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Isolation and characterization of two novel ethanol-tolerant facultative-anaerobic thermophilic bacteria strains from waste compost

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Abstract In a search for potential ethanologens, waste compost was screened for ethanol-tolerant thermophilic microorganisms. Two thermophilic bacterial strains, M5EXG and M10EXG, with tolerance of 5 and 10% (v/v) ethanol, respectively, were isolated. Both isolates are facultative anaerobic, non-spore forming, non-motile, catalase-positive, oxidase-negative, Gram-negative rods that are capable of utilizing a range of carbon sources including arabinose, galactose, mannose, glucose and xylose and produce low amounts of ethanol, acetate and lactate. Growth of both isolates was observed in fully defined minimal media within the temperature range 50–80°C and pH 6.0–8.0. Phylogenetic analysis of the 16S rDNA sequences revealed that both isolates clustered with members of subgroup 5 of the genus *Bacillus*. G + C contents and DNA–DNA relatedness of M5EXG and M10EXG revealed that they are strains belonging to *Geobacillus thermoglucosidasius*. However, physiological and biochemical differences were evident when isolates M5EXG and M10EXG were compared with *G. thermoglucosidasius* type strain (DSM 2542^T). The new thermophilic, ethanol-tolerant strains of *G. thermoglucosidasius* may be candidates for ethanol production at elevated temperatures.

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Introduction

Growing concerns on environmental issues and finite fossil fuel supplies have stimulated an increasing interest in microbial fuel ethanol production using renewable raw materials (Olsson and Hahn-Hagerdal 1996; Wheals et al. 1999; Wyman 1999; Cook and Beyea 2000; Zaldivar et al. 2001; Galbe and Zacchi 2002; Dien et al. 2003; Doi 2003; Demain et al. 2005). More specifically, elevated temperature fermentation for ethanol production has received increased attention in recent years due to the potential use of thermophilic microorganisms as biocatalysts (Edwards 1990; Klapatch et al. 1994; Banat and Marchant 1995; Banat et al. 1998; Sommer et al. 2004). The ability to utilize a wide range of sugars, including pentoses, at high temperature by most thermophilic microorganisms (Larsen et al. 1997) renders them potential hosts for ethanol production from cheap lignocellulosic materials (Olsson and Hahn-Hagerdal 1996). Lignocellulosic materials, which account for approximately 50% of the biomass in the world, are comprised of cellulose, hemicellulose and lignin, and with the exception for lignin, can be converted to hexose and pentose sugars via pretreatment and enzymatic hydrolysis (Lynd 1990; Kuhad and Singh 1993; Gong et al. 1999; Ingram et al. 1999). The sustainable provision and low cost of lignocellulosic agricultural waste (Wheals et al. 1999; Zaldivar et al. 2001) has further potential economic production of ethanol employing microbial systems.

Various thermophilic microorganisms capable of producing low amounts of ethanol have been isolated and characterized in the past two decades from different environments, including farm soils, sewage plants, riverbanks, thermal springs, sediments, as well as waste composts, with the intention of their evaluation and development for large-scale ethanol production. These bacteria include *Thermoanaerobacter ethanolicus* (Kannan and Mutharasan 1985; Wiegel and Ljungdahl

1986), *Thermoanaerobacter thermohydrosulfuricus* (Wiegel et al. 1979), *Thermoanaerobacter mathranii* (Larsen et al. 1997), *Thermoanaerobacter brockii* (Zeikus et al. 1979; Lamed and Zeikus 1980a; Sonnleitner et al. 1984) (formerly *Thermoanaerobium brockii*) (Lee et al. 1993), *Clostridium thermosaccharolyticum* (Vancanneyt et al. 1987a, b) and *C. thermocellum* (Herrero and Gomez 1980; Lamed and Zeikus 1980b; Ng et al. 1981).

In the search for potential thermophilic ethanologens for subsequent genetic manipulation, waste compost was screened in our laboratory, resulting in the isolation of 17 bacterial strains. The present paper reports the isolation, morphology, physiological, biochemical and phylogenetic analyses of two of these strains, M5EXG and M10EXG, which demonstrated high ethanol tolerance.

Materials and methods

Source and isolation of bacterial strains

Compost samples were collected from the University of New South Wales Waste-Composting Unit, Sydney, Australia. The samples were obtained at a depth of 30 cm below the surface of a 3-week old composting heap. The temperature at the point of sampling was between 60 and 65°C. Compost samples were mixed with thermophile minimum medium (TMM) within 24 h upon collection under aerobic condition. TMM contained the following sterile solutions (per litre): Ten Salts Solution (TSS), 920 ml; 1 M MOPS (pH 8.2), 40 ml; 1 mM $FeSO_4$ in 0.4 M Tricine, 10 ml; 0.132 M K_2HPO_4, 10 ml; 0.953 M NH_4Cl, 10 ml; MilliQ water, 10 ml. TSS contained (per litre): NaCl, 5 g; Na_2SO_4, 1.47 g; $NaHCO_3$, 0.08 g; KCl, 0.25 g; KBr, 0.04 g; $MgCl_2{\cdot}6H_2O$, 1.87 g; $CaCl_2{\cdot}2H_2O$, 0.41 g; $SrCl_2{\cdot}6H_2O$, 0.008 g; H_3BO_3, 0.008 g; $NaNO_3$, 0.9 g. TSS was autoclaved, whereas the other constituents of TMM were filter sterilized. Compost solids and compost supernatants, which were used as inocula, were obtained after settling of solid compost particles. Strain isolation was carried out under aerobic condition in various agar media, including brain heart infusion medium (BHI) (Oxoid) with the addition of 1% (w/v) glucose, 1% (w/v) xylose, 5 or 10% (v/v) ethanol and TMM supplemented with 1% (w/v) glucose, 1% (w/v) xylose and with and without addition of 5 or 10% (v/v) ethanol. Ethanol was used to select for ethanol-tolerant thermophilic microorganisms. Tryptone Soya Broth (TSB) (Oxoid) was used in routine culture of *Geobacillus thermoglucosidasius* (DSM 2542^T) and the purified thermophilic isolates at 60°C under aerobic condition unless otherwise specified.

