Lutispora thermophila gen. nov., sp. nov., a thermophilic, spore-forming bacterium isolated from a thermophilic methanogenic bioreactor digesting municipal solid wastes

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A novel anaerobic, moderately thermophilic, spore-forming, rod-shaped bacterium (strain EBR46^T) was isolated from an enrichment culture derived from an anaerobic thermophilic (55 °C) methanogenic bioreactor treating artificial solid wastes. Phylogeny based on 16S rRNA gene sequence analysis placed strain EBR46^T within a distinct lineage between *Clostridium* clusters II and III. The closest recognized relative of strain EBR46^T was *Gracilibacter thermotolerans* DSM 17427^T (85.3% 16S rRNA gene sequence similarity). The DNA G+C content of strain EBR46^T was 36.2 mol%. The novel strain grew optimally at 55–58 °C and at pH 7.5–8.0 and was able to grow on peptone, tryptone, Casamino acids, casein hydrolysate, methionine, threonine, tryptophan, cysteine, lysine and serine in the presence of 0.2% yeast extract. Carbohydrates were not utilized. The main products from tryptone utilization were acetate, iso-butyrate, propionate and iso-valerate. Strain EBR46^T produced hydrogen sulfide from cysteine. The major fatty acids were iso-C_{15:0}, C_{14:0}, C_{16:0} DMA (dimethyl acetal) and iso-C_{15:0} DMA. Based on its unique phylogenetic and physiological features, strain EBR46^T is considered to represent a novel species of a new genus, for which the name *Lutispora thermophila* gen. nov., sp. nov. is proposed. The type strain of the type species is EBR46^T (=NBRC 102133^T=DSM 19022^T).

The genus *Clostridium*, comprising Gram-positive species, was first proposed by Prazmowski in 1880. At the time of writing, the names of 188 species of the genus have been validly published (<http://www.bacterio.cict.fr>). Most members of the genus are chemo-organotrophs that can utilize carbohydrates and/or proteinaceous compounds as an energy source. In classical taxonomy, an organism only had to meet four criteria to be classified as a member of the genus *Clostridium*, i.e. be endospore-forming, be obligately anaerobic, have a Gram-positive cell wall and lack the ability to carry out dissimilatory reduction of sulfate. The genus was thus highly divergent and included psychrophilic, mesophilic and thermophilic species with a wide range of DNA G+C content (21–54 mol%).

To clarify the phylogenetic incoherence within the genus *Clostridium*, Collins *et al.* (1994) proposed its reclassification into newly established taxa based on 16S rRNA gene

sequence analysis. Following this, Wiegel *et al.* (2006) proposed a new criterion: members of the *Clostridium* Cluster I, including the type species, comprise *Clostridium sensu stricto*; those species remaining outside this cluster have been reclassified within different phylogenetic groups such as *Caloramator*, *Oxobacter* and *Thermobrachium*. Based on this new criterion, Lee *et al.* (2006) proposed a new genus, *Gracilibacter*, which is placed between *Clostridium* clusters II and III.

In the present study, we investigated the taxonomy of a novel member of the family *Clostridiaceae* (strain EBR46^T), isolated from thermophilic methanogenic sludge. In a previous study, we successfully determined the 16S rRNA gene sequence of this organism but failed to isolate it owing, to a large part, to its inefficient recovery from long-term storage at –80 °C (Shiratori *et al.*, 2006). Following long-term successive cultivations, we succeeded in isolating and stably culturing strain EBR46^T. The distinct phylogenetic lineage represented by this isolate fell within a position close to members of the genus *Gracilibacter* outside the cluster of members of *Clostridium sensu stricto*. Both phylogenetic and

Abbreviation: DMA, dimethyl acetal.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain EBR46^T is AB186360.

physiological characteristics indicate that strain EBR46^T represents a novel species of a new genus.

