***Saccharococcus caldoxylosilyticus* sp. nov., an obligately thermophilic, xylose-utilizing, endospore-forming bacterium**

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Several closely related, xylanolytic, thermophilic bacilli were isolated from local soils on xylose-containing minimal medium. On the basis of morphology and biochemical characteristics, one of the isolates, designated strain S1812^T (T = type strain), was studied further. Strain S1812^T was a xylanolytic, sporulating, Gram-positive, rod-shaped bacterium. Its Gram-positive nature was confirmed by electron microscopic examination of thin sections of the cells. The isolate was a thermophilic (optimum temperature for growth, 65 °C), facultative anaerobe that grew on a wide range of carbon sources including glucose, lactose, starch and xylose. It expressed high levels of both xylose isomerase and xylulokinase on xylose and also on glucose. The DNA G+C content was 44 mol%. rRNA gene sequence analysis placed strain S1812^T in *Bacillus* cluster 5; it was more closely related to *Saccharococcus thermophilus* than to thermophilic *Bacillus* species. DNA–DNA hybridization also indicated its close relationship to *S. thermophilus*. Based on the evidence presented, it is proposed that strain S1812^T be designated *Saccharococcus caldoxylosilyticus* sp. nov. Strain S1812^T is the type strain (= ATCC 700356^T = DSM 97-987^T).

Keywords: *Saccharococcus caldoxylosilyticus*, *Saccharococcus*, obligate thermophile, phylogeny, xylanolytic

INTRODUCTION

It is now over a century since thermophiles were first reported (Miquel, 1888). Over the years, a number of spore-forming thermophiles have been reported, mainly in the genera *Bacillus* and *Clostridium*. Many strains of the thermophile *Bacillus stearothermophilus* have been described, and proteins isolated from them have been extensively characterized. Most such strains have temperature optima below 60 °C, though some less-characterized bacilli grow successfully at temperatures up to 75 °C (e.g. Guagliardi *et al.*, 1996).

In the search for a suitable thermophilic species that would ferment pentose sugars such as those in lignocellulose hydrolysates, local soils were sampled for strains that would grow at 65 °C on a minimal medium supplemented with xylose. A suitable strain would preferably convert the sugars to ethanol or would have

the potential to be modified by genetic manipulation to produce ethanol (Neale *et al.*, 1988). The most rapidly growing colonies were selected and an aerobic bacillus was isolated. Based on preliminary experiments, this strain appeared to differ from other thermophilic bacilli, and therefore was characterized further. The present paper describes its isolation, biochemical pattern, 16S rRNA sequence, G+C content, morphology and DNA–DNA hybridization with close relatives and proposes that it be designated a new species of the genus *Saccharococcus*.

METHODS

Sample source. Samples were collected from local soil (Melbourne, Australia) in small vials and stored at 5 °C until inoculation in the medium.

Enrichment and isolation. Enrichments were initiated by adding 0.1–0.2 g sample to minimal medium (MM) that contained 1% xylose as the sole carbon source followed by incubation at 65 °C for up to 24 h. MM contained (g l⁻¹): K₂HPO₄, 4.0; KH₂PO₄, 1.0; NH₄NO₃, 1.0; NaCl, 1.0; and MgSO₄, 0.25; it also contained trace mineral solution

The GenBank accession number for the 16S rDNA sequence of strain S1812^T is AF067651.

(10 ml l⁻¹) and the pH was adjusted to 6.8. Trace mineral solution contained (g l⁻¹): EDTA, 5.0; CaCl₂·2H₂O, 6.0; FeSO₄·7H₂O, 6.0; MnCl₂·4H₂O, 1.15; CoCl₂·6H₂O, 0.8; ZnSO₄·7H₂O, 0.7; CuCl₂·2H₂O, 0.3; H₃BO₃, 0.3; and (NH₄)₆Mo₇O₂₄·4H₂O, 0.25.