Phenotypic and physiological characterization

Gram-stains (Doetsch 1981) and Schaeffer-Fulton sporulation stains (Murray et al. 1994) were carried out on cells harvested at various stages of growth under aerobic condition. Gram-stains and sporulation stains were also carried out on M5EXG and M10EXG after 4 days of growth at both 60 and 80°C. Tests were carried out for the presence of catalase and oxidase as described previously (Smibert and Krieg 1981). Motility was assessed by testing the ability of the strains to migrate away from the point of inoculation on TSB agar medium. Antibiotic sensitivity tests (MIC) and ethanol tolerance experiments were carried out in TSB broth, at 60°C with 200 rpm orbital shaking for 2 days under aerobic condition. Sealed glass culture tubes were used in ethanol tolerance experiments to prevent evaporation of ethanol and ethanol tolerance (cultures maintaining viability) was determined in TSB broth containing various concentrations of ethanol. Sugar utilization experiments were carried out in TTV medium [TMM supplemented with trace elements and vitamins (Eguchi et al. 1996)] with the addition of 1% (w/v) of arabinose, galactose, glucose, lactose, maltose, mannitol, mannose, sucrose or xylose as a sole carbon source at 60°C with 200 rpm orbital shaking for 2 days. Inocula for Erlenmeyer flask studies were prepared in 20 ml TSB broth, inoculated with a single colony from TSB agar plate culture, grown overnight at 60°C, 200 rpm. Growth was monitored by measuring the optical density (OD) of the liquid cultures at 660 nm. Temperature (50–85°C) and pH (6.0–8.0) growth effects were determined in TSB broth. Media were adjusted with 50% (v/v) HCl or 10 N NaOH to achieve the desired initial pH. Anaerobic growth experiments were carried out in TSB broth at 60°C with 200 rpm orbital shaking in an anaerobic jar containing an anaerobic gas pack (Oxoid). End products were analysed from regular samples (1 ml) taken from cultures grown in 500 ml Erlenmeyer flasks containing 100 ml of TGTV at 60°C with 200 rpm orbital shaking under aerobic conditions. TGTV contained TMM supplemented with trace elements and vitamins and the addition of 1% (w/v) glucose. Cells were pelleted and supernatants were analysed using high-pressure liquid chromatography (HPLC) with an HPX-87H ion-exchange column (Bio-Rad) and 5 mM H_2SO_4 as the mobile phase.

Electron microscopy

Transmission electron microscopy (TEM) on whole-cell and thin-sections was performed on cultures grown overnight at 60°C in TGTV medium. Cultures for whole-cell TEM were negatively stained with uranyl acetate for 5 min. TEM on thin-sections was carried out on cells fixed with 2.5% glutaraldehyde (in 0.1 M sodium cacodylate buffer) for 2 min, 2% aqueous sodium acetate for 2 min, 2% uranyl acetate (in 50% ethanol) for 2 min, dehydrated with ethanol of increasing concentrations (50, 75, 95 and 100% v/v), washed with acetone, and embedded in epoxy resin. TEM microscopy was performed using a Hitachi H 7000 Transmission Electron Microscope.

16S rDNA sequencing

Total genomic DNA was extracted using a modified C-TAB method (Ausubel et al. 1994). Prior to the addition of 10 mg/ml proteinase K, the cells were incubated with 10 mg/ml of lysozyme for 1 h at 37°C. The 16S rDNA was amplified from extracted genomic DNA using primers 27F1 (UFP), 1494Rc (URP) (Neilan et al. 1997). Thermal cycling was performed at 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 60°C for 20 s and 74°C for 40 s, and a final extension step of 74°C for 2 min. PCR products were purified using the Wizard® DNA Clean-Up System (Promega) and the nucleotide sequences determined using BigDye® Terminator V3.1 Cycle Sequencing Kit and an ABI 373A sequencer (Applied Biosystems). The 16S rDNA universal primers 27F1 (UFP) and 1494Rc (URP) and the thermal cycling conditions used for sequencing have been previously described (Neilan et al. 1997).

Phylogenetic analysis

Alignment of the DNA sequences was performed with the program PILEUP (Wisconsin Genetics Computer Group) and manual confirmation of the sequence alignment was checked against both primary- and secondary-structure considerations of the 16S rRNA molecule. The aligned sequences were studied using genetic distance and maximum-parsimony methods for phylogenetic inference. Ambiguous characteristics, where a deletion, insertion, or unidentified state was recorded for any strain, were removed from the aligned data. For all multiple sequence alignments and phylogenetic inference programs, the input order of taxa was randomized. Pairwise evolutionary distances were calculated as described by Jukes and Cantor (1969). The neighbour-joining method of phylogenetic reconstruction (Saitou and Nei 1987) was applied to the data using the programs DNADIST, NEIGHBOR, CONSENSE and SEQBOOT, which are part of the PHYLIP package (version 3.57c) (Felsenstein 1989). All sequence manipulation and phylogeny programs were made available by the Australian National Genome Information Service (ANGIS).

G + C content and DNA hybridization studies

The G + C content of the genome and DNA–DNA relatedness were determined according to methods described previously (Bowman et al. 1998), with the addition of lysozyme (1 mg/ml) and incubation at 37°C for 2 h during DNA extraction. Mol% G + C values are the average of three replicates, DNA hybridization values are the average of four or five replicates.

Bacterial culture stock and nucleotide sequence accession numbers

The bacterial strains B10EXG, C1, M5EXG and M10EXG have been deposited in the Culture Collection of The University of New South Wales (World Type Culture Collection Number 248 UNSW) under the accession numbers UNSW 097800, UNSW 098200, UNSW 098000 and UNSW 097900, respectively, as well as in American Type Culture Collection (ATCC) under the accession numbers ATCC BAA-1068, ATCC BAA-1070, ATCC BAA-1069 and ATCC BAA-1067, respectively. Bacterial strains M5EXG and M10EXG have also been deposited in the Bacillus Genetic Stock Center (BGSC) under the accession numbers W9A43 and W9A44, respectively (Zeigler 2005). The nearly full-length 16S rDNA sequences of B10EXG, C1, M5EXG and M10EXG have been deposited in the GenBank database under the accession numbers AF537293, AF537294, AF537295 and AF537296, respectively.

Results

Isolation and cultivation

A total of 17 thermophilic isolates were obtained through enrichment cultures set up with compost solids and supernatants and incubated at 60°C for 2 days in various agar media under aerobic condition. Single colonies were identified and pure cultures were isolated by repeated streaking on BHI agar plates incubated at 60°C under aerobic condition. Isolates M5EXG and M10EXG originated from compost supernatant on TMM supplemented with 1% (w/v) glucose, 1% (w/v) xylose, and 5 and 10% (v/v) ethanol, respectively. Strain B10EXG was isolated from compost supernatant on BHI supplemented with 1% (w/v) glucose, 1% (w/v) xylose and 10% (v/v) ethanol, whereas isolate C1 was from compost solids grown on TMM supplemented with 1% (w/v) glucose and 1% (w/v) xylose. During preliminary screening, all four isolates produced small quantities of ethanol and were subsequently included in the phylogenetic analysis of the present study. However, in preliminary conjugation experiments isolates B10EXG and C1 did not yield transconjugants (unpublished observations) and these isolates were not studied as comprehensively as M5EXG and M10EXG.

