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Clostridium peptidivorans sp. nov., a peptide-fermenting bacterium from an olive mill wastewater treatment digester

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A new peptide-degrading, strictly anaerobic bacterium, designated strain TMC4^T, was isolated from an olive mill wastewater treatment digester. Cells of strain TMC4^T were motile, rod-shaped (5–10 × 0·6–1·2 µm), stained Gram-positive and formed terminal to subterminal spores that distended the cells. Optimal growth occurred at 37 °C and pH 7 in an anaerobic basal medium containing 0·5% Casamino acids. Arginine, lysine, cysteine, methionine, histidine, serine, isoleucine, yeast extract, peptone, Biotrypcase, gelatin and crotonate also supported growth, but not carbohydrates, organic acids or alcohols. The end-products of degradation were: acetate and butyrate from lysine and crotonate; acetate, butyrate, H_2 and CO_2 from Biotrypcase, gelatin and peptone; acetate, alanine, H_2 and CO_2 from cysteine; acetate, H_2 and CO_2 from serine, cysteine and yeast extract; acetate and formate from histidine; propionate from methionine; methyl 2-butyrate, H_2 and CO_2 from isoleucine; acetate and ethanol from arginine; and acetate, propionate, butyrate, methyl 2-butyrate, H_2 and CO_2 from Casamino acids. The DNA G+C content of strain TMC4^T was 31 mol%. Phylogeny based on 16S rRNA sequence analysis showed that strain TMC4^T was a member of the low-G+C-content Gram-positive genus *Clostridium*, with the closest relative being *Clostridium pascui* (sequence similarity of 96 %). Due to considerable differences in genomic and phenotypic properties between strain TMC4^T and those of its nearest relative, strain TMC4^T is proposed as a new species of the genus *Clostridium*, *Clostridium peptidivorans* sp. nov. Strain TMC4^T has been deposited in the DSMZ as strain DSM 12505^T.

Keywords: *Clostridium peptidivorans*, peptides, amino acids, olive mill wastewater, digester

INTRODUCTION

Olive mill wastewater is a dark liquid that contains high concentrations of biopolymers such as proteins, polysaccharides and polyphenols and is a cause of severe pollution in the Mediterranean basin. Agro-industrial wastewater has been shown to be treated successfully in anaerobic methanogenic digesters (Lettinga, 1995). Hamdi (1996) has proposed the use of similar systems to treat olive mill wastewater. In order to understand the anaerobic digestion processes occurring in olive oil waste treatment, we have recently isolated and characterized several new species of anaerobic microbes that degrade aromatic compounds (Mechichi *et al.*, 1999). In this paper, we report the isolation and characterization of a new peptide-fermenting bacterium, *Clostridium peptidivorans* sp. nov.

METHODS

Sample source. Strain TMC4^T was enriched from samples collected from an anaerobic methanogenic digester (Sfax, Tunisia) fed with olive mill wastewater. The samples were

The GenBank accession number for the 16S rDNA sequence of strain TMC4^T is AF156796.

collected anaerobically using a N_2-flushed syringe and transferred directly to a sterile anaerobic serum bottle, which was maintained at room temperature until use. The digester temperature was set at 37 °C with no pH control.

Culture medium. The basal medium used for enrichment, isolation and cultivation contained (l^{-1} distilled water): 1 g yeast extract, 1 g NH_4Cl, 0·3 g K_2HPO_4, 0·3 g KH_2PO_4, 0·6 g NaCl, 0·1 g $CaCl_2.2H_2O$, 0·2 g $MgCl_2.6H_2O$, 0·1 g KCl, 1·5 ml trace element solution (Widdel & Pfennig, 1981) and 1 mg resazurin. The pH was adjusted to 7 with 10 M KOH solution and the medium was made anaerobic using techniques described previously (Hungate, 1969; Macy *et al.*, 1972; Miller & Wolin, 1974).

Enrichment, isolation and cultivation. Enrichment, isolation and cultivation were performed in 5 ml basal medium containing 0·5% Casamino acids followed by incubation at 37 °C, as described previously (Mechichi *et al.*, 1999). Pure cultures were obtained by picking single well-isolated colonies from dilution series by using the roll-tube method (Hungate, 1969). Studies of optimum growth conditions and electron acceptor utilization were performed with 0·5% Casamino acids as the substrate.