The operation of a thermophilic (55 °C) methanogenic bioreactor from which strain EBR46^T was isolated has been described previously (Shiratori *et al.*, 2006). M solution used for the cultivation of *Clostridium* species was prepared by mixing 980 ml of basal solution with 10 ml each of vitamin solution and mineral solution. The basal solution had the following composition (per litre distilled water; all chemicals were purchased from Kokusan unless indicated otherwise): 0.4 g KH₂PO₄, 0.4 g K₂HPO₄·3H₂O, 1.0 g NH₄Cl, 0.1 g MgCl₂·6H₂O, 0.2 g yeast extract (Difco Laboratories), 6.0 g NaHCO₃, 0.5 g cysteine-HCl·H₂O, 0.25 g Na₂S·9H₂O (Yoneyama) and 0.001 g resazurin. The vitamin solution had the following composition (per litre distilled water): 2.0 mg biotin, 2.0 mg folic acid, 10.0 mg pyridoxine hydrochloride, 5.0 mg thiamine HCl, 5.0 mg riboflavin, 5.0 mg nicotinic acid, 5.0 mg DL-calcium pantothenate, 0.1 mg vitamin B12, 5.0 mg *p*-aminobenzoic acid and 5.0 mg lipoic acid. The mineral solution had the following composition (per litre distilled water): 4.0 g nitrilotriacetic acid, 0.4 g FeCl₂·4H₂O, 0.12 g CoCl₂·6H₂O, 0.01 g AlK(SO₄)₂, 1.0 g NaCl, 0.02 g CaCl₂, 0.01 g Na₂MoO₄·2H₂O, 0.1 g MnCl₂·4H₂O, 0.1 g ZnCl₂, 0.01 g H₃BO₃, 0.01 g CuSO₄·5H₂O and 0.02 g NiCl₂.

M solution was supplied with various cellulosic substrates to prepare each culture medium. Mpc medium contained 5 % (w/v) cellulose powder (Type D; Advantec) and 1 % (w/v) cellobiose in M solution. The Mf medium contained a filter paper strip (1 cm × 8 cm, approximately 0.05 g; Grade 1, Whatman) in 10 ml M solution per tube. Mc medium contained 0.5 % (w/v) cellobiose and 1.5 % (w/v) agar (for solid medium; Kokusan) in M solution. Mt liquid medium contained 1.0 % (w/v) tryptone (Difco) and 0.2 % (w/v) yeast extract (Difco) in M solution.

A methanogenic sludge sample was collected from the reactor, inoculated into Mpc medium and semi-continuously cultured with periodic supply of propionate (loading rate, 20 mM for 2 weeks). Culturing was performed at 55 °C with continuous shaking at 60 r.p.m. and culture broth was routinely sampled and plated onto Mc agar medium after appropriate dilution. Agar plates were incubated at 55 °C for about 2 weeks in an Anoxomat Mark II anaerobic cultivation system (Mart Microbiology). Anaerobic manipulation was carried out by using a Concept 400 anaerobic chamber (Ruskin). As a result, pale yellow spherical colonies (about 1.0 mm in diameter) were obtained. A single colony was then collected, suspended in sterile M solution and inoculated into Mc liquid medium containing 50 µg neomycin ml⁻¹ after successive dilution to establish a final pure culture of strain EBR46^T. The addition of neomycin was effective in removing contaminant organisms. Unless stated otherwise, cultivation of strain EBR46^T was performed by using Mt liquid medium without shaking at 55 °C in a screw-capped test tube under an N₂/CO₂ atmosphere (4:1, v/v).

Cells of strain EBR46^T were observed under an Axioskop 2 optical microscope (Carl Zeiss Microimaging) and a JEM-1200EX electron microscope (JEOL). Cells were straight or slightly curved rods (0.6–0.8 × 5.0–8.5 µm). Spores were oval and subterminal (Fig. 1a). The cells were laterally flagellated (Fig. 1b). Weak motility was exhibited by cells grown on SIM agar medium (Blazevic, 1968). Although cells stained Gram-negative by the method of Bartholomew & Mittwer (1952), the cell-wall ultrastructure resembled that of Gram-positive bacteria (Fig. 1c). Colonies grown on Mt solid medium (Mt medium solidified with 1.5 % agar) were pale yellow, round and 1.0–1.5 mm in diameter.