16S rDNA sequence and phylogenetic analyses

The levels of similarity of the nearly full-length 16S rDNA sequences (27-1494 in *Escherichia coli* numbering) of isolates M5EXG, M10EXG, B10EXG, C1 and their closest phylogenetic relatives are shown in Table 1. Evolutionary distance and bootstrap analyses showed that both isolates M5EXG and M10EXG clustered with

Table 1 Levels of 16S rDNA (%) similarity between isolates M5EXG, M10EXG, B10EXG, C1 and their closest phylogenetic relatives

Strain	1	2	3	4	5	6	7
1. M5EXG (AF537295)	100						
2. M10EXG (AF537296)	98.7	100					
3. *Geobacillus thermoglucosidasius* (AB021197)	99.5	98.9	100				
4. B10EXG (AF537293)	89.5	89.1	89.9	100			
5. *Bacillus* sp. S13 (AF403022)	89.7	89.3	90.1	99.3	100		
6. C1 (AF537294)	96.8	96.4	97.4	89.7	89.8	100	
7. *Geobacillus caldoxylosilyticus* (AF067651)	96.8	96.4	97.3	89.7	89.7	98.9	100

members of *Bacillus* subgroup 5 (Fig. 1) and were most-closely related to *G. thermoglucosidasius* (AB021197) (Claus and Berkeley 1986; Fortina et al. 2001; Nazina et al. 2001) having 16S rDNA sequence similarities of 99.5 and 98.9%, respectively. Isolate C1 also phylogenetically grouped with members of *Bacillus* subgroup 5 [closest relative is *Geobacillus caldoxylosilyticus* (AF067651) (Ahmad et al. 2000; Fortina et al. 2001)] with 98.9% 16S rDNA sequence similarity whereas isolate B10EXG clustered with members of the subgroup 2 *Bacillus* [most closely related to *Bacillus* sp. S13 (AF403022) (Gagne et al. 2001)] with 99.3% 16S rDNA sequence similarity. Together, these results suggest that M5EXG, M10EXG, B10EXG and C1 belong to the genus *Bacillus*/*Geobacillus* and that M5EXG and M10EXG are likely to be different strains of the same species.

G + C content and genome relatedness

The values for the G + C content and DNA relatedness of isolates M5EXG, M10EXG, *G. thermoglucosidasius* (DSM 2542^T) and the control strain *Bacillus subtilis* (ATCC 6051^T) were determined. Isolates M5EXG and M10EXG shared high DNA–DNA similarity with each other (95%) as well as with *G. thermoglucosidasius* (DSM 2542^T) (86 and 81%, respectively). DNA relatedness between isolate M10EXG and the control *B. subtilis* (ATCC 6051^T) was low (10%). Values of the G + C content were significantly different when comparing the new isolates M5EXG [53.8 (±0.3) mol%] and M10EXG [54.0 (±0.2) mol%] to *B. subtilis* (ATCC 6051^T) [45.0 mol%] but were similar to that of *G. thermoglucosidasius* (DSM 2542^T) [53.5 (±0.4) mol%]. These results indicate that M5EXG and M10EXG are strains of *G. thermoglucosidasius*.

Morphological, biochemical and physiological characterization

The morphological, biochemical and physiological characteristics of isolates M5EXG and M10EXG are shown in Table 2. On TSB agar plates, both isolate M5EXG and M10EXG exhibited translucent tan/dark orange coloured colonies. The colonies of both isolates M5EXG and M10EXG were raised, dome-shaped, with an irregular lobate edge. Gram-stained cells from early, mid- and late growth phases showed that isolates B10EXG, M5EXG and M10EXG were Gram-negative, while isolate C1 was Gram-positive. M5EXG and M10EXG also stained Gram-negative after 4 days of growth at both 60 and 80°C. Under the conditions studied, spores were not observed in isolates M5EXG and M10EXG and they were non-motile, catalase-positive, and oxidase-negative. Both isolates M5EXG and M10EXG grew in TSB broth within the temperature range, 50–80°C, with optimum growth at 60°C. No growth was observed at 85°C. They also grew in media of pH range, 6.0–8.0, with an optimal pH of 7.0. The MICs of M5EXG and M10EXG for various antibiotics in TSB broth were as follows: 5 μg/ml of kanamycin and streptomycin, and 10 μg/ml of chloramphenicol.

Both M5EXG and M10EXG had identical substrate utilization profiles for the substrates tested (Table 2). They utilized galactose, glucose, maltose, mannose, sucrose and xylose but grew poorly in media containing arabinose, lactose and mannitol as a sole carbon source. Growth was observed for both isolates at 60°C in rich TSB medium under aerobic and anaerobic conditions. Major end products identified in cultures of both isolates M5EXG and M10EXG under aerobic conditions in TGTV medium (10% w/v glucose) include ethanol (0.1 and 0.4 g/l, respectively), acetic acid (0.1 and 0.9 g/l, respectively) and lactic acid (0.4 and 2.5 g/l, respectively) (Table 3). In TSB at 60°C, both isolates M10EXG and B10EXG were tolerant (maintain viability) to 10% (v/v) ethanol, while M5EXG and C1 showed tolerance to 5 and 4% (v/v) ethanol, respectively.

Transmission electron microscopy of stationary phase whole-cells revealed that both isolates M5EXG and M10EXG were short rods, 0.6–0.8 μm wide, 3.1–5.0 μm in length and 0.6–1.0 μm wide, 2.8–3.1 μm in length, respectively (Fig. 2). TEM of thin-sections also revealed the Gram-negative two layer-structures of M5EXG and M10EXG cell envelopes (Fig. 2c, d inserts).

Discussion

The phylogenetic analysis, based on the 16S rDNA sequences of B10EXG, C1, M5EXG and M10EXG,