Substrate utilization. Substrate utilization studies were performed in basal medium containing complex substrates at 0·5% (Biotrypcase, yeast extract, gelatin and peptone), carbohydrates at 20 mM (glucose, fructose, xylose, ribose, sorbose, sorbitol, sucrose, melibiose, raffinose, galactose, *myo*-inositol, lactose, cellobiose, mannitol, mannose, arabinose, arabitol, cellulose and xylan), organic acids at 20 mM (formate, acetate, propionate, butyrate, valerate, crotonate, fumarate, malonate, malate, lactate, citrate and succinate) or amino acids at 10 mM (arginine, alanine, proline, histidine, serine, aspartate, glycine, threonine, glutamate, glutamine, leucine, isoleucine, aspartate, asparagine, valine, methionine, lysine, tyrosine, phenylalanine and tryptophan), as described by Mechichi *et al.* (1999).

Cellular studies. Gram staining was performed using a kit purchased from Sigma. Light microscopy and electron microscopy were performed as described by Fardeau *et al.* (1997).

Analytical methods. Growth was determined by measuring changes in optical density at 580 nm by inserting anaerobic Hungate tubes directly into the cuvette holder of a Shimadzu model UV 160A spectrophotometer. Acetate, propionate, butyrate, formate, isobutyrate, ethanol and methyl 2-butyrate were measured by HPLC (Spectra Series 100 model; Thermo Separation Products) equipped with an Aminex HPX-87X 300 × 7·8 (inside diameter) mm column (Bio-Rad) connected to a differential refractometer (RID-6A; Shimadzu). Analysis was performed using a CR-6A Shimadzu integrator. The mobile phase was 0·0025 M H_2SO_4 at a flow rate of 0·6 ml min^{-1} and the column temperature was 35 °C. The volume of the injection loop was 20 µl. Amino acid concentrations were determined by HPLC according to Moore *et al.* (1958). Sulfide was measured photometrically as colloidal CuS by using the method of Cord-Ruwisch (1985).

H_2 and CO_2 were measured using a gas chromatograph GC-8A (Shimadzu) equipped with a thermal conductivity detector, a C-R6A integrator (Shimadzu) and a Chromosorb WAW 80/100 mesh sp100 column (Alltech). N_2 at a pressure of 100 kPa was used as the carrier gas. The detector and the injector temperature was 200 °C, the column temperature was 150 °C.

Determination of G+C content. The G+C content of DNA was determined at the DSMZ by HPLC (Mesbah *et al.*, 1989). Non-methylated lambda DNA (Sigma) was used as the standard.

DNA extraction, amplification and sequencing of the 16S rDNA gene. DNA extraction, 16S rDNA amplification and sequencing were performed as described previously (Redburn & Patel, 1993; Andrews & Patel, 1996; Mechichi *et al.*, 1999). The universal primers Fd1 and Rd1 were used to obtain a PCR product of approximately 1·5 kb corresponding to base positions 28–1539 of the numbering of *Escherichia coli* 16S rDNA (Winker & Woese, 1991). The new sequence data that were generated, consisting of 1481 nucleotides, were aligned and an almost full-length consensus 16S rRNA gene sequence was assembled and checked for accuracy manually using the ae2 alignment editor (Maidak *et al.*, 1999). These were compared with other sequences in the GenBank database (Benson *et al.*, 1993) by using BLAST (Altschul *et al.*, 1997) and in the Ribosomal Database Project, version 7.0, by using SIMILARITY-RANK and SUGGEST-TREE (Maidak *et al.*, 1999). Reference sequences most closely related to our newly generated sequences were extracted from these databases and aligned. Positions of sequence and alignment uncertainty were omitted from the analysis. Pairwise evolutionary distances based on 1128 unambiguous nucleotides were computed by using DNADIST (Jukes and Cantor option) and neighbour-joining programs that form part of the PHYLIP suite of programs (Felsenstein, 1993). TREECON was used extensively for bootstrap analysis (Van de Peer & De Wachter, 1993).

