular Biosciences, University of Tokyo, Japan] included *Pseudomonas aeruginosa* LMG 1242^T, *Pseudomonas alcaligenes* DSM 50342^T, *Pseudomonas fragi* LMG 2191^T, *Pseudomonas mendocina* LMG 1223^T, *Pseudomonas oleovorans* LMG 2229^T, *Pseudomonas pseudoalcaligenes* DSM 50188^T, *Pseudomonas stutzeri* LMG 11199^T, and ‘*Pseudomonas nitroreducens* subsp. *thermotolerans*’ IAM 12401. The colony and cell morphology of strain CM3^T were examined using standard protocols (Doetsch, 1981). Cell morphology, Gram-staining reaction, production of spores and accumulation of polyhydroxybutyrate granules of strain CM3^T were determined by microscopic examination, following procedures described previously (Doetsch, 1981; Smibert & Krieg, 1981). The number and position of flagella were visualized 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 were carried out at 42 °C, unless stated otherwise, as described by Smibert & Krieg (1981), using overnight cultures on LA. The pH tolerance range 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 nutritional pattern was characterized using mineral medium supplemented with 25 mM of the respective filter-sterilized carbon source.

For the determination of DNA base composition of strain CM3^T, genomic DNA was isolated as described by Cashion *et al.* (1977) and the G+C content was analysed by HPLC (Mesbah *et al.*, 1989).

Genomic DNA of strain CM3^T was prepared from individual colonies grown on LA medium, as described in detail elsewhere (Moore *et al.*, 1999). 16S rRNA genes were specifically amplified by PCR (Weisburg *et*

al., 1991; Moore *et al.*, 1996) and the PCR products were purified and sequenced directly (Moore *et al.*, 1996). The PCR and sequencing primers have been described previously (Lane, 1991). The PCR-amplified, nearly complete 16S rDNA sequence of strain CM3^T was aligned with reference rRNA and rRNA gene sequences (Maidak *et al.*, 2001; Stoesser *et al.*, 2001; Wheeler *et al.*, 2001), using evolutionarily conserved primary sequence and secondary structure data as references (Gutell *et al.*, 1985). Sequence similarities were calculated from the number of identical nucleotides at homologous positions of unmasked sequence pairs (gaps and insertions were considered to be mismatches). Sequence dissimilarities were converted to evolutionary distances (Jukes & Cantor, 1969) and dendrograms of inferred phylogenetic relationships were generated using the DNADIST, SEQBOOT and FITCH programs in PHYLIP (version 3.5c) (Felsenstein, 1989).

Polar lipids and total fatty acid methyl esters of the type strains of eight species of the genus *Pseudomonas*, cultured under the same nutrient and temperature conditions, were characterized concurrently with strain CM3^T. The cultures for polar lipid analysis were grown in LB medium at 25 °C until the exponential phase of growth was reached. Lipid extractions were performed as described previously (Prado *et al.*, 1988). The individual polar lipids were separated by one-dimensional TLC on silica gel G plates (Merck; 0.25 mm thickness) using a solvent system of chloroform/methanol/water (65:25:4, by vol.).

All strains were grown on LA medium at 25, 37 or 50 °C, as stated, for the analysis of methylated fatty acids. After 24 h growth, approximately 50 mg (wet wt) cells was harvested from the agar quadrant exhibiting the most dilute confluent growth. Fatty acid methyl esters were obtained from freshly harvested biomass by saponification, methylation and extraction, as described by Kuykendall *et al.* (1988).

