***Thermobacillus xylanilyticus* gen. nov., sp. nov., a new aerobic thermophilic xylan-degrading bacterium isolated from farm soil**

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An aerobic, thermophilic, xylanolytic, spore-forming bacterium, XE^{TP} (T = type strain; P = patent strain), has been isolated from farm soil situated underneath a manure heap in northern France. Strain XE^{TP}, which stained negative in the Gram test, occurs as short rods which sometimes form chains. Its spores are ellipsoidal, central to subterminal and occur in swollen sporangia. It grows at temperatures up to 63 °C and in the pH range 6.5–8.5. When grown on glucose in optimal conditions, its doubling time was found to be 33 min. CO₂ was observed to have a growth-stimulating effect at the start of the culture. In addition to glucose, the isolate utilizes xylose, arabinose, mannose, cellobiose, galactose, maltose, sucrose, xylan and starch. Growth is inhibited by 5 % NaCl. The G+C content of strain XE^{TP} is 57.5 mol %. The 16S rDNA sequence analysis indicated that strain XE^{TP} falls into the radiation of the *Bacillus*–*Lactobacillus*–*Streptococcus* subdivision of the Gram-positive phylum. Its three closest phylogenetic relatives are '*Bacillus viscosus*', *Paenibacillus curdlanolyticus* and *Bacillus popilliae* with identity values of 91.15, 90.94 and 90.92 %, respectively. The major cellular fatty acids are 14-methyl pentadecanoic acid (16:0 iso), hexadecanoic acid (16:0) and 14-methyl hexadecanoic acid (17:0 anteiso). On the basis of 16S rRNA sequence and chemotaxonomic characteristics, the isolate is different enough for it to be considered as a member of a new genus. It is therefore proposed that this isolate represents a new genus and species: *Thermobacillus xylanilyticus*. Strain XE^{TP}, the type strain of *Thermobacillus xylanilyticus*, has been deposited in the Collection Nationale de Cultures Microbiennes (CNCM I-1017) as a patent strain.

Keywords: *Thermobacillus xylanilyticus*, thermophile, xylanolytic bacterium

INTRODUCTION

Thermophilic micro-organisms are of special interest to enzymologists both at the fundamental and industrial level as a natural source of enzymes that are active and stable at high temperatures (Zeikus, 1979; Bergquist *et al.*, 1989). Enzymes that take part in the biodegradation of plant cell-wall polymers, i.e. cellulose and hemicellulose have attracted much attention in past decades because they can be useful in fields as diverse as production of fuels or chemicals

such as ethanol or lactate from plant biomass (Lamed *et al.*, 1988), bio-bleaching of paper pulps (Mora *et al.*, 1986; Noé *et al.*, 1986; Viikari *et al.*, 1994; Wong *et al.*, 1996), animal feed additives (Annison, 1992; Classen, 1996) and breadmaking (Maat *et al.*, 1992; Rouau *et al.*, 1994). Moreover, the interest in enzymes with adequate thermostability is of importance for industrial applications (Wassermann, 1984).

During the course of our search for aerobic thermophilic bacteria able to degrade xylans, such a bacterium was isolated from a soil sample taken from beneath a manure heap in a farm in northern France. It was enriched in several steps using liquid medium containing xylan from oat spelts as the main carbon source

The EMBL accession number for the 16S rDNA sequence of *Thermobacillus xylanilyticus* strain XE^{TP} is AJ005795.

(Samain *et al.*, 1992). This strain, called XE^{TP}, is unique in that it is able to produce high levels of an extracellular 20 kDa endoxylanase in about 16 h of cultivation at 55 °C (Samain *et al.*, 1997). As an aerobic spore-former, the isolate was tentatively identified as *Bacillus* sp. The isolate has been patented together with a mutant thereof, the strain D3, obtained by mutagenesis with ethylmethanesulfonate (Samain *et al.*, 1991). This paper deals with the characterization and taxonomy of the strain XE^{TP}, which does not appear to fit into any of the new genera arising from the splitting of the genus *Bacillus*.