RESULTS

Morphology

Several pure cultures were obtained and one of the cultures was designated strain TMC4^T and studied further. As strain TMC4^T did not grow on glucose, basal medium containing glucose was routinely used to check culture purity. Cells of strain TMC4^T were rod-shaped (0·6–1·2 × 5–10 µm). Spores were oval and were located terminally or subterminally, and they distended the cells (Fig. 1a). The cells stained Gram-positive and possessed a thick Gram-positive-type cell wall ultrastructure (Fig. 1b). Cells were motile and possessed peritrichous flagella.

Physiology

Strain TMC4^T was a strictly anaerobic and chemo-organotrophic bacterium. Growth occurred between 20 and 42 °C with the optimum temperature for growth at 37 °C. The optimum pH for growth was 7 and no growth occurred below pH 6·0 or above pH 9·0. NaCl was not required for growth, but was tolerated up to 4%.

Substrate utilization and fermentation end-products

Arginine, lysine, cysteine, methionine, histidine, serine, isoleucine, Biotrypcase, yeast extract and Casamino acids were fermented but carbohydrates, organic acids and other single amino acids (alanine, proline,

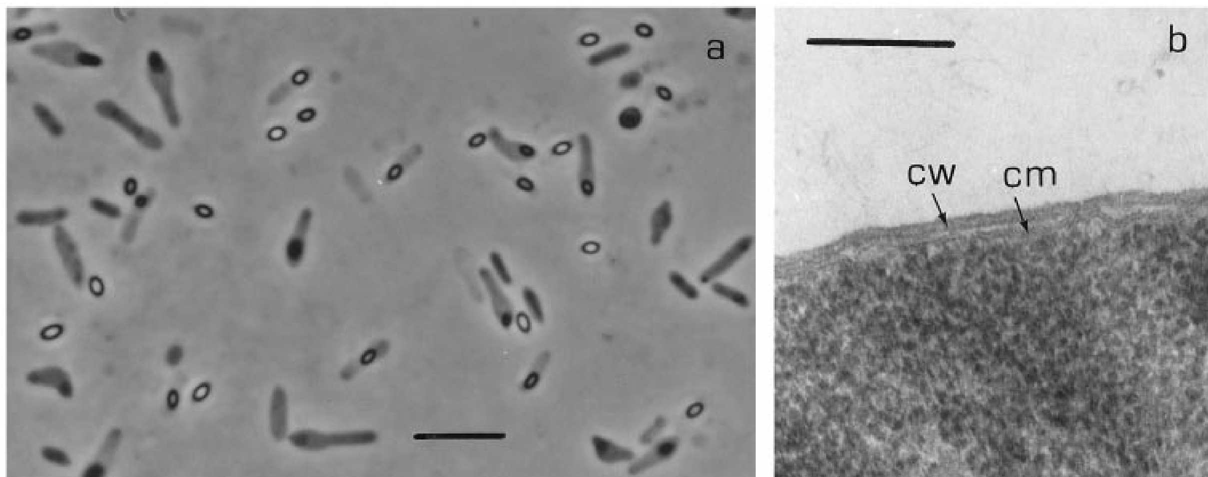


Fig. 1. (a) Phase-contrast micrograph of cells of strain TMC4^T in the exponential growth phase. Bar, 10 μm. (b) Transmission electron micrograph of the cell wall ultrastructure of strain TMC4^T, showing the presence of a cytoplasmic membrane (cm) and a thick, electron-dense cell wall layer (cw). Bar, 0·1 μm.

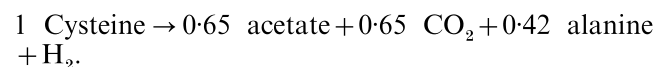
Table 1. Fermentation of amino acids by strain TMC4^T

Amino acids tested but not used were threonine, aspartate, asparagine, leucine, alanine, valine, glutamate, glycine, glutamine, phenylalanine, tyrosine, tryptophan and proline.