Fatty acid methyl ester analysis was carried out using a Varian CP-3800 GC equipped with a Varian Saturn 2000 mass-selective detector and the Saturn GC/MS workstation software version 5.3. The column used was a 60 m STABILWAX-DA fused silica (RESTEK) 0.2 mm i.d. and 0.25 µm film thickness. The injector port was heated to 220 °C. The split vent was opened after 30 s. The carrier gas was He C-60 (GASIN) and the constant flow was 1 ml min⁻¹. The oven temperature was set at 40 °C (for 1 min) and then ramped at 2 °C min⁻¹ to 220 °C and maintained at this temperature for 30 min. All mass spectra were acquired in the electron impact mode. The ion trap detector was set as follows. The transfer line, manifold and trap temperatures were respectively 230, 45 and 170 °C. The mass range was 33–350 *m/z*, with a scan rate of 6 scans s⁻¹. The emission current was 50 µA and the electron multiplier was set in relative mode to the autotune procedure. The maximum ionization time was 25000 µs, with an ionization storage level of 35 *m/z*. The injection volume was 1 µl and the analysis was performed in full scan mode. To identify the

Fig. 1. Effect of temperature on the growth rate of strain CM3^T.

substances present, the mass spectra obtained from the sample were compared to those present in the NIST 98 MS Library Database and to those of the standards injected previously, under the same conditions. Fatty acid methyl esters were extracted and analysed at least twice. For analysis of respiratory quinones, cells were

cultured on LA medium, harvested, freeze-dried and extracted, according to Tindall (1989), and the extracts were analysed as described by Moreira *et al.* (2000). Cultivation at 50 °C in mineral medium supplemented with hexadecane was used to enrich moderate thermophiles capable of using alkanes as the sole source of carbon and energy. The inoculum for this enrichment was taken from water used to boil cork in a cork-processing factory. The enrichment procedure resulted in a mixed culture and strain CM3^T was obtained through the purification of organisms that could degrade hexadecane in pure culture. Strain CM3^T was maintained on LA medium.

Strain CM3^T grew on hexadecane as the sole source of carbon and energy. Liquid cultures, under the growth conditions described above, reached an OD₆₁₀ of approximately 0.6 within 48 h. Strain CM3^T also grew on standard heterotrophic liquid media, e.g. LB medium, such that cultures reached the stationary phase of growth within 3–4 h. At the optimal growth temperature, the doubling time in LB medium was approximately 0.5 h. Cultured on complex agar media, e.g. LA medium, strain CM3^T produced non-pigmented colonies of 1–2 mm after 24 h growth.

Table 1. Phenotypic characteristics of strain CM3^T

+, Positive result or growth; –, negative result or no growth.

Character	Result
Presence of:	
Catalase, oxidase	+
Reduction of nitrate	–
Anaerobic growth with nitrate	–
Urease	–
Arginine dihydrolase	+
Polar flagellum	+
Hydrolysis of:	
Aesculin, gelatin, starch, skimmed milk	–
Tweens 20 and 80	+
Growth at/in:	
3% NaCl	+
4% NaCl (37 °C)	–
4% NaCl (50 °C)	+
pH 11	–
pH 10, pH 6	+
pH 5	–
Utilization of carbon sources:	
D-Mannose, D-trehalose, D-cellobiose, L-arabinose, D-glucose, L-sorbose, D-sucrose, D-xylose, D-maltose	–
Gluconate, phenylacetate, citrate, adipate, D-mannitol, glycerol, pyruvate, succinate	–
Acetate, propionate, caproate, malate, betaine	+
L-Lysine, glycine, L-cysteine, L-phenylalanine, L-histidine	–
L-Asparagine, L-arginine, L-serine, L-glutamine, L-alanine, L-isoleucine, L-proline	+
N-Acetylglucosamine	–
Decane	–
Dodecane, tridecane, hexadecane, nonadecane, eicosane	+
Palmitic acid, octanoic acid, octanal, 1-octanol, lauryl alcohol, Tween 80, SDS	+
Benzoate, salicylate, naphthalene, anthracene, phenanthrene	–

Table 2. Characteristics that differentiate strain CM3^T from *Pseudomonas* species of the different phylogenetic lineages of the '*P. aeruginosa* intrageneric cluster'

Taxa: 1, strain CM3^T; 2, *P. aeruginosa* (data from Palleroni, 1984); 3, *P. stutzeri* (Palleroni, 1984); 4, *P. mendocina* (Palleroni, 1984); 5, *P. balearica* (Bennasar *et al.*, 1996). +, Positive; –, negative; d, variable within the group; NA, data not available. All taxa are positive for growth at 41 °C.

