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ORIGINAL PAPER

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Isolation and characterization of a cellulolytic *Geobacillus thermoleovorans* T4 strain from sugar refinery wastewater

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Abstract A novel, cellulolytic, bacterial thermophilic strain, T4, was isolated from sugar refinery wastewater in southern Taiwan. This isolate, a Gram-negative, motile, aerobically growing sporulating rod, can secrete thermostable endocellulase (endo-1,4-β-D-glucanase, EC 3.2.1.4) and hydrolyze carboxymethylcellulose (CMC), phosphoric acid-swollen cellulose, Avicel, filter paper, and salicin. When strain T4 was grown in CMC medium, the cellulolytic enzyme activity in culture supernatants was stable up to 70°C. More than 10% of the original activity was still detectable after heating to 100°C with a pH 7.0 for 1 h. Based on 16S rDNA sequence analysis, DNA base composition, phenotypic and physiological characteristics, as well as DNA–DNA hybridization, strain T4 was classified as *Geobacillus thermoleovorans* T4 (DSM 14791 = CCRC 17200). We also demonstrated that the type species *G. stearothermophilus* (DSM 22 = ATCC 12980) could hydrolyze amorphous and crystalline (filter paper) celluloses at a rate of 13 and 14%, respectively, in comparison with strain T4.

Keywords Carboxymethylcellulose · Cellulolytic · Endocellulase · *Geobacillus thermoleovorans* · Hybridization · 16S rDNA · Thermostable

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Introduction

Cellulases have a wide range of industrial applications, such as the manufacture of textile, food, animal feed, and fuel. Because more than half of the industrial enzymatic reactions operate above 50°C, the isolation of cellulase from thermophilic bacterial strains is potentially valuable for industrial application (Godfrey and Reichelt 1983).

Based on polyphasic characteristics, Nazina et al. (2001) transferred some related thermophilic *Bacillus* group 5 (Ash et al. 1991) to the novel genus *Geobacillus* and presented two new species, *G. subterraneus* and *G. uzenensis*. The genus *Geobacillus* has a high 16S rDNA sequence homology within its members. Its major fatty acids are iso-fatty acids but differ in both quality and quantity among its species (Nazina et al. 2001). This chemotaxonomic profile was also supported in subsequent reports (Fortina et al. 2001; Sung et al. 2002).

The presence of cellulase activity in *Geobacillus* (formerly *Bacillus*) *stearothermophilus* has been suggested previously (Kalogridou-Vassiliadou 1992; Zahran et al. 1992). However, no thermostable endoglucanase activity was reported. In this study, thermophilic bacterial strains were isolated from sugar refinery wastewater. Based on 16S rRNA gene sequence analysis, these isolates have a close phylogenetic relationship with the genus *Geobacillus*. A novel isolate, T4, is very similar to *G. thermoleovorans* and is the first reported *Geobacillus* strain exhibiting thermostable endoglucanase activity.

Materials and methods

Media and growth conditions

For the cellulase enzyme activity assay, strain T4 and other strains were grown in a modified, chemically defined carboxymethylcellulose (CMC) medium (Mandels and Reese 1957) that consisted of 10 g CMC, 1 g

peptone, 1.4 g $(NH_4)_2$ SO_4, 0.3 g urea, 2 g KH_2 PO_4, 0.34 g $CaCl_2$, 0.3 g $MgSO_4 \cdot 7H_2O$, 0.005 g $FeSO_4 \cdot 7H_2O$, 0.0016 g $MnSO_4 \cdot H_2O$, 0.0014 g $ZnSO_4 \cdot 7H_2O$, and 0.002 g $CoCl_2 \cdot 6H_2O$ in 1 l distilled water (pH 7.0). For all other studies, all strains were incubated in nutrient broth or on nutrient agar plates. The substrates used were CMC, phosphoric acid-swollen cellulose, Avicel type PH-101, Whatman No. 1 filter paper, and salicin.

Organisms

The novel strain T4 was isolated from sugar refinery wastewater (55–60°C) in Kaohsiung, Taiwan. The sample was inoculated (3% v/v) into 200-ml vials containing sterilized distilled water and incubated in a water bath at 60°C for 3 days. Then the culture was subcultured several times under the same conditions in CMC medium. Cellulolytic thermophiles were isolated from CMC plates after growing at 60°C under aerobic conditions. The bacterial strain was then purified by repeated streaking onto CMC plates and further cultivating in liquid CMC culture at 60°C, pH 7.0. The purity of the isolate was checked microscopically. The type strain *Geobacillus thermoleovorans* (DSM 5366 = ATCC 43513) used in this study was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). *G. kaustophilus* (type strain ATCC 8005), *G. stearothermophilus* strains (ATCC 7953, ATCC 10149, ATCC 12976, and type strain DSM 22 = ATCC 12980) were purchased from the Culture Collection and Research Center (CCRC, Hsinchu, Taiwan).