Amino acid	Fermentation end-products	Maximum OD_{580}
Isoleucine	Methyl 2-butyrate, H_2, CO_2	0·30
Histidine	Acetate, formate	0·32
Methionine	Propionate	0·40
Lysine	Acetate, butyrate	0·28
Arginine	Acetate, ethanol	0·41
Cysteine	Acetate, alanine, H_2, CO_2	0·36
Serine	Acetate, H_2, CO_2	0·37
Casamino acids	Acetate, butyrate, methyl 2-butyrate, H_2, CO_2	0·55

aspartate, glycine, threonine, glutamate, glutamine, leucine, aspartate, asparagine, valine, tyrosine, phenylalanine and tryptophan) were not utilized. The following amino acids were utilized from Casamino acids: serine, glycine, leucine, phenylalanine, histidine, tyrosine, lysine, arginine and isoleucine. Strain TMC4^T was not able to perform the Stickland reaction when cultured on the following mixtures of amino acids: histidine/aspartate, histidine/proline, valine/cysteine, valine/proline and leucine/proline. The fermentation end-products from single amino acids and Casamino acids are shown in Table 1. The fermentation end-products from other substrates were: acetate and butyrate from crotonate; acetate, butyrate, H_2 and CO_2 from Biotrypcase, gelatin and peptone; and acetate, H_2 and CO_2 from yeast extract.

The carbon balance from cysteine fermentation was:



$$1 \text{ Cysteine} \rightarrow 0{\cdot}65 \text{ acetate} + 0{\cdot}65\ CO_2 + 0{\cdot}42 \text{ alanine} + H_2.$$

Electron acceptor utilization

During Casamino acid degradation, thiosulfate, but not sulfate, elemental sulfur, nitrate or fumarate, was reduced to H_2S and an increase was observed in the concentrations of the end-products acetate, propionate, butyrate, methyl 2-butyrate, H_2 and CO_2.

DNA base composition

The DNA G+C content of strain TMC4^T was 31 mol%, as determined by HPLC.

Phylogeny

Analysis of the 16S rDNA sequence showed that strain TMC4^T was a member of the low-G+C-content Gram-positive bacteria and a member of cluster I (Collins *et al*., 1994) and, hence, was not related to most of the previously described peptide-fermenting bacteria (Baena *et al*., 1998, 1999a, 1999b). Cluster I is similar to the rRNA group I of Johnson & Francis (1975), consisting of some 78 species, including *Clostridium pascui* and *Clostridium acetireducens*, and is currently the largest group of the low-G+C-content Gram-positive clostridia. Further in-depth phylogenetic analysis indicated that *C. pascui* (sequence similarity of 96%), rather than *C. acetireducens* (sequence similarity of 93%), was the closest relative of strain TMC4^T. A dendrogram showing the relationship of strain TMC4^T to *C. pascui* and other representative members of cluster I is depicted in Fig. 2.