For 16S rRNA gene sequence analysis and determination of the genomic G + C content, DNA was extracted by using a bacterial genomic DNA purification kit (Edge Biosystems). A nearly-complete 16S rRNA gene fragment of strain EBR46^T was amplified by PCR by using primers B8F (5'-AGAGTTTGATCCTGGCTCAG; nucleotides 8–27 based on the *Escherichia coli* numbering system) and B1492R (5'-GGTTACCTTGTTACGACTT; nucleotides 1510–1492). The PCR was performed in a T1 Thermocycler (Biometra) with Ex Taq polymerase (Takara-shuzo). The PCR protocol included an initial denaturation period of 4 min at 94 °C; 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min; 72 °C for 3 min; and incubation at 4 °C until further processing. The 16S

Fig. 1. Micrographs of cells of strain EBR46^T. (a) Phase-contrast micrograph of a sporulating cell (bar, 5 µm). (b) Electron micrograph of a negatively stained cell forming a lateral flagellum (bar, 2 µm). (c) Electron micrograph of a cross-section of a cell (bar, 1 µm); inset: longitudinal section (bar, 0.2 µm). S, S-layer; P, peptidoglycan; CM, cytoplasmic membrane.

rRNA gene fragment was sequenced directly by using the BigDye terminator v3.1 cycle sequencing kit on an ABI 3130 Genetic Analyzer (Applied Biosystems).

The 16S rRNA gene sequence of strain EBR46^T, comprising 1438 bp (corresponding to nucleotides 27–1556 based on the *E. coli* numbering system), was compared with those from the GenBank/EMBL/DDBJ nucleotide sequence database by using the BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST/>). The nucleotide sequence was identical to that determined in our previous study (Shiratori *et al.*, 2006), and placed strain EBR46^T within the subphylum *Clostridia*. The closest GenBank relatives of strain EBR46^T were uncultured clones derived from: a packed-bed reactor that degraded household waste (GenBank accession no. AB274491) (Sasaki *et al.*, 2006), a mesophilic anaerobic reactor (GenBank accession no. AB248637) and a methanogenic consortium that degraded propionate (GenBank accession no. AB232818), with 16S rRNA gene sequence similarities of 93–98 %.

Phylogenetic trees were constructed according to the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) methods. An evolutionary distance matrix for the neighbour-joining method was generated according to the model of Jukes & Cantor (1969). To evaluate the topology of the resultant tree, bootstrap analysis (Felsenstein, 1985) of 1000 resamplings was used. Alignment and phylogenetic analysis were performed by using CLUSTAL W (Thompson *et al.*, 1994), SeaView (Galtier *et al.*, 1996), MEGA 3.1 (Kumar *et al.*, 2004) and the PHYLIP package (Felsenstein, 1989). In an inferred phylogenetic

tree constructed based on the neighbour-joining method (Fig. 2), strain EBR46^T was placed between *Clostridium* clusters II and III (Collins *et al.*, 1994); however, the novel bacterium formed a distinct lineage of descent within the neighbouring clostridial groups. The trees constructed based on the other two methods showed a similar topology (data not shown). The closest recognized relative of strain EBR46^T was *Gracilibacter thermotolerans* DSM 17427^T (85.3 % 16S rRNA gene sequence similarity), an anaerobic chemo-organotroph isolated from a sediment sample of a constructed wetland receiving acid sulfate water (Lee *et al.*, 2006). The G+C content of the genomic DNA of strain EBR46^T was 36.2 mol% (mean of three replicates), as determined by an HPLC method (Mesbah & Whitman, 1989).

The potential for growth of strain EBR46^T was determined by using Mt liquid or solid medium anaerobically supplied with reducing agents and resazurin. To study electron acceptor availability, M solution was modified as follows: CuSO₄ · 5H₂O (0.01 g l⁻¹) was replaced with CuCl₂ · 2H₂O (0.01 g l⁻¹) and thioglycollate and ascorbate were added (each at 100 mg l⁻¹). To study H₂S and indole production, SIM broth (Blazevic, 1968) was used.

