Brachybacterium zhongshanense sp. nov., a cellulose-decomposing bacterium from sediment along the Qijiang River, Zhongshan City, China

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A cellulose-decomposing bacterium, strain JB^T, was isolated from sediments along the Qijiang River, Zhongshan City, China. Results of morphological, biochemical and chemotaxonomic characterization and 16S rRNA gene sequence analysis revealed that strain JB^T belonged to the genus *Brachybacterium*. Insertion sequence-PCR fingerprinting patterns, DNA base ratio analysis and DNA–DNA hybridization data showed that strain JB^T differed from recognized species of the genus *Brachybacterium*. Based on polyphasic analysis, strain JB^T represents a novel species of the genus *Brachybacterium*, for which the name *Brachybacterium zhongshanense* sp. nov. is proposed. The type strain is JB^T (=LMG 23926^T=CGMCC 1.6508^T=DSM 18832^T).

Cellulosic materials are among the Earth's most abundant, renewable resources and micro-organisms play an important role in their degradation and utilization in nature. More and more researchers are becoming interested in cellulose degradation because of the potential for converting large amounts of photosynthetically produced cellulosic materials into industrial substrates. Many cellulolytic bacteria from soils and sediments secrete cellulases and xylanases that associate into high molecular mass complexes (Gal *et al.*, 1997; Veiga *et al.*, 1983).

During the course of collecting environmental micro-organisms from various sources, sediments from along the Qijiang River, Zhongshan City, China, were sampled and used to isolate micro-organisms that decompose the cellulosic compounds of municipal solid waste. A sucrose manufacturing plant is located near the river and the sediment is rich in cellulose. One of the cellulose-decomposing micro-organisms was characterized. The taxonomic position of the isolate was determined. On the basis of morphological, biochemical, chemotaxonomic and molecular taxonomic characteristics, it was concluded that the isolate represents a novel species of the genus *Brachybacterium*.

The sediment samples were shaken for 2 h on a rotary shaker at 250 r.p.m. to disperse them and then suspensions were serially diluted with 0.85 % (w/v) NaCl solution. Each dilution (0.1 ml) was plated into Congo red agar medium, pH 7.0, modified from Hendrick *et al.* (1995) containing

(g l⁻¹): KH₂PO₄ (0.5), MgSO₄·7H₂O (0.25), cellulose powder (1.88), Congo red (0.2), gelatin (2.0) and agar (16). After 7 days incubation at 30 °C, isolated colonies showing distinct red circles were selected and purified further on Congo red plates. Pure cultures were stored in 15 % (w/v) glycerol at -20 °C and used for further testing. A number of the isolates had nearly identical results in Biolog GP2 MicroPlate assays after incubation for 1 day; one of these was selected as the type strain.

The reference strains used were: *Brachybacterium rhamnosum* LMG 19848^T, *Brachybacterium fresconis* LMG 20336^T, *Brachybacterium nesterenkovi* LMG 19549^T (=DSM 9573^T), *Brachybacterium paraconglomeratum* LMG 19861^T and *Brachybacterium sacelli* LMG 20345^T, all obtained from the Laboratorium voor Microbiologie, Rijksuniversiteit Gent, Ledeganckstraat 35, B-9000, Gent, Belgium; and *Brachybacterium muris* DSM 15460^T, which was obtained from the DSMZ, Braunschweig, Germany.