Physiological tests

All assays were performed either in duplicates or triplicates. Adonitol and citrate utilization, nitrate reduction, aesculin and gelatin hydrolysis, tryptophan deamination, indole and H_2S formation, the Voges–Proskauer reaction, and carbohydrates fermentation were determined as described by Logan and Berkeley (1984) and Nazina et al. (2001). The β-galactosidase, arginine dihydrolase, and lysine and ornithine decarboxylases activities were determined by using API 20 E tests. Temperature and pH growth ranges were determined in nutrient broth.

16S rDNA sequencing, phylogenetic analysis, and strain identification

Two oligonucleotide primers, 27f and 1492r, were used to amplify 16S rDNA as described by Kim et al. (2000). The 1,454-nucleotide (nt) PCR product was then sequenced. The 16S rDNA sequence of the new isolate was aligned manually with the 16S rDNA sequences of the genus *Geobacillus* and related validly described members of the genus *Bacillus* extracted from GenBank databases and the Ribosomal Database Project. Pairwise evolutionary distances, based on 1,371 nt positions, were computed by the method of Jukes and Cantor (1969), and a neighbor-joining phylogenetic tree was inferred using the PHYLIP package (Felsenstein 1989). One thousand bootstrapped data sets were performed using SEQBOOT, which was implemented in PHYLIP programs. The 16S rRNA gene sequence of strain T4 had submitted to GenBank databases and had been assigned an accession number of AY074879. The accession numbers of the reference organisms used in the 16S rDNA sequences analysis are shown in Fig. 1. The G + C content of the purified DNA was identified by HPLC. DNA–DNA hybridizations were performed with photobiotin-labeled probes in microplate wells as described by Ezaki et al. (1989). Cellular fatty acids were analyzed by gas chromatography (Microbial ID, Newark, Del., USA).

Cellulolytic enzyme assay

Strain T4 was incubated (1% v/v) aerobically in 250-ml flasks containing CMC medium at 60°C in a rotary shaker (120 rpm). Cultures were centrifuged (8,000 *g*, 10 min) and the supernatants were used for enzyme activity assay. Crude cellulase activity was examined by a modified method of Acebal et al. (1986). Culture supernatant (0.5 ml) and 0.5 ml 0.1 M Na_2HPO_4–NaH_2PO_4 buffer, pH 7.0, containing 1% (w/v) substrate was incubated at 60°C for 1 h. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 μmol reducing sugar (expressed in glucose equivalents) per min (Miller 1959).

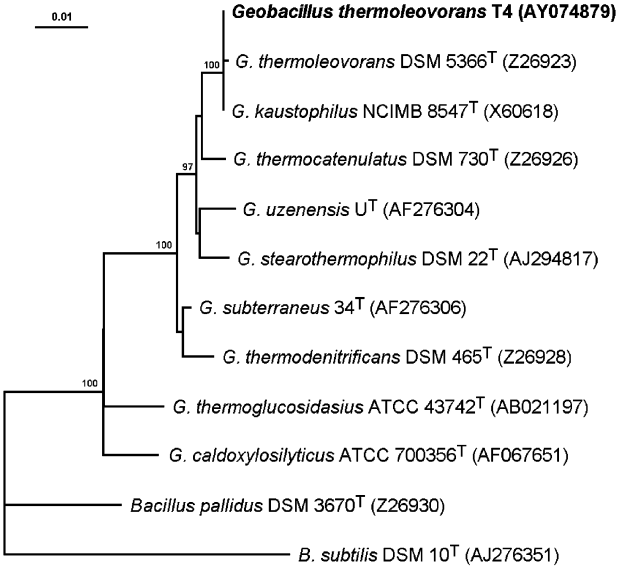

Fig. 1 Phylogenetic tree showing the evolutionary position of strain T4 among the genus *Geobacillus* and two related genus *Bacillus*. GenBank accession numbers are given in *parentheses*. The *bar* represents one substitution per 100 nucleotides. Bootstrap percentage values from 1,000 resamplings of the dataset have been included at *branch points*, but only for those that were statistically supported to 85% or more

Results and discussion

Morphological studies

The vegetative cells of strain T4 stained Gram negative, were rod-shaped, motile, single or short chain, and 0.7–1×1–5 μm in size. An ellipsoidal spore was located terminally or subterminally within a swollen sporulating cell. Colonies on nutrient agar plate were circular, mucous, convex, and white. The diameter of these colonies was slightly smaller than 1 mm.