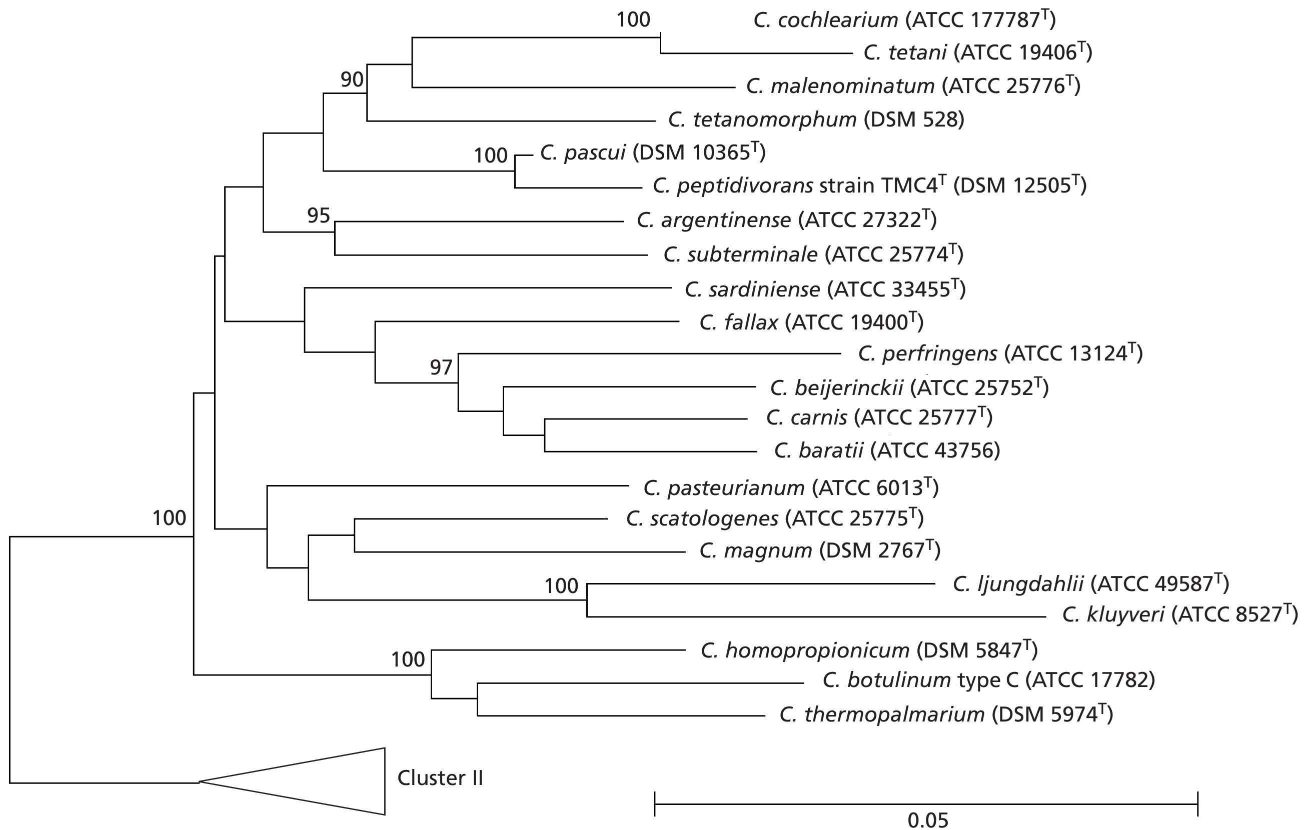


Fig. 2. Unrooted phylogenetic dendrogram based on 16S rRNA sequence data indicating the position of *C. peptidivorans* strain TMC4^T within the radiation of representative members of the genus *Clostridium* (cluster I) according to Collins *et al*. (1994). All sequences used in the analysis were obtained from the Ribosomal Database Project, version 7 (Maidak *et al*., 1999). The triangle indicates representative members of cluster II (*Clostridium histolyticum* ATCC 19401^T, *Clostridium limosum* ATCC 25620^T and *Clostridium proteolyticum* ATCC 49002^T). Evolutionary distances (based on 1128 unambiguous nucleotides) and bootstrap analysis (100 data sets) were computed using programs that form part of the PHYLIP package (Felsenstein, 1993). Only values greater than 90% were considered significant and are therefore reported. Bar, 5 nucleotide substitutions per 100 nucleotides.

DISCUSSION

The trait of degrading proteinaceous compounds but not carbohydrates is a characteristic shared by strain TMC4^T with only a few members of the low-G+C-content Gram-positive bacteria, including *C. pascui*, *C. acetireducens*, *Clostridium litorale*, *Clostridium sticklandii*, *Clostridium aminophilum*, *Clostridium hydroxybenzoicum*, *Peptostreptococcus anaerobius*, *Acidaminobacter hydrogenoformans*, *Eubacterium acidaminophilum*, *Anaeromusa acidaminophila* (formerly '*Selenomonas acidaminophila*'), *Dethiosulfovibrio peptidovorans*, *Aminobacterium colombiense* and *Aminomonas paucivorans*. However, with the exception of *C. pascui*, with which it shares a sequence similarity of 96%, all the other bacteria are phylogenetically distantly related. In addition, strain TMC4^T and *C. pascui* share the ability to ferment histidine but not carbohydrates, have similar optimum conditions for growth (37 °C and pH 7), form spores and are motile. However, numerous phenotypic differences also exist between the two taxa (Table 2). *C. pascui* stained Gram-negative and fermented glutamate, whereas strain TMC4^T stained Gram-positive and did not ferment glutamate. Strain TMC4^T is a proteolytic bacterium and utilized a large number of proteinaceous substrates, whereas *C. pascui* is not proteolytic and used only amino acids. The G+C content of TMC4^T is 31 mol%, whereas that of *C. pascui* is much lower (27 mol%). The habitats from which these two strains were isolated are also different; strain TMC4^T was isolated from an anaerobic digester whereas *C. pascui* was isolated from intestinal tracts of donkeys. A different spectrum of end-products from histidine fermentation was produced by strain TMC4^T (acetate and formate) when compared with *C. pascui* (acetate, butyrate, ethanol, CO_2 and H_2).