Characteristic	1	2	3	4	5
Flagella	1*	1	1*	1*	1
Growth at:					
46 °C	+	–	–	–	+
50 °C	+	–	–	–	–
Arginine dihydrolase	+	+	–	+	–
Denitrification	–	+	+	+	+
Tween 80 hydrolysis	+	d	+	+	NA
Utilization of:					
Hexadecane	+	d	–	–	NA
Glucose	–	+	+	+	NA
Xylose	–	–	–	–	+
Pyruvate	–	+	+	d	NA
Betaine	+	+	–	+	NA

* A short lateral flagellum may be observed.

Strain CM3^T was Gram-negative, non-spore-forming and had rod-shaped cells with a single polar flagellum, although sometimes a short lateral flagellum was also observed. No visible deposits of poly- β -hydroxybutyrate were observed. Likewise, no prosthecae or cell sheaths were observed by microscopic examination. Strain CM3^T was strictly aerobic, did not reduce nitrate under anaerobic conditions, tested positive for oxidase and produced a positive reaction for arginine dihydrolase. The bacterium did not produce fluorescent pigments on King B medium.

The optimal growth temperature of strain CM3^T in LB medium was 47 °C, with a maximal growth temperature of 55 °C (Fig. 1).

The nutritional pattern of strain CM3^T revealed an inability to use the sugars that were tested and a preference for carbon sources with long hydrocarbon chains, i.e. alkanes shorter than 11 carbons were not degraded (Table 1). With the exception of lipase (Tweenase), no extracellular enzymes were detected (Table 1). Phenotypic characteristics that differentiate strain CM3^T from other representative species of the genus *Pseudomonas* are summarized in Table 2.

The G + C content of genomic DNA of strain CM3^T was 66.4 mol %. This level of G + C content is within the range recognized for species of *Pseudomonas* (59–68 mol %; Palleroni, 1984). Although the G + C content of strain CM3^T is at the high end of the range observed for *Pseudomonas*, a relatively high genomic DNA G + C content is characteristic for the different species of the '*P. aeruginosa* lineage' (Palleroni, 1984).

PCR amplification and sequence determination of the 16S rDNA enabled the determination of 1492 nt positions between positions 27 and 1524 (*Escherichia coli* 16S rRNA gene sequence numbering), comprising more than 93 % of the complete 16S rRNA gene sequence, as estimated by comparison with the previously published complete 16S rRNA gene sequence of *P. aeruginosa* (Toschka *et al.*, 1988). The nearly complete 16S rDNA sequence of strain CM3^T was confirmed by determining contiguous overlapping sequences of PCR amplicons produced by two separate reactions. The sequence was determined in the forward and reverse directions.

Pairwise sequence comparisons with reference sequence data demonstrated that the 16S rDNA sequence of strain CM3^T is most similar to the 16S rRNA gene sequences of species of the genus *Pseudomonas sensu stricto*. In particular, among the type strains of validly published species, the 16S rDNA sequence strain CM3^T possessed highest sequence similarity (95.9 %) to that of *P. aeruginosa* LMG 1242^T (accession no. Z76651) and slightly less similarity to the sequences of *P. stutzeri*, *P. balearica*, *Pseudomonas resinovorans* and other species of the '*P. aeruginosa* and *P. resinovorans* phylogenetic lineages' (Moore *et al.*, 1996). The similarities of the 16S rDNA sequence of strain CM3^T to those of *Pseudomonas* species were within the range of 16S rRNA gene sequence similarities observed between the species of *Pseudomonas* (93.7–99.9 %) (Moore *et al.*, 1996; Anzai *et al.*, 1997, 2000).