Cellulose-degrading ability was measured by following the loss in dry weight of either rice straw stalk or its powder. Either 2 g rice straw stalk or 2 g powder was mixed into 100 ml zymolytic medium, pH 7.2, containing (g l⁻¹): peptone, 0.5; yeast extract, 0.5; (NH₄)₂SO₄, 2.0; KH₂PO₄, 4.0; CaCl₂·2H₂O, 0.3; and MgSO₄·7H₂O, 0.3. A 5 % inoculum was added and cultures were incubated at 30 °C with shaking at 150 r.p.m. (three replicates). After 6 days, the residual rice straw stalk or powder was harvested by centrifugation and washed repeatedly. The cellulose content was determined colorimetrically with K₂Cr₂O₇ as described by Halliwell (1974). The cellulose-decomposing ratio (%) was calculated according to the following formula: cellulose decomposed (%)=[(A-B)/A]×100, in

Abbreviation: IS-PCR, insertion sequence-PCR.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain JB^T is EF125186.

which A is the initial weight of straw stalk (or powder) $\times$ cellulose content and B is straw stalk (or powder) remaining $\times$ cellulose content of residual straw stalk (or powder).

For morphological, physiological and biochemical analysis, strain JB^T was grown aerobically on Luria–Bertani (LB) plates (Sambrook *et al.*, 1989) overnight at 30 °C. Microaerophilic conditions were maintained in an oxygen range varying between 0.01 and 0.5 mg l⁻¹ without aeration in flasks at 30 °C for 36 h. Anaerobic growth (80 % N₂, 10 % CO₂, 10 % H₂) on LB plates at 30 °C for 36 h was analysed by using a Bug Box anaerobic workstation (Ruskin). The Hucker method (Murray *et al.*, 1994) was used for Gram staining. Gram staining was observed by normal light microscopy and cell morphology was examined by transmission electron microscopy after negative staining with 4 % phosphotungstic acid, pH 7.0. Oxidase activity was determined by using 1 % (w/v) tetramethyl-*p*-phenylene-diamine as the substrate. Catalase activity was determined by the detection of bubble formation in 3 % (w/v) hydrogen peroxide solution after incubation in LB medium for 18–24 h (Smibert & Krieg, 1994). Carbon source utilization was tested by using Biolog GP2 MicroPlates. Overnight cultures were used to inoculate the GP2 MicroPlates, according to the manufacturer's instructions, and they were incubated at 30 °C for 24 h; the colour result in each well was recorded by the Biolog MicroStation. Some biochemical tests were performed by using the Microbial Biochemical Identification Tube System (GuangDong Huankai Microbial Sci & Tech). Tolerance to different NaCl concentrations (0–15 %, w/v) and pH values (4–14) was measured spectrophotometrically (Bachman U 640) at OD₅₅₀ in LB medium after 18–24 h incubation at 30 °C; results were scored as positive if the change in OD₅₅₀ was greater than 0.300 (Heyrman *et al.*, 2002).

For analysis of cellular fatty acids, strain JB^T was incubated for 36 h in LB medium at 30 °C. Sample preparation was performed by the method of Song *et al.* (2000); extracts and analysis of cellular fatty acid methyl esters were as described by Xu *et al.* (2005).

The 16S rRNA gene was amplified from genomic DNA of the isolate by using forward primer 27f (5'-AGAGTTT-GATCCTGGCTCAG-3') and reverse primer 1492r (5'-TACGGYTACCTTGTTACGACTT-3'). PCR conditions were as described by Hurek *et al.* (1997). The purified PCR product was sequenced directly by using the sequencing primers 35f (5'-CTKAAGAGTTTGATCMTG-GCTCAGATTGAACG-3'), 342f (5'-CTCCTACGGGAG-GCAG-3') and 930f (5'-GGTTAAACTYAAKGAAT-TGACGGGGA-3'). Sequencing was carried out by Shanghai Invitrogen Biotechnology and the nearly full-length 16S rRNA gene sequence was compiled using SEQMAN software (DNASTAR). The determined sequence and some related sequences selected from GenBank with the program BLAST were aligned by using the RDP program

(Maidak *et al.*, 1999). Alignment gaps and ambiguous bases were excluded from similarity calculations. Tree topology was inferred by using the neighbour-joining method (Saitou & Nei, 1987) and the phylogenetic tree was visualized and bootstrapped by using the TREECON software package (Van de Peer & De Wachter, 1994). The topology of the phylogenetic tree was evaluated by performing bootstrap analysis with 100 replicates.