METHODS

Culture conditions. For stock maintenance and experimental studies, strains were cultivated in a basal medium having the following composition (l⁻¹): 0.3 g KH₂PO₄, 0.6 g NaCl, 0.12 g MgSO₄·7H₂O, 0.08 g CaCl₂·2H₂O, 1 g NH₄Cl, 2 g yeast extract, 10 ml vitamin solution and 10 ml trace mineral solution. Vitamin and trace mineral solution were prepared according to Zeikus & Wolfe (1972). An appropriate carbon source was added at a concentration of 2 g l⁻¹. The pH was brought to 7 with NaOH, then 5 g KHCO₃ l⁻¹ was added. The medium was dispensed in 10 ml aliquots in 125 ml serum bottles equipped with rubber septa and crimped aluminium seals. Before sealing and autoclaving, 10 ml CO₂ was injected into the headspace of bottles with a syringe. Inoculation (1%, v/v) was made by injection with a syringe through the rubber septum. Cultures were grown overnight at 55 °C on a rotary shaker at 200 r.p.m. Liquid stock cultures grown on glucose remained viable when kept at 4 °C for periods exceeding one year. When required, the basal medium was solidified with 17 g agar l⁻¹; the Petri dishes were incubated in a sealed container with about 10% CO₂ in the gas phase. J-agar was also used as an alternative solid medium for observing the colony shape.

Growth experiments, physiological and biochemical characterization. Substrate utilization analysis was performed in duplicate in 125 ml serum bottles containing 10 ml basal medium supplemented with different carbon sources, each to a final concentration of 2 g l⁻¹. All cultures were incubated at 55 °C for up to 3 d. Growth was monitored by OD measurements at 540 nm. The basal medium containing 2 g glucose l⁻¹ was used for testing growth in the presence of NaCl. The same medium was used for the determination of temperature and pH growth ranges. Due to the peculiar requirement for CO₂, the different pH values ranging from 6.5 to 8.6 at 55 °C at the start of the culture were obtained by varying the concentration of KHCO₃. Tests for swimming motility, oxygen relationships, catalase, oxidase, urease, aesculin hydrolysis and nitrate reduction were conducted according to Smibert & Krieg (1994).

Gram reaction and general morphology. Smears for Gram staining were performed using overnight cultures. The Bacto 3-Step Gram Stain Set-S (Difco) was used according to the manufacturer's instructions. Cell morphology was examined by phase-contrast microscopy (Nikon Optiphot-2). Colonies were observed using a stereoscopic microscope (Nikon SMZ-10A). The presence of flagella was sought using scanning electron microscopy with cells from overnight cultures as the sample. These cells were fixed for 30 min with 2.5% glutaraldehyde in 0.2 M PBS (0.145 M NaCl, 0.15 M sodium phosphate, pH 7.2), deposited onto a polylysine-coated glass slide and post-fixed for 1 h in 2% (w/v) osmium

tetroxide in water. After critical-point drying, the preparation was metal-shadowed with gold/palladium and examined using a model JSM-5400LV (JEOL) scanning electron microscope at 15 kV.

Fatty acids and quinone analysis. Analyses were performed by the DSMZ Identification Service (German Collection of Microorganisms, Braunschweig, Germany) using the extraction procedure of Minnikin *et al.* (1984). Fatty acids were determined by GC and menaquinone by HPLC (Kroppenstedt, 1985) on freeze-dried cells grown on glucose in the standard medium.

DNA isolation and base composition. DNA was isolated as described by Touzel *et al.* (1992). The G+C content was determined by melting point analysis (Marmur & Doty, 1962), with *Escherichia coli* DNA (50 mol% G+C) as a reference.

16S rRNA gene sequencing. 16S rDNA was amplified from XE^{TP} genomic DNA by PCR (Mullis & Faloona, 1987; Saiki *et al.*, 1988). The primers employed were 5'-AGA GTT TGA TCC TGG CTC AG-3' and 5'-AAG GAG GTG ATC CAG CC-3' (Wisotzkey *et al.*, 1992) which correspond to positions 8–27 and 1541–1525 in the 16S rRNA (*E. coli* numbering; Brosius *et al.*, 1978). The sequences of both strands of the PCR-amplified 16S DNA (1554 bp) were determined using the dideoxy sequencing method (Sanger *et al.*, 1977) supplied in kit form (Ladderman; Takara Shuzo). Sequence assembly of the various contiguous sequences was performed using the SeqMan module of the DNASTAR Lasergene package.