The inferred phylogenetic relationships of strain CM3^T, derived from evolutionary distance estimations and least-squares clustering, neighbour-joining, maximum-likelihood and analysis of the 16S rRNA gene sequences from representative species of the major phylogenetic lineages within the γ -*Proteobacteria* (Woese *et al.*, 1985) (Fig. 2), demonstrated that strain CM3^T clusters within the phylogenetic radius of the genus *Pseudomonas* and is most closely related to species of the '*P. aeruginosa* and *P. resinovorans* phylogenetic lineages'. However, the 16S rRNA gene sequence branching order observed between strain CM3^T and those of species comprising the '*P. aeruginosa* and *P. resinovorans* phylogenetic lineages' suggests that strain CM3^T comprises a separate phylogenetic 'lineage' within the '*P. aeruginosa* intrageneric cluster' (Moore *et al.*, 1996).

16S rDNA sequence similarities and cluster analyses demonstrated that strain CM3^T belongs to the genus *Pseudomonas*. The levels of 16S rDNA sequence similarity also demonstrated that strain CM3^T is not a strain of a previously described species. While bacterial species are not defined on the basis of 16S rRNA gene sequence analysis alone, member strains of a bacterial species are recognized to exhibit 16S rRNA gene sequence similarities of not less than 98–99 % (Roselló-Mora & Amann, 2001). Moreover, in an earlier study, Stackebrandt & Goebel (1994) observed that organisms exhibiting 16S rRNA gene sequence simi-

Fig. 2. A rooted dendrogram of the inferred phylogenetic relationships between strain CM3^T and representative species of the genotypic lineages of *Pseudomonas* (*sensu stricto*) derived from 16S rDNA pairwise sequence comparisons and calculated evolutionary distances. Bar, estimated evolutionary distance (i.e. a mean of 1 substitution per 100 nucleotide positions). Bootstrap proportions of confidence were generated from 1000 resamplings and are shown for the relevant branchings.

larities below a more conservative value of 97% are unlikely to have genomic DNA–DNA reassociation similarities greater than 60–70%. Analysis of the 16S rDNA sequence of strain CM3^T, in consideration of these observations, clearly demonstrated that strain CM3^T represents a previously unrecognized species of *Pseudomonas*, with a level of differentiation that made further genotypic characterization, e.g. DNA–DNA hybridization, unnecessary. The genotypic data of the genomic DNA G + C content and 16S rDNA sequence analysis supported the placement of strain CM3^T in the genus *Pseudomonas*, as the genus *Pseudomonas* is currently recognized. Furthermore, the levels of similarities observed between the 16S rDNA sequence of strain CM3^T and those of the validly published species of *Pseudomonas*, together with the phenotypic data, demonstrated that strain CM3^T represents a species that has not previously been described or validly published.

The polar lipid pattern of strain CM3^T, determined by TLC, revealed the presence of phosphatidylethanolamine and phosphatidylglycerol as the major phospholipids, with other minor phospholipids. The same polar lipid pattern was detected in all type strains of *Pseudomonas* species analysed and agreed with previous reports (Wilkinson, 1988) of the polar lipid compositions of *Pseudomonas* species. The reference *Pseudomonas* species analysed comprised the type strains of representative species of each of the distinct 16S rDNA phylogenetic lineages (Moore *et al.*, 1996).

The fatty acid composition of strain CM3^T was analysed at a relatively low (25 °C) growth temperature and compared with those of the type strains of *P. aeruginosa*, *P. alcaligenes*, *P. fragi*, *P. mendocina*, *P.*

oleovorans, *P. pseudoalcaligenes* and *P. stutzeri* grown under the same conditions. The fatty acids 3-OH C10:0 and 3-OH C12:0, considered to be important for delineating the species of rRNA group I *Pseudomonas* (Oyaizu & Komagata, 1983), were detected in all strains (see the table in supplementary material in IJSEM Online at <http://ijs.sgmjournals.org/>). This analysis revealed a noted similarity among the strains of the tested species and supports inclusion of CM3^T in the genus *Pseudomonas*. Ubiquinone 9, which is typical of *Pseudomonas sensu stricto* (Suzuki *et al.*, 1993), was the only respiratory quinone detected in strain CM3^T. The polar lipid pattern, the characteristic fatty acids and the type of respiratory quinone also supported classification of strain CM3^T as a species of *Pseudomonas sensu stricto*.