Growth of strain EBR46^T occurred at between 40 and 60 °C (optimum growth at 55–58 °C) and at pH 6.0–9.0 (optimum growth at pH 7.5–8.0). No growth was detected at 65 °C or at temperatures below 30 °C, and no growth was observed at pH 5.5 or 10.0. The pH was adjusted by using HCl (pH 6.0 and 6.5), NaOH (pH 7.0–9.0), as well as 50 mM each of MES (pH 5.5), HEPES (pH 7.5) and CAPS (pH 10.0). Growth of strain EBR46^T was observed at

Fig. 2. Phylogenetic position of strain EBR46^T within the family *Clostridiaceae* based on 16S rRNA gene sequences. The tree was constructed by using the neighbour-joining method. Branching points supported by the neighbour-joining, maximum-likelihood and maximum-parsimony methods (based on 1000 resampled data sets) are indicated by filled (>95 % bootstrap values) and open (>75 % bootstrap values) circles. Roman numerals are *Clostridium* cluster designations. Bar, 0.02 substitutions per nucleotide position.

NaCl concentrations ranging from 0 to 2.0 % (w/v), with no growth observed at 3.0 % (w/v) NaCl. Colony formation was not observed under an aerobic atmosphere ($O_2 \geq 2\%$). Strain EBR46^T required yeast extract ($\geq 0.02\%$) for growth. Utilization of substrates was judged based on the increase in optical density or decrease in pH value of the culture broth over 7 days cultivation. Strain EBR46^T utilized peptone, tryptone, Casamino acids, casein hydrolysate (each supplied at 1.0 %, w/v) and pyruvate (20 mM) as carbon and energy sources.

Amino acid utilization in casein hydrolysate medium (containing 3.0 % N-Z-amine in M solution) was studied by two-dimensional TLC by using a cellulose plate (Munier *et al.*, 1975) in the same medium incubated without inoculation as a reference. The result showed that strain EBR46^T was able to utilize methionine, threonine, tryptophan, cysteine and lysine effectively and was able to degrade serine slightly. Strain EBR46^T was able to degrade these amino acids supplied at 20 mM in M solution containing 0.2 % (w/v) yeast extract and showed marked growth. Under these conditions, strain EBR46^T degraded cysteine to form H_2S , which was detected via a spectrometric assay (Cord-Ruwisch, 1985). The novel strain did not perform the Stickland reaction (Nisman, 1954) when utilizing phenylalanine, leucine and glutamic acid (each at 20 mM) as electron donors and proline (10 mM) and methionine (20 mM) as electron acceptors.

Strain EBR46^T did not utilize the following carbohydrates (added at 1.0 % to media): adonitol, amygdalin, arabinose, cellobiose, cellulose, dulcitol, erythritol, fructose, galactitol, galactose, glucose, glycerol, glycogen, inositol, inulin, lactose, maltose, mannitol, mannose, melibiose, melezitose, raffinose, rhamnose, ribose, salicin, sorbitol, sorbose, starch, sucrose, trehalose, xylitol or xylose. Electron acceptor utilization tests (Cord-Ruwisch, 1985) showed that strain EBR46^T was unable to utilize acetate, lactate, formate, butyrate, propionate or ethanol (each supplied at 20 mM to media) either with or without sulfate (20 mM) as an electron acceptor. None of the following substances served as an electron acceptor in culture media supplemented with 1.0 % tryptone: fumarate, nitrate, thiosulfate, elemental sulfur, iron (III) or anthraquinone 2,6-disulfonate (each supplied at 20 mM to media). The main fermentation products from tryptone utilization were acetate, iso-butyrate, propionate and iso-valerate.

Activities of the following enzymes (Holdeman *et al.*, 1977) were negative: caseinase, catalase, lectinase, lipase and urease. Indole was produced in SIM broth. Hydrogen sulfide was formed from cysteine. Aesculin hydrolysis and gelatin liquefaction were weakly positive.