After two transfers of 1 ml growing culture into fresh medium, culture purification was performed by isolating single colonies on MM agar plates (MM containing 1.5% agar). The incubations were carried out at 65 °C for 24 h.

Routine culturing and strain maintenance. Routine culturing of strain S1812^T was performed in Luria broth (LB) containing 0.5 or 1.0% xylose. The strain was stored in 30% glycerol at -20 °C. For routine use, it was preserved on LB agar slants containing 0.5% xylose at 5 °C.

Cellular and colony characteristics. Colony characteristics were determined from 12 h cultures grown at 65 °C. Phase-contrast microscopy was used to determine the morphology of cells that had been cultured under different conditions. Electron microscopy of thin sections of strain S1812^T was carried out as described previously (Love *et al.*, 1993).

Substrate utilization, biochemical and growth characterization. MM and LB containing different substrates were used to determine the range of substrates utilized by strain S1812^T. Substrates were sterilized separately either by filtration or autoclaving and added to the medium prior to inoculation. Unless indicated otherwise, all incubations were performed at 65 °C. Growth was measured at 580 nm for up to 72 h. Acid production from carbohydrates was determined by measuring the change in pH after 72 h growth.

Temperature growth range (38–75 °C) and pH growth range (4.0–8.0) were determined in LB containing 1% xylose. Media were adjusted to the initial pH indicated with either 1 M NaOH or 1 M HCl.

Anaerobic growth was determined according to Gordon *et al.* (1973). Growth on citrate, xylan, xylose and other pentoses was monitored on MM agar plates supplemented with 1% of the test substance. Anaerobic growth was also tested in degassed LB medium containing 1% of the test substance.

Catalase and oxidase were detected by the method of Cowan & Steel (1974).

Antibacterial inhibition tests. Inhibition of growth of strain S1812^T was determined in LB in the presence of sodium azide (0.02%), sodium chloride (1–3%), chloramphenicol (25 µg ml⁻¹), streptomycin sulphate (25 µg ml⁻¹) and ampicillin (15 µg ml⁻¹). Growth was recorded after 3 d incubation at 65 °C.

DNA base composition. The DNA base composition was determined by thermal denaturation as described previously (Rees *et al.*, 1995). The base composition was calculated following thermal denaturation of the DNA (Mandel & Marmur, 1968). *Escherichia coli* DNA was used as the standard.

16S rRNA sequence analysis. Purification of genomic DNA, and amplification and purification of the 16S rRNA gene (16S rDNA) from strain S1812^T were performed by using a technique described previously (Love *et al.*, 1993; Redburn & Patel, 1993). The purified PCR product was sequenced directly on an ABI automated DNA sequencer using a Prism dideoxy terminator cycle sequencing kit and protocols recommended by the manufacturer (Applied Biosystems).

Using the sequence editor, ae2, the 16S rDNA sequence of strain S1812^T was aligned with the 16S rRNA sequences of various members of the bacterial phyla obtained from the rRNA Database Project (Maidak *et al.*, 1994) and from GenBank. Positions of sequence and alignment uncertainty were omitted from the analysis, and pairwise evolutionary distances for 1332 unambiguous nucleotides were computed. Phylogenetic analysis was performed using programs which form part of the PHYLIP package and include DNADIST (Jukes & Cantor, 1969), NEIGHBOR-JOINING and DNAPARS (Felsenstein, 1993). Tree topology was re-examined by using 100 bootstrapped data sets for which a script file with the following PHYLIP programs was used: SEQBOOT, DNADIST, FITCH and CONSENSE. Programs in the phylogenetic package MEGA (Kumar *et al.*, 1993) were also used. PHYLIP programs were run on a Sun SPARC work station, and MEGA was run on a Compaq notebook (Contura model 410CX).

DNA–DNA hybridization. The DNA was isolated by chromatography on hydroxyapatite (Cashion *et al.*, 1977). DNA–DNA hybridization was determined at the DSMZ as described by De Ley *et al.* (1970) with modifications described by Huß *et al.* (1983) and Escara & Hutton (1980). A Gilford System model 2600 spectrophotometer equipped with Gilford model 2527-R thermoprogrammer and plotter was used. Renaturation rates were computed with the TRANSFER.BAS program (Jahnke, 1992).