The change in fatty acid composition with varying growth temperature was also evaluated for strain CM3^T. An increase of growth temperature was observed to lead to a gradual shift in fatty acid composition, characterized by a marked rise in the relative percentage of C16:0, with a concomitant decrease in the relative percentages of C16:1 and C18:1. Another long chain fatty acid, *cyclo*-C19, was observed only at growth temperatures above 37 °C. The decrease in the degree of chain unsaturation and the corresponding increase in the mean chain length are presumably necessary for the stabilization of the cell membrane at higher temperatures (Russell, 1983). Production of the hydroxy fatty acids 3-OH C10:0 and 3-OH C12:0 was not observed to be temperature-dependent in strain CM3^T.

The thermotolerance of strain '*P. nitroreducens* subsp. *thermotolerans*' IAM 12401, the subspecies designation (not validly published) suggesting a capacity for growth at elevated temperatures (Watanabe *et al.*, 1977), was also analysed during this study. The maximal growth temperature was between 40 and 45 °C, with an optimal growth temperature of approximately 35 °C. The fatty acid compositions of this organism and strain CM3^T at 25 and 37 °C were very similar (see the table in supplementary material in IJSEM Online at <http://ijs.sgmjournals.org/>). It is well known that growth conditions may have pronounced effects on some bacterial chemotaxonomic markers, such as polar lipids and fatty acids and, for that reason, the use of standardized cultivation conditions is indispensable for proper analyses and reliable results (Suzuki *et al.*, 1993). The temperature-induced variations in the fatty acid composition observed for strain CM3^T agreed with such observations. In this respect, it is interesting to note the limitations of fatty acid methyl ester profiling for comparing organisms that, despite their estimated taxonomic and phylogenetic proximities, exhibit markedly different growth requirements, for example in terms of temperature.

It is assumed that strain CM3^T possesses mechanisms that permit a preference for growth at higher temperatures than those observed for mesophilic *Pseudomonas*

species. The dramatic change in the fatty acid profile, observed when strain CM3^T was cultivated at higher temperatures, may be important in such mechanisms as a way of maintaining the integrity of the cell membrane.

The isolation of thermotolerant and thermophilic bacteria affiliated to genera that are comprised typically of mesophiles may lend insight into the mechanisms involved in temperature adaptation, from the biochemical and evolutionary points of view.

Description of *Pseudomonas thermotolerans* sp. nov.

Pseudomonas thermotolerans (ther.mo.to'le.rans. Gr. n. *therme* heat; L. pres. part. *tolerans* tolerating; N.L. adj. *thermotolerans* able to tolerate high temperatures).

Forms rod-shaped cells, 1.0–1.5 × 0.4–0.7 µm. Gram reaction is negative. Cells are motile by a single polar flagellum. Colonies grown on LA medium are not pigmented and are 1–2 mm in diameter after 24 h growth; fluorescence is not produced on King B medium. Growth occurs above 25 °C and below 56 °C; the optimal growth temperature for strain CM3^T is approximately 47 °C. Growth occurs at pH 6 and pH 10. Major fatty acids are C16:0, C16:1 and C18:1; *cyclo*-C19 is present at temperatures above 37 °C. The hydroxylated fatty acids 3-OH C10:0 and 3-OH C12:0 are also present. Ubiquinone 9 is the major respiratory quinone. Strictly aerobic and positive for cytochrome oxidase, catalase and arginine dihydrolase. Does not reduce nitrate. The nutritional pattern reveals an inability to use sugars and a preference for carbon sources with long hydrocarbon chains. The DNA G+C content of strain CM3^T is 66.4 mol %. Isolated from cooking water of industrial cork transformation. Isolate CM3^T (= DSM 14292^T = LMG 21284^T) is the type strain.

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