Sequence analysis. The 16S rRNA gene sequence was compared to sequences available in the prokaryote subset (EMPRO) and the latest entries (EMNEW) of the EMBL database using FASTA3 (Pearson & Lipman, 1988; Pearson, 1990) through on-line access to the European Bioinformatics Institute World Wide Web server. The sequence was also analysed using Sequence Match, Distance Matrix and other on-line services provided by the RDP-II server (Maidak *et al.*, 1997). A phylogenetic analysis was performed on a set of selected sequences spanning all the groups of the former *Bacillus* genus including the three closest neighbours of strain XE^{TP} found by FASTA3 and *E. coli*. A total of 18 sequences were aligned using PILEUP [Wisconsin Package Version 9.0, Genetics Computer Group (GCG), Madison, WI, USA]. Nucleotide positions that could be unambiguously aligned for all 16S rRNA genes compared were included in the analysis. The final data comprised 1421 nt positions corresponding to positions 46–1466 of the alignment. The distances were calculated using DISTANCES (GCG) with the two-parameter correction method (Kimura, 1980). A tree was built using TREECON 1.2 for Windows (Van de Peer & De Wachter, 1994) by the neighbour-joining method. Other nucleotide sequences, obtained from the EMBL database, had the following accession numbers: X76436 (*Bacillus alcalophilus* DSM 485^T); D78312 (*Bacillus circulans* IAM 12462^T); X68415 (*Bacillus globisporus* DSM 4^T); X60618 (*Bacillus kaustophilus* NCIMB 8547^T); X60631 (*Bacillus pasteurii* NCIMB 8841^T); AF071859 (*Bacillus popilliae* ATCC 4706^T); K00637 (*Bacillus subtilis* W168-PY79); X60641 (*Bacillus thermoglucosidasius* ATCC 43742^T); X77792 (*Bacillus viscosus* ATCC 51155); X60612 (*Brevibacillus brevis* NCIMB 9372^T); X60620 (*Brevibacillus laterosporus* NCDO 1763); X60627 (*Virgibacillus pantothenicus* NCDO 1765); X60606 (*Paenibacillus amylolyticus* NCIMB 8144); D78466 (*Paenibacillus curdolanolyticus* IFO 15724^T); X57306 (*Paenibacillus macerans* ATCC 8244^T); and D16276 (*Paenibacillus polymyxa* IAM 13419^T).

RESULTS

Morphological and physiological characteristics

Cells from cultures grown for 7 h at 55 °C were observed as thin rods measuring 0.4–0.5 by 2.0–2.8 µm. (Fig. 1a). They occurred singly, in pairs and occasionally in chains. After about 15 h, they produced terminal or subterminal ellipsoidal spores in swollen sporangia. (Fig. 1b, c). Colonies exhibited an irregular, flat morphology and had rather undulate margins (Fig. 1d). Strain XE^{TP} was found to be strictly aerobic, Gram-negative and non-motile. The measurement of catalase activity for this strain was positive, but no

oxidase or urease activity was measured. Strain XE^{TP} was able to grow in the presence of NaCl up to 3% (w/v) and was found to degrade both starch and aesculin but not gelatin or casein. Nitrate is not reduced. This strain grew optimally at pH 7.8 and 55 °C whereas its pH and temperature limits are pH 6.5 (lower), pH 8.5 (higher) and 63 °C, respectively. CO₂ and hydrogen carbonate were found to be necessary for normal growth. Their omission resulted in an increased lag phase and overall poor growth. The following compounds were assimilated within 24 h: cellobiose, galactose, lactose, mannose, melibiose, raffinose, starch, trehalose and xylose. Fructose,

Fig. 1. Microscopic images of strain XE^{TP}. Phase-contrast micrographs of (a) cells in the vegetative state after a 7 h growth period in a liquid medium containing xylan and (b) sporulating cells from the same culture after a 15 h growth period; bar, 10 µm. (c) SEM image of cells in late-exponential growth phase displaying the swollen sporangia; bar, 1 µm. (d) Colonies grown for 2 d on J-agar; bar, 2 mm.