Insertion sequence (IS)-PCR fingerprinting patterns were analysed to evaluate genotypic differences between strains JB^T, DSM 15460^T and DSM 9573^T. The primer and reaction mixtures were as described by Peng *et al.* (2006). PCR began with denaturation at 94 °C for 7 min followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 68 °C for 1 min and extension at 68 °C for 2 min. The final extension cycle was at 65 °C for 15 min. PCR products were separated in 1.2 % agarose gels in 1 $\times$ TAE buffer (40 mM Tris/acetate and 1 mM EDTA at pH 8.3).

Genomic DNA from strain JB^T and reference strains was isolated and purified as described by Marmur (1961) and DNA base composition was determined spectrophotometrically (De Ley *et al.*, 1970). DNA from *Escherichia coli* K-12 was used as the standard for estimation of G+C content. DNA–DNA relatedness was determined by using the initial renaturation rate method (De Ley *et al.*, 1970) in 2 $\times$ SSC as modified by Tan *et al.* (2001). Hybridization experiments were carried out under optimal and stringent temperatures calculated from the melting temperature (T_m) based on the G+C content of each test strain (three replicates for each test).

Cells of strain JB^T were Gram-positive and mainly short rods, 0.48–0.65 μ m wide and 0.57–0.92 μ m long; any coccoid cells were 1 μ m in diameter (Fig. 1). Colonies on LB plates at 30 °C for 24 h were cream-coloured, smooth, glistening and grew well in an anaerobic chamber on LB plates under microaerophilic conditions at 30 °C for 36 h.

Strain JB^T showed cellulose-decomposing ratios of rice straw stalk and straw powder of 3.64 ± 1.29 and 1.62 ± 2.73 %, respectively, after 6 days incubation at 30 °C.

Phenotypic properties of strain JB^T were consistent with its classification in the genus *Brachybacterium*. The major phenotypic properties of the isolate and the type strains of closely related species of the genus *Brachybacterium* (i.e. those with 16S rRNA gene sequence similarity above 97 %) are listed in Table 1; other characters are given in the species description.

The fatty acid profile of strain JB^T contained predominantly anteiso-C_{15:0} (37.38 %) and anteiso-C_{17:0} (28.17 %), which is similar to profiles of other known species of the genus *Brachybacterium* (Collins *et al.*, 1988; Gvozdyak *et al.*, 1992; Takeuchi *et al.*, 1995; Schubert *et al.*, 1996; Heyrman *et al.*, 2002; Buczolits *et al.*, 2003). Additionally, considerable amounts of iso-C_{16:0}

Fig. 1. Morphological features of cells of strain JB^T observed by transmission electron microscopy. Cells in exponential (a; bar, 100 nm) and stationary (b; bar, 62.5 nm) phases are shown.

(13.13 %), iso-C_{15:0} (9.22 %), C_{16:0} (2.23 %), C_{20:1ω7c} (2.20 %), iso-C_{14:0} (1.45 %) and C_{14:0} (1.42 %) and minor amounts of an unknown lipid (0.57 %) were also present.

16S rRNA gene sequence analyses clearly demonstrated that strain JB^T was affiliated to the genus *Brachybacterium*, as shown in Fig. 2. Data show that strain JB^T formed a close

Table 1. Differential characteristics of strain JB^T (*Brachybacterium zhongshanense* sp. nov.) and closely related species of the genus *Brachybacterium*

Strains: 1, JB^T; 2, *B. muris* DSM 15460^T; 3, *B. nesterenkovi* DSM 9573^T; 4, *B. rhamnosum* LMG 19848^T; 5, *B. sacelli* LMG 20345^T; 6, *B. fresconis* LMG 20336^T; 7, *B. paraconglomeratum* LMG 19861^T. +, Positive; (+), weakly positive; –, negative; ND, not determined. All strains are catalase-positive and oxidase-negative. Except where indicated, all data are from this study and obtained under the same conditions as data from the other studies.