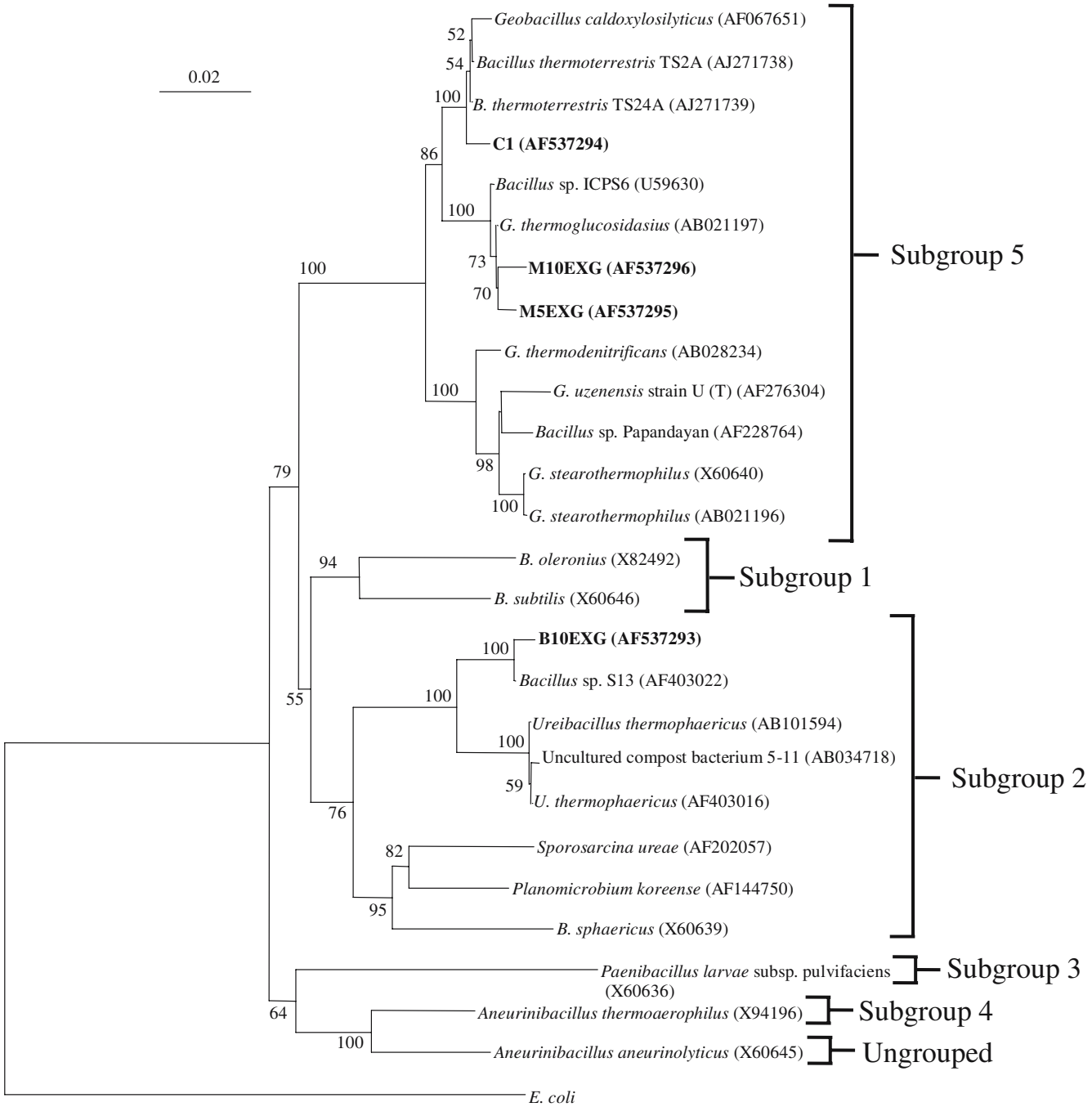

Fig. 1 Phylogenetic tree based on 16S rDNA sequence analyses, showing relationships of isolates M5EXG, M10EXG, B10EXG and C1 amongst members of the *Bacillus*. Database accession numbers are shown in parentheses after species, strain, or clone name. Bootstrap values greater than 50% are shown at the nodes. *Bar* indicates a distance of 0.02 nucleotide difference per sequence position

showed that these isolates were phylogenetically related to the Gram-positive/Gram-variable, rod-shaped, aerobic/facultative anaerobic and spore-forming genus *Bacillus* (Table 1, Fig. 1). A recent report also placed isolates M5EXG and M10EXG in the same cluster as strains of *G. thermoglucosidasius* based on their 16S rRNA and *recN* gene sequences (Zeigler 2005). It is not entirely surprising that the Gram-negative isolate B10EXG and the Gram-negative and non-spore forming isolates M5EXG and M10EXG were phylogenetically positioned among species of *Bacillus*/*Geobacillus* as several non-spore-forming organisms such as *Planococcus citreus* and *Filibacter limicola* (Ash et al. 1991) and the Gram-negative bacterium *Bacillus oleronius*

Table 2 Morphological, physiological and biochemical characteristics of isolates M5EXG, M10EXG, *Geobacillus thermoglucosidasius* (DSM 2542^T) and the type species of the five sub-groups of the genus *Bacillus*

Characteristics[b]	Strain[a]							
	1	2	3	4	5	6	7	8
Morphology								
Colour of colonies	Translucent tan/dark orange	Translucent tan/dark orange	Translucent brown	Dull, opaque, cream-coloured or brown	ND	Un-pigmented, translucent to opaque, cream-coloured or light brown	Translucent creamy greyish	ND
Cell shape	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods
Cell size								
Width (μm)	0.6–0.8	0.6–1.0	0.5–1.2	0.7–0.8	0.6–1.0	0.3–0.6	1.0–1.2	0.6–1.0
Length (μm)	3.1–5.0	2.8–3.1	3.0–7.0	2.0–3.0	1.5–5.0	1.5–3.0	3.5–5.5	2.0–3.5
Gram staining	–	–	+	+	V	+	+	V
Motility	–	–	+	+	+	+	+	+
Spore formation	–	–	+	+	+	+	+	+
Physical growth conditions								
Temperature range (°C)	50–80	50–80	37–68	10–50	10–40	20–45	40–60	37–65
pH range	6.0–8.0[c]	6.0–8.0[c]	6.0–8.0	5.5–8.5	ND	ND	7.0–8.0	6.0–8.0
Anaerobic growth	+	+	–	–	–	+	–	–
Aerobic growth	+	+	+	+	+	+	+	+
Biochemical pattern								
Catalase	+	+	+	+	+	–	V	V
Sugars utilized								
Arabinose	±	±	–	+	–	ND	–	V[d]
Galactose	+	+	V[d]	ND	ND	+	–	–
Glucose	+	+	+	+	–	+	+	+
Lactose	±	±	–	–	ND	ND	–	–
Maltose	+	+	+	ND	ND	+	–	+
Mannitol	±	±	+	+	–	+	+	V[d]
Mannose	+	+	+	ND	ND	+	–	ND
Sucrose	+	+	+	ND	ND	ND	–	ND
Xylose	+	+	+	+	–	ND	+	V[d]

[a]Taxa are identified as: *1* M5EXG, *2* M10EXG, *3 Geobacillus thermoglucosidasius* (DSM 2542^T) (Claus and Berkeley 1986; Fortina et al. 2001; Nazina et al. 2001), *4 Bacillus subtilis* (ATCC 6051^T) (subgroup 1) (Gibson and Gordon 1974; Claus and Berkeley 1986; Roberts et al. 1996), *5 B. sphaericus* (ATCC 14577^T) (subgroup 2) (Gibson and Gordon 1974; Claus and Berkeley 1986), *6 B. pulvifaciens* (ATCC 13537) (subgroup 3) (Katznelson 1950; Gibson and Gordon 1974; Claus and Berkeley 1986), *7 Aneurinibacillus thermoaerophilus* (DSM 10154^T) (subgroup 4) (Heyndrickx et al. 1997) and *8 Geobacillus stearothermophilus* (DSM 22^T) (subgroup5) (Gibson and Gordon 1974; Claus and Berkeley 1986; Nazina et al. 2001)

[b]+ positive, – negative, *V* variable results, ± poor growth, *ND* no data

[c]Growth response at highest and lowest pH tested

[d]11–89% of strains are positive (Nazina et al. 2001)

(Kuhnigk et al. 1995) have been reported to be closely related to some *Bacillus* species (Fortina et al. 2001; Nazina et al. 2001). As preliminary conjugation experiments only resulted in transconjugants from M5EXG and M10EXG (unpublished data), these isolates were further characterized. For comparison, B10EXG and C1 were included in the phylogenetic analyses.