Table 1. Cellular fatty acid composition (%) of strain XE^{TP} and some representatives of the genus *Paenibacillus*

The three major fatty acids are shown in bold.

Fatty acid	Short designation	Fatty acid composition (% w/w)	
		Strain XE ^{TP}	<i>Paenibacilli</i> *
12-Methyl tridecanoic acid	14:0 iso	1.0	0.6–7.2
Tetradecanoic acid	14:0	1.2	1.2–3.3
13-Methyl tetradecanoic acid	15:0 iso	0.9	10.5–34.3
12-Methyl tetradecanoic acid	15:0 anteiso	8.7	28.2–57.3
Pentadecanoic acid	15:0	1.8	0.6–6.7
14-Methyl pentadecanoic acid	16:0 iso	48.0	2.9–4.5
Hexadecanoic acid	16:0	21.4	8.1–28.0
15-Methyl hexadecanoic acid	17:0 iso	1.8	1.0–6.9
14-Methyl hexadecanoic acid	17:0 anteiso	12.4	1.3–12.8
Heptadecanoic acid	17:0	0.9	
17-Methyl heptadecanoic acid	18:0 iso	1.1	
Octadecanoic acid	18:0	0.7	

* Data obtained from Heyndrickx *et al.* (1996). The range for four different species (*P. larvae*, *P. pulvificiens*, *P. alvei* and *P. laterosporus*) is given.

Fig. 2. Phylogenetic tree based on 16S rDNA sequences comparisons of strain XE^{TP} and selected bacteria from the *Bacillus* group. Bar, 10 base substitutions per 100 nucleotides. The numbers at each node are bootstrap values based on 1000 resamplings.

melezitol and tributyrin were assimilated within 48 h, whereas adonitol, casein, citrate, dextrin, dulcitol, erythritol, gelatin, inulin, salicin, sorbitol and succinate remained unassimilated after 72 h.

Chemotaxonomic characteristics and DNA base composition

The major isoprenoid quinone found in strain XE^{TP} was MK-7, a 2-methyl 1,4 naphthoquinone showing a polyisoprenoid chain (7 units) in position 3. MK-7 is the major menaquinone generally found in aerobic, endospore-forming rods. The major cellular fatty acids were 14-methyl pentadecanoic acid (16:0 iso), hexadecanoic acid (16:0) and 14-methyl hexadecanoic acid (17:0 anteiso) (Table 1).

The G+C content of strain XE^{TP} was 57.5 mol %.

Phylogenetic analysis

An almost complete 16S rDNA sequence (1554 bp), which corresponds to a region between positions 8 and 1525 by comparison with the *E. coli* 16S rRNA gene (Brosius *et al.*, 1978), was directly sequenced. 16S rDNA alignments showed that the closest relatives of strain XE^{TP} were '*Bacillus viscosus*', *Paenibacillus curdlanolyticus* and *Bacillus popilliae* with identity values of 91.15, 90.94 and 90.92%, respectively. The phylogenetic tree (Fig. 2), constructed from the se-

quence data, shows the position of strain XE^{TP} within the group of selected species of *Bacillus sensu lato* including its three closest relatives.

DISCUSSION

The definitive taxonomic position of strain XE^{TP} was impossible to establish by traditional methods. Indeed, this approach led to the classification of strain XE^{TP} as a thermophilic member of the genus *Bacillus* where all aerobic, endospore-forming organisms have been classified (Yoon *et al.*, 1998). For many years, the genus *Bacillus* was considered to be taxonomically heterogeneous (Sneath, 1986). Recently this genus was split, giving rise to several genera which were defined using 16S rDNA sequence data. To date, five new genera have been recognized. They are: *Alicyclobacillus* (Wisotzkey *et al.*, 1992), *Aneurinibacillus* (Shida *et al.*, 1996), *Brevibacillus* (Shida *et al.*, 1996), *Paenibacillus* (Ash *et al.*, 1993) and *Virgibacillus* (Heyndrickx *et al.*, 1996).