On the basis of the phenotypic, genotypic and phylogenetic evidence presented and based on the current taxonomic guidelines, we propose that strain TMC4^T should be designated as a new member of cluster I, which encompasses the true members of the genus *Clostridium* (Collins *et al*., 1994).

Table 2. Characteristics of strain TMC4[T] and *C. pascui*

Data for *C. pascui* were taken from Wilde *et al*. (1997).

Characteristic	Strain TMC4[T]	*C. pascui*
Habitat	Olive mill wastewater digester	Intestinal tract of donkey
Morphology	Rods, 5–10 × 0·6–1·2 µm	Rods, 3·2–8 × 0·75–1 µm
Spores	Oval, terminal to subterminal; distend the cell	Elliptical, subterminal; distend the cell
Temperature for growth (°C):		
Range	20–42	10–43
Optimum	37	37–40
pH for growth:		
Range	6–9	5·5–9
Optimum	7·0	6·4–7·8
Substrates utilized	Arginine, lysine, cysteine, methionine, histidine, serine, isoleucine, Casamino acids, gelatin, yeast extract, peptone, Biotrypcase	Glutamate, histidine, ribose (weakly)
End-products of histidine fermentation	Acetate, formate	Acetate, *n*-butyrate, ethanol, CO_2, H_2
DNA G+C content (mol%)	31	27

Description of *Clostridium peptidivorans* sp. nov.

Clostridium peptidivorans (pep.ti.di.vo′rans. N.L. n. *peptidum* peptide; L. v. *vorare* to devour; N.L. part. adj. *peptidivorans* peptide consuming).

Cells are motile, stain Gram-positive, are rod-shaped (0·6–1·2 × 5–10 µm) and form spores. Spores are terminal to subterminal and oval and distend the cell. Strictly anaerobic and grows optimally at 37 °C over a temperature range of 20–42 °C. No growth is observed at or below 15 °C or above 45 °C. The pH range for growth is between 6 and 9 with an optimum at 7·0. Grows in the presence of NaCl concentrations less than 4%. Grows on Biotrypcase, yeast extract, Casamino acids, gelatin, peptone, arginine, lysine, cysteine, methionine, histidine, serine, isoleucine and crotonate but not on carbohydrates (glucose, fructose, xylose, sorbose, sorbitol, sucrose, melibiose, raffinose, galactose, *myo*-inositol, sucrose, lactose, cellobiose, mannitol, mannose, arabinose, arabitol, raffinose, cellulose or xylan ribose), organic acids (formate, acetate, propionate, *n*-butyrate, valerate, fumarate, malonate, malate, lactate, citrate or succinate) or other amino acids (alanine, proline, aspartate, glycine, threonine, glutamate, glutamine, leucine, aspartate, asparagine, valine, tyrosine, phenylalanine or tryptophan). Thiosulfate is reduced but not sulfate, elemental sulfur, nitrate or fumarate. The fermentation end-products are: acetate and butyrate from lysine and crotonate; acetate, butyrate, H_2 and CO_2 from Biotrypcase, gelatin and peptone; acetate, alanine, H_2 and CO_2 from cysteine; acetate, H_2 and CO_2 from serine, cysteine and yeast extract; acetate and formate from histidine; propionate from methionine; methyl 2-butyrate, H_2 and CO_2 from isoleucine; acetate and ethanol from arginine; and acetate, propionate, butyrate, methyl 2-butyrate, H_2 and CO_2 from Casamino acids. The G+C content of DNA is 31 mol%, as determined by HPLC. The type strain is TMC4[T] (= DSM 12505[T]). Isolated from an olive mill wastewater treatment digester.

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