It has been suggested that organisms with more than 97.5% similarity in their 16S rDNA sequences are likely to be members of the same species (Stackebrandt and Goebel 1994). Furthermore, DNA–DNA similarity of ≥70% is used to place bacteria into the same species (Amann and Schleifer 2001) while bacteria with DNA–DNA similarity of less than 60% should be considered as genetically independent (Johnson 1984; Ezaki et al. 1988). As such, isolates M5EXG and M10EXG are likely different strains of *G. thermoglucosidasius* based on their 16S rDNA sequence similarity and DNA relatedness (Table 1). Both isolates exhibit identical sugar utilization profiles and growth conditions for the sugars tested but show some differences in the levels of ethanol tolerance and end products formation (Tables 2, 3). Both isolates also appear as identical rods under TEM with similar cell size dimensions (Fig. 2). Base on 16S rDNA similarities, isolate B10EXG may be a different strain of *Bacillus* sp. S13 of the genus *Ureibacillus* (Gagne et al. 2001) while isolate C1 is likely to be a new strain of *G. caldoxylosilyticus*.

Although the values of 16S rDNA sequence similarity, G+C content and DNA–DNA relatedness of isolates M5EXG and M10EXG are very similar to those of *G. thermoglucosidasius* (DSM 2542^T), several differences in their biochemical and physiological characteristics were observed. These include Gram staining, spore formation, ability for anaerobic

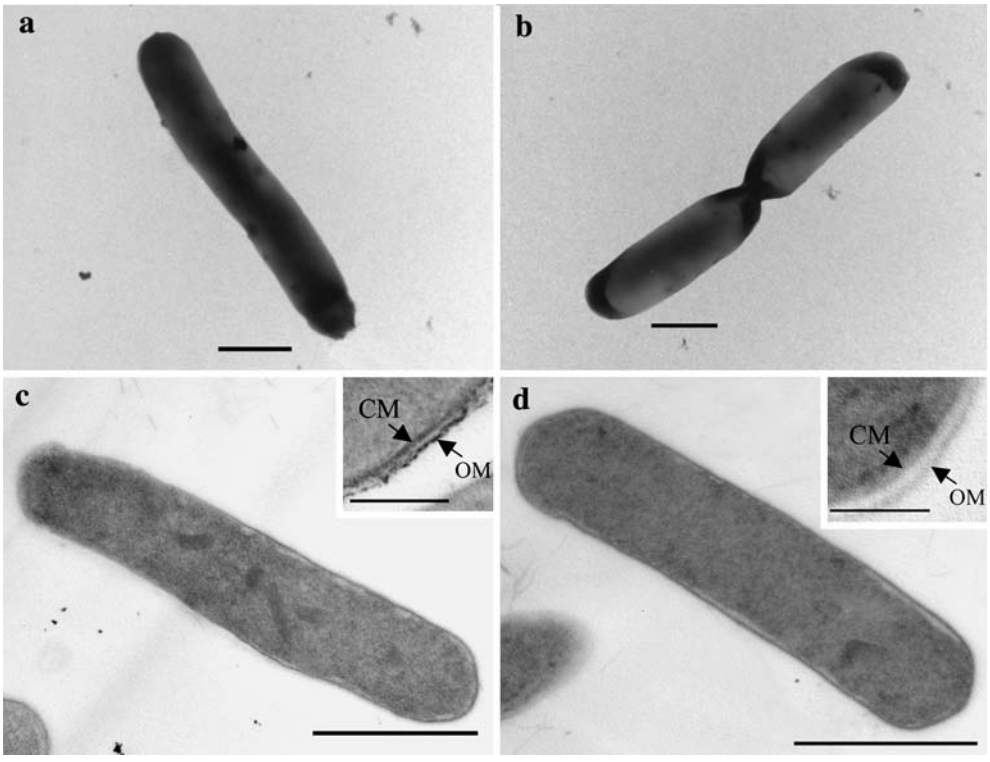

Fig. 2 Transmission electron micrographs of negatively stained whole-cell of M5EXG (**a**) and M10EXG (**b**) and thin-sectioned M5EXG (**c**) and M10EXG (**d**). Cell envelope structures of M5EXG and M10EXG are shown in the inserts. *CM* cytoplasmic membrane, *OM* outer membrane. *Bars* represent 1 (**a**–**d**) and 0.2 μm (*inserts*)

growth, growth temperature range as well as sugar utilization profile (Table 2). As such, it is obvious that isolates M5EXG and M10EXG are different when compared to the type strain (DSM 2542^T) of *G. thermoglucosidasius*. Similar differences between isolates M5EXG and M10EXG and the type species of the five subgroups of *Bacillus* were also evident. Most obvious differences include growth temperature range, motility and spore formation (Table 2). While current phylogenetic convention places isolates M5EXG and M10EXG in subgroup 5 of Bacillus as strains of *G. thermoglucosidasius* (DSM 2542^T), data presented in this study clearly highlighted the need for a more satisfactory system in phylogenetic classification of members of the phylum Bacillus. Such observation has also been noted by others (Ash et al. 1991; Zeigler 2005).

Unlike most thermophilic bacteria isolated to date, isolates M5EXG and M10EXG are tolerant (maintained viability) to high levels of ethanol (5 and 10% v/v, respectively) in TSB at 60°C (Table 3). Ethanol tolerance in wild-type thermophilic microorganisms has been reported to be relatively low (1–5%) (Table 3) although ethanol tolerance as high as 8% (Wiegel 1980) has been reported for *Clostridium thermocellum* ethanol-tolerant mutants. Reports to date on ethanol tolerance levels are also limited to obligate anaerobes. In addition to low ethanol tolerance, organic acids production in microorganisms also hinders their development into efficient ethanologens. This formation of metabolic by-products in wild-type thermophilic microorganisms is unavoidable. Major end products detected in cultures of isolates M5EXG and M10EXG were acetic and lactic acids, within the range of reported values of other thermophilic ethanologens (Table 3). Low amounts of ethanol were also detected in aerobic cultures of both isolates M5EXG and M10EXG. Increased ethanol yields in mutants lacking lactate dehydrogenase have been suggested (Lamed and Zeikus 1980a; Payton and Hartley 1985) and achieved in *G. stearothermophilus* (Payton and Hartley 1985). Generations of ethanol-producing mesophilic bacterial strains including lactic acid bacteria (Gold et al. 1996; Nichols et al. 2003), *Escherichia coli* (Ingram et al. 1987; Neale et al. 1988) and cyanobacteria (Deng and Coleman 1999) via the expression of *Zymomonas mobilis* enzymes pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) have been reported. Recently, the expressions of PDC from *Z. mobilis* and *Zymobacter palmae* in the halophilic archaea *Heloferax volcanii* (Kaczowka et al. 2005) and *Lactococcus lactis* (Liu et al. 1988), respectively, have also been reported. It may be possible to apply similar strategies to isolates M5EXG and M10EXG in developing them as more efficient ethanologens for ethanol production at elevated temperatures.