Physiological characteristics

Strain T4 was a thermophilic, aerobically growing bacterium. Growth of this isolate was observed at 40–65°C (optimum: 55–60°C); no growth could be found at or above 70°C. The isolate grew well between pH 6.5 and 8.0, with optimum growth at pH 7.0–7.5. Other biochemical properties are listed in Table 1.

16S rDNA sequences

The 16S rDNA sequence of isolate T4 was compared with the sequences of nine *Geobacillus* and two related *Bacillus* strains (Fig. 1). Strain T4 was located in a monophyletic clade with *G. thermoleovorans* and *G. kaustophilus*, and this relationship was strongly supported by 100 bootstrap values. The degree of sequence similarity of strain T4 to *G. thermoleovorans* and *G. kaustophilus* was over 99.6%. This high homology suggested that *G. kaustophilus* be combined into the emended species *G. thermoleovorans* (Sunna et al. 1997). Other related species of the genus *Geobacillus*, such as *G. stearothermophilus* and *G. thermoglucosidasius*, exhibited 98.7 and 96.3% sequence similarity to strain T4, respectively.

Taxonomy

The DNA G + C content of strain T4 was 53.3 mol%, which is similar to *G. thermoleovorans* and *G. stearothermophilus*. The phenotypic and physiological characteristics of three *Geobacillus* species and the isolate T4 are demonstrated in Table 1. Using DNA–DNA hybridization, isolate T4 exhibits 76.7% similarity with *G. thermoleovorans* and 43.4% with the type species *G. stearothermophilus*. This result reaffirms that the isolate T4 is closely related to *G. thermoleovorans*.

From the cellular fatty acid analysis, we found that 13-methyl tetradecanoic acid (iso-15:0, 20.02%), 14-methyl pentadecanoic acid (iso-16:0, 47.47%), and 15-methyl hexadecanoic acid (iso-17:0, 13.74%) were the major components (81.23% of total) of strain T4. This result is very similar to the previous reports on *B. thermocloaceae*, *B. caldolyticus*, *B. caldovelox*, *B. caldotenax*, *Bacillus* strain HSR, *G. thermodenitrificans* (DSM 465), and *G. thermoleovorans* IHI-91 (Demharter and Hensel 1989; Fortina et al. 2001; Heinen and Heinen 1972; Markossian et al. 2000; Sunna et al. 1997; Weerkamp and Heinen 1972). Markossian et al. (2000) demonstrated that an increase in the relative amount of the high-melting point iso-fatty acids and a decrease of the

Table 1 Comparison of *Geobacillus* species. + Positive, − negative, *w* weakly positive, *ND* not determined

Character	T4[a]	*G. thermoleovorans*[b]	*G. kaustophilus*[c]	*G. stearothermophilus*[d]
G + C (mol%)	53.3	53.7[e]	51–58	52.2[e]
Growth				
70°C	−	+	+	−
pH 6.0	−	−	−	+
Fermentation of glucose	+	+	−	ND
Citrate utilization	−	−	+	W
Acid production from				
Glycerol	+	+	ND	+
L-Arabinose	−	−	ND	ND
Ribose	ND	ND	+	ND
D-Xylose	+	−	W	W
Adonitol	+	ND	−[f]	−[f]
Rhamnose	+	−	−	−
Denitrification	ND	ND	+	−
Hydrolysis				
Aesculin	+	−	+[f]	ND
Starch	−	+	+	+
Gelatin	+	−	+	W
Motility	+	−	−	+

[a]Strain T4 was negative for the Voges–Proskauer reaction, nitrate reduction, lysine and ornithine decarboxylases, arginine dihydrolase, urease, H_2S, indole, D-fructose, D-mannose, mannitol, xylan, sorbitol, α-methyl-D-glucoside, amygdalin, arbutin, salicin, acetoin, maltose, sucrose, trehalose and D-arabitol, but not for galactose, erythritol, L-xylose, β-methylxyloside, L-sorbose, dulcitol, α-methyl-D-mannoside, *N*-acetylglucosamine, lactose, melibiose, inulin, melezitose, D-raffinose, glycogen, β-gentiobiose, D-lyxose, D-tagatose, D-fucose, D-arabitol, gluconate or 2-ketogluconate

[b]Zarilla and Perry (1987), Markossian et al. (2000)

[c]Priest et al. (1988), White et al. (1993)

[d]Claus and Berkeley (1986)

[e]Data from Nazina et al. (2001)

[f]Data from this study

Table 2 Cellulolytic activities tested in the culture supernatants of strain T4 and some related *Geobacillus* strains. Assays were performed at 60°C in 0.1 M Na_2HPO_4–NaH_2PO_4 buffer (pH 7.0) for 1 h. *G. kaustophilus* (ATCC 8005) and three *G. stearothermophilus* strains (ATCC 7953, ATCC 10149, and ATCC 12976) show negative activities toward all five substrates

Substrate (1.0%, w/v)	Relative activity (%)		
	T4	*G. thermoleovorans* (DSM 5366)	*G. stearothermophilus* (DSM 22)
CMC	100	0	16
Phosphoric acid-swollen cellulose	63	0	13
Avicel	36	0	0
Filter paper	36	0	14
Salicin	20	0	34

low-melting point anteiso-fatty acids was due to adaptation to high growth temperatures.