Enzyme assays. D-Xylose, D-xylulose, L-xylulose, D-xylulose 5-phosphate, ribose 5-phosphate, erythrose 4-phosphate, fructose 6-phosphate, ATP, NADH and phosphoenolpyruvate were purchased from Sigma. Pyruvate kinase was from *Zymomonas mobilis*; lactate dehydrogenase, triose phosphate isomerase and glycerol phosphate dehydrogenase were from rabbit muscle and were purified in our laboratory.

The activities of pentose metabolic enzymes (xylose isomerase, xylulokinase, transaldolase and transketolase) were determined in extracts of strain S1812^T. For this experiment, the cells were cultivated in LB medium containing either 0.5% or 1.0% xylose in a 21 Biostat or in 101 Braun fermenters under pH and temperature control at pH 6.5 and 65 °C, respectively. Cells were lysed by adding 5 ml of a 25 mM phosphate buffer, pH 7.0, containing lysozyme (0.2 mg ml⁻¹), DNase (5 µg ml⁻¹) and Triton X-100 (0.1%) to every g (wet weight) of cells. The mixture was stirred gently for 1–3 h at room temperature. The lysate was centrifuged (20000 g for 20 min) and the clear brownish-red extract was used for enzyme assay. Protein content of the lysate was measured as described by Sedmak & Grossberg (1977).

Xylulokinase activity was measured using a reaction mixture containing 50 mM Tris/HCl, pH 8.0, 10 mM MgCl₂, 1 mM ATP and 2 mM xylulose. After incubation of a sample for 5 min in 1 ml assay mixture at 60 °C, 50 µl cold 60% perchloric acid was added and the denatured protein was removed by centrifugation. After neutralization with 250 µl 2 M KOH containing 50 mM K₂CO₃, the amount of ADP that had been formed, compared with a zero time control, was determined with NADH/phosphoenolpyruvate/pyruvate kinase/lactate dehydrogenase. Xylose isomerase was measured as described by Lee *et al.* (1990). Transketolase and transaldolase were measured by stopped procedures at 60 °C, similar to that described above for xylulokinase. For transketolase, 1 mM xylulose 5-phosphate and 2 mM ribose 5-phosphate, and for transaldolase, 1 mM erythrose 4-phosphate and 2 mM fructose 6-phosphate, were

incubated at pH 8 with extract sample for 5 min before adding the perchloric acid. Formed glyceraldehyde 3-phosphate was measured using NADH/triose phosphate isomerase/glycerol phosphate dehydrogenase.

Alcohol dehydrogenase was measured at ambient temperature in 50 mM potassium phosphate buffer containing 100 mM ethanol and 1 mM NAD⁺. All enzyme units are expressed as $\mu\text{mol min}^{-1}$.

RESULTS

Enrichment and isolation

Enrichment cultures, derived from diverse soil samples from garden and compost heaps, were obtained in MM containing xylose following incubation at 65 °C for 72 h. After several transfers, the enrichment cultures were plated onto LB agar plates. After incubation, several well-isolated colonies that developed were picked. The purified clones were tested individually for their xylose utilization capability and the most efficient strain was selected for further study.

Biochemical characteristics

Strain S1812^T grew well aerobically, but relatively slowly in MM containing xylose as sole carbon source. Because of this slow growth, the bacterium was routinely cultivated in LB medium containing xylose. Strain S1812^T grew well at pH 5.5–8.0, with optimum growth at 6.8. Under optimum growth conditions, the generation time was between 24 and 30 min. The strain was a facultative anaerobe, catalase-positive and oxidase-negative. The strain was nutritionally versatile and used a wide variety of carbohydrates when grown on MM, both aerobically and anaerobically. It did not grow on citrate and grew poorly on lactate (Table 1).