We report here the isolation and characterization of two new thermophilic, ethanol-tolerant strains of *G. thermoglucosidasius* (strains M5EXG and M10EXG) from waste compost that may be candidates for ethanol production at elevated temperatures.

Table 3 Characteristics of M5EXG, M10EXG and other potential thermophilic ethanologens

Characteristics[b,c]	Strain[a]								
	1	2	3	4	5	6	7	8	9
Aerobe or anaerobe	Facultative anaerobe	Facultative anaerobe	Obligate anaerobe	Obligate anaerobe	Obligate anaerobe	Obligate anaerobe	Obligate anaerobe	Obligate anaerobe	Obligate anaerobe
Gram staining	–	–	V	V	V	+	V	–	–
Temperature optimum (°C)	~60	~60	69	65	70	70	67–69	55–60	62–65
PH optimum	~7.0	~7.0	5.8–8.5	6.5–7.5	7.0	7.5	6.9–7.5	ND	6.4–7.4
Ethanol tolerance	5% (v/v)	10% (v/v)	1.4% (v/v)	2% (v/v)[d]	5% (v/v)	< 1%[e]	ND	5% (v/v)	5% (v/v)
Fermentation end products (g/l)									
Ethanol	0.1	0.4	3.6	2.0–2.5	0.8	1.0	1.3	0.2	0.7
Acetate	0.1	0.9	0.3	0.2–0.4	0.8	0.2–0.3	0.8	0.2	0.8–1.0
Lactate	0.4	2.5	0.4	0.4–0.5	0.2	1.5–3.2	1.5	0.1	0.2–0.3

[a]Taxa are identified as: *1* M5EXG, *2* M10EXG, *3 Thermoanaerobacter ethanolicus* JW200^T (ATCC 31550) (Wiegel and Ljungdahl 1981), *4 T. ethanolicus* 39E (ATCC 33223) (Zeikus et al. 1980; Ng et al. 1981) [formally *Clostridium thermohydrosulfuricum* (Lee et al. 1993)], *5 T. mathranii* A3^T (DSM 11426) (Larsen et al. 1997), *6 T. brockii* HTD4^T (ATCC 33075) (Zeikus et al. 1979; Lamed and Zeikus 1980a, b) [formally *Thermoanaerobium brockii* (Lee et al. 1993)], *7 T. thermohydrosulfuricus* E100-69^T (DSM 567) (Wiegel et al. 1979) [formally *Clostridium thermohydrosulfuricum* (Lee et al. 1993)], *8 Clostridium thermosaccharolyticum* strain 3814 (Lee and Ordal 1967) and *9 C. thermocellum* LQRI (Lamed and Zeikus 1980b; Ng et al. 1981)

[b]Data were also taken from (McBee 1950; Smith and Hobbs 1974; Sonnleitner 1983; Esser and Karsch 1984; Kannan and Mutharasan 1985; Lovitt et al. 1988)

[c]+ Gram-positive, – Gram-negative, *V* Gram-variable, *ND* No data

[d]1.6% (w/v) ethanol tolerance on glucose complex medium, 5% (w/v) on pyruvate medium (Lovitt et al. 1988)

[e]Cell toxicity occurred at ethanol concentration higher than 1% (Lamed and Zeikus 1980a)

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References

Ahmad S, Scopes RK, Rees GN, Patel BKC (2000) *Saccharococcus caldoxylosilyticus* sp. nov., an obligately thermophilic, xylose-utilizing, endospore-forming bacterium. Int J Syst Evol Microbiol 50:517–523

Amann R, Schleifer KH (2001) Nucleic acid probes and their application in environmental microbiology. In: Garrity GM, Boone DR, Castenholz RW (eds) Bergey's manual of systematic bacteriology. The William and Wilkins Co., Baltimore, pp 67–82

Ash C, Farrow JAE, Wallbanks S, Collins MD (1991) Phylogenetic heterogeneity of the genus *Bacillus* revealed by comparative analysis of small-subunit-ribosomal RNA sequences. Lett Appl Microbiol 13:202–206

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (1994) Preparation and analysis of DNA. In: Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (eds) Current protocols in molecular biology. Wiley, New York, pp 2.0.1–2.4.5

Banat IM, Marchant R (1995) Characterization and potential industrial applications of five novel, thermotolerant, fermentative, yeast strains. World J Microbiol Biotechnol 11:304–306

Banat IM, Nigam P, Singh D, Marchant R, McHale AP (1998) Review: ethanol production at elevated temperatures and alcohol concentrations: Part I. Yeasts in general. World J Microbiol Biotechnol 14:809–821

Bowman JP, McCammon SA, Brown JL, McMeekin TA (1998) *Glaciecola punicea* gen. nov., sp. nov. and *Glaciecola pallidula* gen. nov., sp. nov.: psychrophilic bacteria from Antarctic sea-ice habitats. Int J Syst Bacteriol 48:1213–1222

Claus D, Berkeley RCW (1986) Genus *Bacillus* Cohn 1872. In: Sneath PHA, Mair NS, Sharpe ME, Holt JG (eds) Bergey's manual of systematic bacteriology. The William and Wilkins Co., Baltimore, pp 1105–1139

Cook J, Beyea J (2000) Bioenergy in the United States: progress and possibilities. Biomass Bioenerg 18:441–455

Demain AL, Newcomb M, Wu JH (2005) Cellulase, clostridia, and ethanol. Microbiol Mol Biol Rev 69:124–154

Deng M, Coleman JR (1999) Ethanol synthesis by genetic engineering in cyanobacteria. Appl Environ Microbiol 65:523–528

Dien BS, Cotta MA, Jeffries TW (2003) Bacteria engineered for fuel ethanol production: current status. Appl Microbiol Biotechnol 63:258–266

Doetsch RN (1981) Determinative methods of light microscopy. In: Gerhardt P et al (eds) Manual of methods for general bacteriology. American Society for Microbiology, Washington, pp 21–33

Doi RH (2003) Microbial conversion of corn stalks to riches. J Bacteriol 185:701–702