Characteristic	1	2	3	4	5	6	7
Growth conditions	Facultative	Aerobic	Facultative	ND	Aerobic	Aerobic	ND
Cellulose-decomposing ability	+	–	–	–	–	–	–
Urease production	+	–	–	+	–	+	+
Nitrate reduction	+	–	+	+	+	+	+
Growth at:							
pH 5.0	+	–	(+)	+	+	+	–
pH 10.0	+	+	–	–	–	+	ND
4 °C	–	–	–	+	–	–	+
30 °C	+	+	+	+	(+)	(+)	+
42 °C	(+)	(+)	–	–	–	–	+
Growth in:							
5 % NaCl	+	+	(+)	+	+	+	+
10 % NaCl	+	+	–	(+)	+	+	(+)
15 % NaCl	–	–	–	–	(+)*	(+)	(+)
Hydrolysis of:							
Tween 20	–	+	+	+	–	–	–
Tween 80	–	(+)	–	–	–	–	–
Starch	–	+	–	+	–	–	+
Acid production from:							
D-Mannose	+	(+)	–	+	(+)	+	+
Sucrose	+	+	+	+	–	–	+
Maltose	+	+	(+)	+	–	–	+
D-Fructose	+	+	–	+	+	+	+
L-Rhamnose	+	+	–	–	(+)	(+)*	–
D-Sorbitol	+	–	–	+†	–	–	–
DNA G + C content (mol%)	71.2	ND	70.0‡	71.5‡	70.3‡	70.4‡	68.6‡

*From Heyrman *et al.* (2002).

†From Takeuchi *et al.* (1995).

‡From Buczolits *et al.* (2003).

Fig. 2. Phylogenetic positions based on 16S rRNA gene sequences of strain JB^T and the type strains of species of the genus *Brachybacterium*. *Dermabacter hominis* was included as an outgroup. Bootstrap values above 50% (expressed as percentages of 100 replications) are indicated at branch points. Bar, 2% sequence divergence.

cluster with *B. muris* (97.9%), *B. nesterenkovi* (97.7%), *B. rhamnosum* (97.7%), *B. sacelli* (97.6%), *B. fresconis* (97.5%) and *B. paraconglomeratum* (97.1%). Sequence similarities between strain JB^T and other type strains of members of the genus *Brachybacterium* were in the narrow range 96.1–96.7%; sequence similarity to another species of the family *Dermabacteraceae*, *Dermabacter hominis* DSM 7083^T, was 93.6% (bootstrap value of 100%). However, the precise position of strain JB^T within the genus is not very clear since the branching order among strain JB^T, *B. muris* and *B. rhamnosum* was not supported by a high bootstrap value (<50%).

The 16S rRNA gene sequence of strain JB^T showed similarities of 97.7 and 97.9% to those of *B. nesterenkovi* DSM 9573^T and *B. muris* DSM 15460^T, respectively, so IS-PCR fingerprinting patterns of three strains were made to distinguish them more precisely (Fig. 3). The three strains had 2–4 fragments between 250 bp and 2000 bp and the pattern for strain JB^T differed distinctly from those of strains DSM 9573^T and DSM 15460^T. This difference in genomic organization supports classification of strain JB^T as a representative of a novel species of the genus *Brachybacterium*.

The G+C content of the genomic DNA of strain JB^T was 71.2±1.7 mol%, which is in the range expected for members of the genus *Brachybacterium* (68–72 mol%).