The fatty acid methyl esters of strain EBR46^T were extracted and analysed according to the standard protocol of the Sherlock Microbial Identification System (version 5.0; MIDI). As detailed in Table 1, the major fatty acids were iso- $C_{15:0}$, $C_{14:0}$, $C_{16:0}$ DMA (dimethyl acetal) and iso- $C_{15:0}$ DMA, which accounted for 64.4 % of the total

Table 1. Whole-cell fatty acid profiles (percentage of total) of strain EBR46^T and *Gracilibacter thermotolerans* JW/YJL-S1^T (data from Lee *et al.*, 2006)

DMA, dimethyl acetal. —, Absent.

Fatty acid	Strain EBR46 ^T	<i>G. thermotolerans</i> JW/YJL-S1 ^T
$C_{12:0}$	0.6	—
iso- $C_{13:0}$	0.6	—
$C_{14:0}$	21.4	2.3
$C_{14:0}$ DMA	6.7	—
iso- $C_{15:0}$	25.6	23.8
anteiso- $C_{15:0}$	3.6	4.9
$C_{15:0}$	0.9	0.5
iso- $C_{15:0}$ DMA	6.7	—
iso- $C_{16:0}$	0.1	0.6
$C_{16:1}$	—	5.4
$C_{16:1\omega7c}$	2.6	—
$C_{16:0}$	3.9	29.0
$C_{16:0}$ DMA	10.7	—
iso- $C_{17:0}$	0.3	15.4
$C_{17:1}$	—	5.4
$C_{17:0}$	—	2.5
$C_{18:0}$	0.4	3.5
$C_{18:1}$	—	0.8
$C_{18:1\omega9c}$	0.8	1.6
$C_{18:1\omega9c}$ DMA	0.3	—

fatty acids. This result was consistent with the fact that thermophilic *Clostridia* contain a high content of saturated and branched-chain iso-fatty acids with C_{15} as the predominant component (Chan *et al.*, 1971). The fatty acid profile of strain EBR46^T differed from that of *G. thermotolerans* DSM 17427^T, its closest recognized relative, in terms of the absence of unsaturated $C_{16:1}$, $C_{17:1}$ and $C_{18:1}$, and the presence of $C_{14:0}$ DMA, iso- $C_{15:0}$ DMA and $C_{16:0}$ DMA (Table 1).

The morphological and physiological characteristics of strain EBR46^T are compared with those of five species of four genera (*Gracilibacter*, *Thermobrachium*, *Caloramator* and *Oxobacter*) that have a phylogenetic position between *Clostridium* clusters II and III (see Fig. 2) in Table 2. According to the reclassification proposals of Wiegand *et al.* (2006), the above four genera are distinct from those affiliated to *Clostridium sensu stricto*. Our 16S rRNA gene-based phylogeny (Fig. 2) indicated that strain EBR46^T occupied a similar position to the above genera and belonged to a lineage branched from a diverging point of *Clostridium* clusters III and IV. Strain EBR46^T could be distinguished readily from members of *Clostridium* clusters III and IV based on the lack of cellulolytic activity.

Strain EBR46^T differed from *G. thermotolerans*, its closest phylogenetic relative, in terms of spore formation, motility, DNA G+C content and temperature range and temperature optimum for growth. Strain EBR46^T produced H_2S in SIM medium whereas *G. thermotolerans* did not. Fatty

Table 2. Differential characteristics between strain EBR46^T and related taxa within the phylum of low-G + C Gram-positive bacteria

Taxa: 1, strain EBR46^T; 2, *Gracilibacter thermotolerans* JW/YJL-S1^T (data from Lee *et al.*, 2006); 3, *Thermobrachium celere* JW/YL-NZ35^T (Engle *et al.*, 1996); 4, *Caloramator fervidus* Rt4-B1^T (Patel *et al.*, 1987); 5, *Caloramator indicus* IndiB4^T (Christostomos *et al.*, 1996); 6, *Oxobacter pfennigii* V5-2^T (Krumholz & Bryant, 1985). +, Positive; –, negative; w, weakly positive; ND, not determined.