Edwards C (1990) Thermophiles. In: Edwards C (eds) Microbiology of extreme environments. McGraw-Hill Publishing Co., New York, pp 1–32

Eguchi M, Nishikawa T, MacDonald K, Cavicchioli R, Gottschal J, Kjelleberg S (1996) Responses to stress and nutrient availability by the marine ultramicrobacterium *Sphingomonas* sp. strain RB2256. Appl Environ Microbiol 62:1287–1294

Esser K, Karsch T (1984) Bacteria ethanol production: advantages and disadvantages. Process Biochem 19:116–121

Ezaki T, Hashimoto Y, Takeuchi N, Yamamoto H, Liu S-L, Miura H, Matsui K, Yabuuchi E (1988) Simple genetic method to identify viridans group Streptococci by colorimetric dot hybridization and fluorometric hybridization in microdilution wells. J Clin Microbiol 29:1708–1713

Felsenstein J (1989) PHYLIP—phylogeny inference package (version 3.2). Cladistics 5:164–166

Fortina MG, Mora D, Schumann P, Parini C, Manachini PL, Stackebrandt E (2001) Reclassification of *Saccharococcus caldoxylosilyticus* as *Geobacillus caldoxylosilyticus* (Ahmand et al. 2000) comb. nov. Int J Syst Evol Microbiol 51:2063–2071

Gagne A, Chicoine M, Morin A, Houde A (2001) Phenotypic and genotypic characterization of esterase-producing *Ureibacillus thermosphaericus* isolated from an aerobic digestor of swine waste. Can J Microbiol 47:908–915

Galbe M, Zacchi G (2002) A review of the production of ethanol from softwood. Appl Microbiol Biotechnol 59:618–658

Gibson T, Gordon RE (1974) Genus I. *Bacillus* Cohn 1872. In: Buchanan RE, Gibbons NE (eds) Bergey's manual of determinative bacteriology. The William and Wilkins Co., Baltimore, pp 529–551

Gold RS, Meagher MM, Tong SX, Hutkins RW, Conway T (1996) Cloning and expression of the *Zymomonas mobilis* "production of ethanol" genes in *Lactobacillus casei*. Curr Microbiol 33:256–260

Gong CS, Gao NJ, Du J, Tsao GT (1999) Ethanol production from renewable resources. Adv Biochem Eng Biotechnol 65:207–241

Herrero AA, Gomez RF (1980) Development of ethanol tolerance in *Clostridium thermocellum*: effect of growth temperature. Appl Environ Microbiol 40:571–577

Heyndrickx M, Lebbe L, Vancanneyt M, Kersters K, Devos P, Logan NA, Forsyth G, Nazli S, Ali N, Berkeley RCW (1997) A polyphasic reassessment of the genus *Aneurinibacillus*, reclassification of *Bacillus thermoaerophilus* (Meier-Stauffer et al. 1996) as *Aneurinibacillus thermoaerophilus* comb. nov., and emended descriptions of *A. aneurinilyticus* corrig., *A. migulanus*, and *A. thermoaerophilus*. Int J Syst Bacteriol 47:808–817

Ingram LO, Conway T, Clark DP, Sewell GW, Preston JF (1987) Genetic engineering of ethanol production in *Escherichia coli*. Appl Environ Microbiol 53:2420–2425

Ingram LO, Aldrich HC, Borges ACC, Causey TB, Martinez A, Morales F, Saleh A, Underwood SA, Yomano LP, York SW, Zaldivar J, Zhou S (1999) Enteric bacterial catalysts for fuel ethanol production. Biotechnol Prog 15:855–866

Johnson JL (1984) Nucleic acids in bacterial classification. In: Krieg NR, Holt JG (eds) Bergey's manual of systematic bacteriology. The William and Wilkins Co., Baltimore, pp 8–11

Jukes TH, Cantor CR (1969) Evolution of protein molecules. In: Munro HN (eds) Mammalian protein metabolism. Academic, New York, pp 21–132

Kaczowka SJ, Reuter CJ, Talarico LA, Maupin-Furlow JA (2005) Recombinant production of *Zymomonas mobilis* pyruvate decarboxylase in the haloarchaeon *Haloferax volcanii*. Archaea 1:327–334

Kannan V, Mutharasan R (1985) Ethanol fermentation characteristics of *Thermoanaerobacter ethanolicus*. Enzyme Microb Technol 7:87–89

Katznelson H (1950) *Bacillus pulvifaciens* sp. nov., an organism associated with powdery scale of honeybee larvae. J Bacteriol 59:153–155

Klapatch TR, Hogsett DAL, Baskaran S, Pal S, Lynd LR (1994) Organism development and characterization for ethanol production using thermophilic bacteria. Appl Biochem Biotechnol 45–46:209–223

Kuhad RC, Singh A (1993) Lignocellulose biotechnology: current and future prospects. Crit Rev Biotechnol 13:151–172

Kuhnigk T, Borst E, Breunig A, Konig H, Collins MD, Hutson RA, Kampfer P (1995) *Bacillus oleronius* sp. nov., a member of the hindgut flora of the termite *Reticulitermes santonensis* (Feytaud). Can J Microbiol 41:699–706

Lamed R, Zeikus JG (1980a) Glucose fermentation pathway of *Thermoanaerobium brockii*. J Bacteriol 141:1251–1257

Lamed R, Zeikus JG (1980b) Ethanol production by thermophilic bacteria: relationship between fermentation product yields of the catabolic enzyme activities in *Clostridium thermocellum* and *Thermoanaerobium brockii*. J Bacteriol 144:569–578

Larsen L, Nielsen P, Ahring BK (1997) *Thermoanaerobacter mathranii* sp. nov., an ethanol-producing, extremely thermophilic anaerobic bacterium from a hot spring in Iceland. Arch Microbiol 168:114–119

Lee CK, Ordal ZJ (1967) Regulatory effect of pyruvate on the glucose metabolism of *Clostridium thermosaccharolyticum*. J Bacteriol 94:530–536

Lee Y, Jain MK, Lee C, Lowe SE, Zeikus JG (1993) Taxonomic distinction of saccharolytic thermophilic anaerobes: Description of *Thermoanaerobacterium xylanolyticum* gen. nov., sp. nov., and *Thermoanaerobacterium saccharolyticum* gen. nov., sp. nov.; reclassification of *Thermoanaerobium brockii*, *Clostridium thermosulfurogenes*, and *Clostridium thermohydrosulfuricum* E100-69 as *Thermoanaerobacter brockii* comb. nov., *Thermoanaerobacterium thermosulfurigenes* comb. nov., and *Thermoanaerobacter thermohydrosulfuricus* comb. nov., respectively; and transfer of *Clostridium thermohydrosulfuricum* 39E to *Thermoanaerobacter ethanolicus*. Int J Syst Bacteriol 43:41–51