Enzymatic properties of crude cellulase

As shown in Table 2, cellulase activities tested in the culture supernatant of strain T4 hydrolyzed CMC, phosphoric acid-swollen cellulose, Avicel, filter paper, and salicin. CMC was hydrolyzed more efficiently than amorphous (phosphoric acid-swollen cellulose) and crystalline (Avicel and filter paper) forms of cellulose. In spite of the fact that the isolate T4 has 76.7% DNA–DNA homology to the type strain *G. thermoleovorans* (DSM 5366), an obvious difference was found in their cellulose hydrolysis reactions. *G. thermoleovorans* (DSM 5366 = ATCC 43513), *G. kaustophilus* (ATCC 8005), and *G. stearothermophilus* strains (ATCC 7953, ATCC 10149, and ATCC 12976) do not utilize cellulose as their carbon and energy source (Table 2). Moreover, the strain T4 was more active than the type species *G. stearothermophilus* (DSM 22 = ATCC 12980) towards these substrates except salicin.

Since strain T4 more efficiently hydrolyzed CMC, the extracellular endoglucanase enzyme activity in culture supernatants of strain T4 grown at 60°C in CMC medium was also examined. The optimum temperature for the reaction with CMC as substrate was detected as 70°C at pH 7.0 in 0.1 M Na_2HPO_4–NaH_2PO_4 buffer (Fig. 2). A maximum endoglucanase activity at optimum temperature corresponds to 11.3 mU/ml. The maximum activity of 60% was observed at 80°C, and 9% of the enzyme activity was left at 90 and 100°C. For thermal stability examination, the enzyme was stable at 70°C, with more than 90% of the activity retained (Fig. 2). More than 10% of the original activity still could be detected after the supernatants were heated to 90 and 100°C for 1 h.

Although strain T4 is very similar to *G. thermoleovorans*, this isolate was differentiated from related validly described *Geobacillus* species by the more efficient cellulose hydrolysis activity—especially for those of

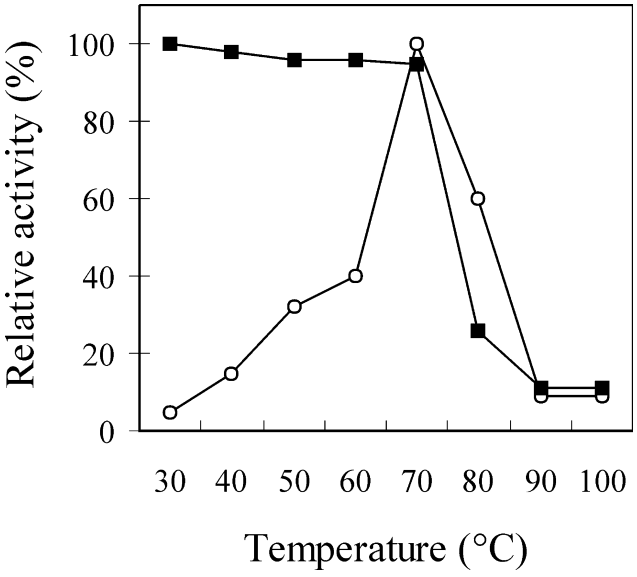

Fig. 2 Effect of temperature on the activity (*open circle*) and stability (*solid square*) of the crude endoglucanase from strain T4. The effect of temperature on the activity (*open circles*) was determined with CMC as substrate at various temperatures in 0.1 M Na_2HPO_4–NaH_2PO_4 buffer (pH 7.0) for 60 min. The enzymatic activities were expressed as percentages of the activity at 70°C. For thermal stability (*solid squares*), the crude enzyme supernatants were preincubated for 1 h at various temperatures. The enzymatic activity, after heating to 30°C for 1 h, was regarded as 100%

CMC, phosphoric acid-swollen cellulose, Avicel, and filter paper—as well as a unique thermostable endoglucanase activity. In this study, we also presented the type species *G. stearothermophilus*, which could hydrolyze amorphous and crystalline (filter paper) celluloses, and we quantified its cellulolytic activities (Table 2). For potential industrial application, the novel strain *G. thermoleovorans* T4 was deposited at DSMZ as DSM 14791 and at CCRC as CCRC 17200. The purification of its thermostable endoglucanase is in progress.

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