The results of 16S rDNA sequencing for strain XE^{TP} showed that its closest relatives are '*Bacillus viscosus*', *Paenibacillus curdlanolyticus* and *Bacillus popilliae* with similarities below 92%. '*Bacillus viscosus*' has never been validly described and, like *Bacillus popilliae*, probably belongs to the genus *Paenibacillus* (Fig. 2). However strain XE^{TP} can be clearly distinguished from the genus *Paenibacillus*: 16S rDNA sequence similarity is low; its G+C content is well out of the range 45–54 mol% for *Paenibacillus* (Shida *et al.*, 1997); and its major cellular fatty acid is 16:0 iso compared to 15:0 anteiso for *Paenibacillus* (Shida *et al.*, 1997). Moreover, the optimum growth temperature of 19 species (all species except *Paenibacillus macquariensis*) is 28–30 °C, the optimum growth temperature being 20–23 °C for *Paenibacillus macquariensis* (Shida *et al.*, 1997). Finally, strain XE^{TP} displays 16:0 iso as its main fatty acid, a feature not only unusual for the genus *Paenibacillus* (Shida *et al.*, 1997) but equally unusual for the genus *Bacillus* as a whole (Kämpfer, 1994).

The phenotypic, chemotaxonomic and phylogenetic data show that strain XE^{TP} does not belong to the genus *Paenibacillus* and is distinct from any other described genus. The creation of a new genus *Thermobacillus* and a new species *Thermobacillus xylanilyticus* to accommodate the new isolate is therefore proposed.

Description of *Thermobacillus* gen. nov.

Thermobacillus (Ther.mo.ba.cil'lus. Gr. adj. *thermos* hot; M.L. dim. n. *bacillus* small rod; M.L. n. *Thermobacillus* a small thermophilic rod).

Thermobacillus cells are Gram-negative, spore-forming, aerobic, non-motile, rod-shaped, thermophilic bacteria. The menaquinone is MK-7. The major fatty acid is C16:0 iso. The G+C content is

57.5 mol%. The type species is *Thermobacillus xylanilyticus* sp. nov.

Description of *Thermobacillus xylanilyticus* sp. nov.

Thermobacillus xylanilyticus (xy.la.ni.ly'ti.cus. Gr. n. *xylon* wood; N.L. n. *xylanum* xylan, a plant polysaccharide; Gr. adj. *lyticus* dissolving; M.L. adj. *xylanilyticus* hydrolysing xylan).

Cells are short rods measuring 0.4–0.5 by 2.0–2.8 µm. Non-motile. Ellipsoidal endospores are formed in swollen sporangia. Aerobic. Gram reaction is negative. Colonies are irregular, flat, with undulate margins. Catalase-positive and oxidase- and urease-negative. Growth occurs in the presence of 3% NaCl. Starch and aesculin are hydrolysed, whereas gelatin and casein are not. Utilizes cellobiose, fructose, galactose, lactose, mannose, melezitol, melibiose, raffinose, trehalose, tributyrin and xylose as sole carbon source for growth. Substrates which are not utilized are adonitol, casein, citrate, dextrin, dulcitol, erythritol, gelatin, inulin, salicin, sorbitol and succinate. Nitrate is not reduced. Thermophilic. Grows optimally at 55 °C; maximum temperature for growth is 63 °C. Grows at pH 6.5–8.5; optimum pH is 7.8. CO₂ is required. Produces large quantities of a xylan-inducible xylanase in the culture medium. The menaquinone is MK-7. The major fatty acid is C16:0 iso. The G + C content is 57.5 mol% (determined by thermal denaturation). According to the complete 16S rDNA sequence of its only member, this genus belongs to the *Clostridium–Bacillus* subphylum of Gram-positive bacteria. The type species is *Thermobacillus xylanilyticus*. The type strain is XE^{TP}, which was isolated in France. The type strain has been deposited in the Collection Nationale de Cultures Microbiennes (CNCM I-1017) as a patent strain.

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