It is necessary to perform DNA–DNA hybridization to judge whether a novel isolate belongs to a species when the

Fig. 3. IS-PCR fingerprinting patterns on 1.2% agarose gel electrophoresis of strain JB^T (lane 1), *B. nesterenkovi* DSM 9573^T (lane 2) and *B. muris* DSM 15460^T (lane 3). Lane M, molecular mass marker (DME-50).

isolate and species representatives share more than 97% 16S rRNA gene sequence similarity (Stackebrandt & Goebel, 1994). Thus, six species (*B. muris*, *B. sacelli*, *B. nesterenkovi*, *B. rhamnosum*, *B. fresconis* and *B. paraconglomeratum*) were selected for DNA–DNA hybridization. Levels of DNA–DNA relatedness at optimal temperatures among these related species were 41–54% (Table 2). All DNA–DNA relatedness values were below the threshold value that has been suggested as delineating a bacterial species (approx. 70%) under optimal hybridization conditions (T_m of 25 °C) (Grimont, 1999; Wayne *et al.*, 1987). This confirmed the view that strain JB^T represents a novel species of the genus *Brachybacterium*.

Table 2. DNA–DNA relatedness (%) between strain JB^T (*Brachybacterium zhongshanense* sp. nov.), *B. muris*, *B. sacelli*, *B. nesterenkovi*, *B. rhamnosum*, *B. fresconis* and *B. paraconglomeratum*

E. coli K-12 was used as a negative control; DNA–DNA relatedness between strain JB^T and *E. coli* K-12 was 8±0.8%.

Type strain	DNA–DNA relatedness with strain JB ^T (%)
JB ^T	100
<i>B. muris</i> DSM 15460 ^T	54±3.2
<i>B. sacelli</i> LMG 20345 ^T	50±0.5
<i>B. nesterenkovi</i> DSM 9573 ^T	48±1.1
<i>B. rhamnosum</i> LMG 19848 ^T	46±2.9
<i>B. fresconis</i> LMG 20336 ^T	43±5.7
<i>B. paraconglomeratum</i> LMG 19861 ^T	41±3.6

Description of *Brachybacterium zhongshanense* sp. nov.

Brachybacterium zhongshanense (zhong.shan.en'se. N.L. neut. adj. *zhongshanense* pertaining to Zhongshan, a city in China, from where the type strain was isolated).

Cells vary in shape from coccoid forms (irregular) in the stationary phase to short rods in the exponential phase. Cells are non-motile, Gram-positive and do not form endospores. Colonies on LB plates are cream-coloured, smooth, glistening and grow well in an anaerobic chamber on LB plates. Temperature range for good growth is 25–40 °C; no growth occurs at 4 or 45 °C. Grows in 0–10% (w/v) NaCl. Grows well at pH 5–8 and weakly at pH 9–11; no growth is observed at pH 12–14. Catalase and urease are produced. Oxidase is not produced. Aesculin and gelatin are hydrolysed, but starch is not. Nitrate is reduced to nitrite. Indole is not produced. Positive for arginine dihydrolase and denitrification. Acid is produced from glucose. Utilizes the following carbon sources: α -cyclodextrin, β -cyclodextrin, dextrin, glycogen, inulin, amygdalin, L-arabinose, arbutin, D-cellobiose, D-fructose, D-galactose, D-galacturonic acid, gentiobiose, α -D-glucose, α -D-lactose, lactulose, maltose, maltotriose, D-mannose, D-melezitose, D-melibiose, methyl α -D-galactoside, methyl β -D-galactoside, methyl β -D-glucoside, palatinose, D-psicose, D-raffinose, stachyose, sucrose, D-tagatose, trehalose, turanose, xylitol, D-xylose, acetic acid, L-lactic acid, pyruvic acid methyl ester, pyruvic acid, glycerol, adenosine, inosine and thymidine. Predominant fatty acids are anteiso-C_{17:0} and anteiso-C_{17:0}.

The type strain is JB^T (=LMG 23926^T=CGMCC 1.6508^T=DSM 18832^T), which was isolated from sediments along the Qijiang River, Zhongshan City, China. The DNA G+C content of strain JB^T is 71.2 mol%.

Acknowledgements

The authors acknowledge the financial support of the funds of Chinese National Programs for High Technology Research and Development (2006106Z3063) and Guangdong Provincial Natural Science Fund (no. 015017).

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