Liu C, Goodman AE, Dunn NW (1988) Expression of cloned *Xanthomonas* D-xylose catabolic genes in *Zymomonas mobilis*. J Biotechnol 7:61–70

Lovitt RW, Shen G, Zeikus JG (1988) Ethanol production by thermophilic bacteria: biochemical basis for ethanol and hydrogen tolerance in *Clostridium thermohydrosulfuricum*. J Bacteriol 170:2809–2815

Lynd LR (1990) Large-scale fuel ethanol from lignocellulose. Appl Biochem Biotechnol 24–25:695–719

McBee RH (1950) The anaerobic thermophilic cellulolytic bacteria. Bacteriol Rev 14:51–63

Murray RGE, Doetsch RN, Robinow CF (1994) Determinative and cytological light microscopy. In: Gerhardt P, Murray RGE, Wood WA, Krieg NR (eds) Methods for general and molecular bacteriology. American Society for Microbiology, Washington, pp 21–41

Nazina TN, Tourova TP, Poltaraus AB, Novikova EV, Grigoryan AA, Ivanova AE, Lysenko AM, Petrunyaka VV, Osipov GA, Belyaev SS, Ivanov MV (2001) Taxonomic study of aerobic thermophilic bacilli: description of *Geobacillus subterraneus* gen. nov., sp. nov. and *Geobacillus uzenensis* sp. nov. from petroleum reservoirs and transfer of *Bacillus stearothermophilus, Bacillus thermocatenulatus, Bacillus thermoleovorans, Bacillus kaustophilus, Bacillus thermoglucosidasius* and *Bacillus thermodenitrificans* to *Geobacillus* as the new combinations *G. stearothermophilus, G. thermocatenulatus, G. thermoleovorans, G. kaustophilus, G. thermoglucosidasius* and *G. thermodenitrificans*. Int J Syst Evol Microbiol 51:433–446

Neale AD, Scopes RK, Kelly JM (1988) Alcohol production from glucose and xylose using *Escherichia coli* containing *Zymomonas mobilis* genes. Appl Microbiol Biotechnol 29:162–167

Neilan BA, Jacobs D, Deldot T, Blackall LL, Hawkins PR, Cox PT, Goodman AE (1997) rRNA sequences and evolutionary relationships among toxic and nontoxic cyanobacteria of the genus *Microcystis*. Int J Syst Bacteriol 47:693–697

Ng TK, Ben-Bassat A, Zeikus JG (1981) Ethanol production by thermophilic bacteria: fermentation of cellulosic substrates by cocultures of *Clostridium thermocellum* and *Clostridium thermohydrosulfuricum*. Appl Environ Microbiol 41:1337–1343

Nichols NN, Dien BS, Bothast RB (2003) Engineering lactic acid bacteria with pyruvate decarboxylase and alcohol dehydrogenase genes for ethanol production from *Zymomonas mobilis*. J Ind Microbiol Biotechnol 30:315–321

Olsson L, Hahn-Hagerdal B (1996) Fermentation of lignocellulosic hydrolysates for ethanol production. Enzyme Microb Technol 18:312–331

Payton MA, Hartley BS (1985) Mutants of *Bacillus stearothermophilus* lacking NAD-linked L-lactate dehydrogenase. FEMS Microbiol Lett 26:333–336

Roberts MS, Nakamura LK, Cohan FM (1996) *Bacillus vallismortis* sp. nov., a close relative of *Bacillus subtilis*, isolated from soil in Death Valley, California. Int J Syst Bacteriol 46:470–475

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Smibert RM, Krieg NR (1981) General characterization. In: Gerhardt P et al (eds) Manual of methods for general bacteriology. American Society for Microbiology, Washington, pp 409–443

Smith LDS, Hobbs G (1974) Genus III. *Clostridium* Prazmowski 1880. In: Buchanan RE, Gibbons NE (eds) Bergey's manual of determinative bacteriology. The William and Wilkins Co., Baltimore, pp 551–572

Sommer P, Georgieva T, Ahring BK (2004) Potential for using thermophilic anaerobic bacteria for bioethanol production from hemicellulose. Biochem Soc Trans 32:283–289

Sonnleitner B (1983) Biotechnology of thermophilic bacteria-growth, products, and application. Adv Biochem Eng Biotechnol 28:69–138

Sonnleitner B, Fiechter A, Giovannini F (1984) Growth of *Thermoanaerobium brockii* in batch and continuous culture at supra-optimal temperatures. Appl Microbiol Biotechnol 19:326–334

Stackebrandt E, Goebel BM (1994) Taxonomic note: a place for DNA–DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int J Syst Bacteriol 44:846–849

Vancanneyt M, De Vos P, De Ley J (1987a) Ethanol production from glucose by *Clostridium thermosaccharolyticum* strains: effect of pH and temperature. Biotechnol Lett 9:567–572

Vancanneyt M, De Vos P, Kersters K, De Ley J (1987b) Isolation characterization and identification of strictly anaerobic thermophilic ethanol producing bacteria from compost. Syst Appl Microbiol 9:293–298

Wheals AE, Basso LC, Alves DMG, Amorium HV (1999) Fuel ethanol after 25 years. TIBTECH 17:482–487

Wiegel J (1980) Formation of ethanol by bacteria. A pledge for the use of extreme thermophilic anaerobic bacteria in industrial ethanol fermentation processes. Experientia 36:1434–1446

Wiegel J, Ljungdahl LG (1981) *Thermoanaerobacter ethanolicus* gen. nov., spec. nov., a new, extreme thermophilic, anaerobic bacterium. Arch Microbiol 128:343–348

Wiegel J, Ljungdahl LG (1986) The importance of thermophilic bacteria in biotechnology. Crit Rev Biotechnol 3:39–108

Wiegel J, Ljungdahl LG, Rawson JR (1979) Isolation from soil and properties of the extreme thermophile *Clostridium thermohydrosulfuricum*. J Bacteriol 139:800–810

Wyman CE (1999) Biomass ethanol: technical progress, opportunities, and commercial challenges. Annu Rev Energy Environ 24:189–226

Zaldivar J, Nielsen J, Olsson L (2001) Fuel ethanol production from lignocellulose: a challenge for metabolic engineering and process integration. Appl Microbiol Biotechnol 56:17–34

Zeigler DR (2005) Application of a *recN* sequence similarity analysis to the identification of species within the bacterial genus *Geobacillus*. Int J Syst Evol Microbiol 55:1171–1179

Zeikus JG, Hegge PW, Anderson MA (1979) *Thermoanaerobium brockii* gen. nov. and sp. nov., a new chemoorganotrophic, caldoactive, anaerobic bacterium. Arch Microbiol 122:41–48

Zeikus JG, Ben-Bassat A, Hegge PW (1980) Microbiology of methanogenesis in thermal, volcanic environments. J Bacteriol 143:432–440