Characteristic	1	2	3	4	5	6
Gram reaction	–	–	+	–	–	+
Spore formation	+	–	–	+	–	+
Motility	+	–	+	+	–	+
DNA G + C content (mol%)	36.2	42.8	31	39	25.6	38
Temperature range (°C)	40–60	25–54	37–75	37–80	37–75	23–39
Temperature optimum (°C)	55–58	42.5–46.5	67	68	60–65	36–38
pH optimum	7.5–8.0	6.8–7.75	8.2	7.0–7.5	7.5–8.1	7.3
Indole production	+	+	ND	ND	ND	ND
Casein digestion	–	ND	+	ND	ND	ND
Gelatin liquefaction	w +	ND	ND	ND	ND	–
Utilization of:						
Casamino acids	+	+	–	–	ND	–
Glucose	–	+	+	+	+	–
Pyruvate	+	–	–	+	ND	+
Tryptone	+	+	ND	+	+	–

acid content profiling clearly differentiated the two organisms (see above). Strain EBR46^T was distinct from its other close relatives based on the following: whereas members of the genus *Caloramator* and *Thermobrachium celere* are saccharolytic, strain EBR46^T did not metabolize most of the carbohydrates tested; *Oxobacter pfennigii* is phenotypically similar to strain EBR46^T in terms of substrate utilization, but the former is mesophilic; and *Caloramator indicus* and *T. celere* are negative for sporulation.

Based on these unique phylogenetic and physiological features, we conclude that strain EBR46^T represents a novel species in a new genus, for which the name *Lutispora thermophila* gen. nov., sp. nov. is proposed.

Description of *Lutispora* gen. nov.

Lutispora (Lu.ti.spo'ra. L. n. *lutum* mud, sludge; Gr. fem. n. *spora* a seed and, in biology, a spore; N.L. fem. n. *Lutispora* a spore-forming organism that lives in anaerobic sludge).

A member of the phylum comprising low-G + C, Gram-positive bacteria. Cells are Gram-reaction-negative, motile, spore-forming rods. Moderately thermophilic. Obligatory anaerobic and catalase-negative. Chemo-organotroph. The type species is *Lutispora thermophila*.

Description of *Lutispora thermophila* sp. nov.

Lutispora thermophila (ther.mo.phi'la. Gr. fem. n. *therme* heat; Gr. adj. *philos* loving; N.L. fem. adj. *thermophila* heat-loving).

Has the following characteristics in addition to those given above for the genus. Cells are 0.6–0.8 µm in width and 5.0–8.5 µm in length and are weakly motile. Colonies

(1.0–1.5 mm in diameter) are round, translucent with smooth surfaces and pale yellow. Major fatty acids are iso-C_{15:0}, C_{14:0}, C_{16:0} DMA and iso-C_{15:0} DMA. Growth occurs at 40–60 °C (optimum growth at 55–58 °C) and at pH 6.0–9.0 (optimum growth at pH 7.5–8.0). Growth occurs at NaCl concentrations of 0–2.0% (w/v), with optimum growth in the absence of NaCl. Yeast extract is required for growth. In the presence of yeast extract, growth and substrate utilization are observed with the following substrates: peptone, tryptone, Casamino acids, casein hydrolysate, pyruvate and some amino acids (methionine, threonine, tryptophan, cysteine, lysine and serine). Carbohydrates are not utilized for growth. Fermentation products from tryptone are acetate, iso-butyrate, propionate and iso-valerate. No growth occurs by utilization of sulfate, fumarate, nitrate, thiosulfate, elemental sulfur, iron (III) or anthraquinone 2,6-disulfonate as electron acceptor. Aesculin hydrolysis and gelatin liquefaction are weakly positive. Indole is produced in SIM broth. Hydrogen sulfide is formed from cysteine. The DNA G + C content is 36.2 mol%. Resistant to neomycin (50 µg ml^{–1}).

The type strain, EBR46^T (=NBRC 102133^T=DSM 19022^T), was isolated from a thermophilic methanogenic bioreactor.

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