Morphology

Strain S1812^T formed small colonies with entire margins after overnight incubation at 65 °C on LB plates with or without xylose. Light microscopy revealed that the strain was a rod-shaped and sporu-

Table 1. Physiological and biochemical properties of *S. caldoxylosilyticus* strain S1812^T and *S. thermophilus*

+, Growth/activity observed; –, no growth/activity observed; w, weak growth; ND, not determined. Both strains produced catalase. Neither strain grew on citrate, but both strains grew on the following substrates: arabinose†, galactose†, glucose†, glycerol, mannitol, mannose†, rhamnose†, sorbitol and xylose† († indicates anaerobic growth observed with S1812^T).

Character	<i>S. caldoxylosilyticus</i> strain S1812 ^T	<i>S. thermophilus</i> CCM 3586*
Morphology:		
Cell shape	Rods	Cocci
Spores	Sporulating	Non-sporulating
G + C content (mol %)	44	48
Physical growth conditions:		
Optimum temperature (°C)	65	70
Optimum pH	6.5	7.0
Anaerobic growth	+	–
Biochemical pattern:		
Oxidase	–	+
NaCl (3 %)	–	ND
Sodium azide (0.02 %)	–	ND
Carbon sources tested (MM):		
Cellobiose†	+	ND
Glycogen	+	–
Lactate	w	ND
Lactose	+	–
Raffinose	+	–
Starch†	+	–
Sucrose	+	ND
Trehalose	+	ND
Xylan†	+	ND

* Data from Nystrand (1984).

† Anaerobic growth observed with S1812^T.

Fig. 1. Phase-contrast photomicrograph of strain S1812^T. Bar, 2 μ m.

Fig. 2. Electron micrograph of an ultrathin section of strain S1812^T showing the cell wall structure. Bar, 2 μ m.

lating *Bacillus*. This was subsequently confirmed by electron microscopy (Figs 1 and 2), which revealed cells 2.1 μ m long and 0.6 μ m wide, and ellipsoidal

spores 1.2 μ m long and 0.6–0.7 μ m wide. The spores were central to terminal in location.

Salt and antibiotic sensitivity

Growth was inhibited in the presence of 3% NaCl, sodium azide (0.02%), chloramphenicol (25 μ g ml⁻¹), streptomycin sulphate (25 μ g ml⁻¹) and ampicillin (15 μ g ml⁻¹).

G + C content of DNA and DNA hybridization studies

The G + C content of this strain is 44.4 mol %, which is significantly lower than that of most other obligately thermophilic *Bacillus* spp., which exhibit G + C values higher than 52 mol % (Sharp *et al.*, 1980). However, some thermophilic *Bacillus* species, such as *Bacillus thermoglucosidasius*, do exhibit G + C values around 44 mol % (White *et al.*, 1993). The value for *Saccharococcus thermophilus* is reported to be 48 mol % (Nystrand, 1984).

The results of the DNA hybridization studies are shown in Table 2. The relationship with *B. stearothermophilus* and '*Bacillus thermodenitrificans*' is fairly distant, but is close with *S. thermophilus*, and at 67%, hybridization is much stronger with *S. thermophilus* than with the *Bacillus* spp. studied.

16S rRNA sequence

A sequence consisting of 1516 nt of the 16S rRNA gene of strain S1812^T was determined. The sequence positions 14–1516 [*E. coli* numbering according to Winker & Woese (1991)] were aligned with representatives of the various phyla of the domain *Bacteria* and phylogenetic analysis was performed. Several data sets which included different representatives from the various phyla of the domain *Bacteria* were also created and, in all cases, strain S1812^T was consistently placed as a peripheral member of the thermophilic *Bacillus* species cluster 5, in the sub-branch of the low-G + C Gram-positive bacteria as defined by Ash *et al.* (1991). The evolutionary distances separating strain S1812^T and its relatives, and the dendrogram derived from evolution distances is depicted in Fig. 3. Transversion analysis did not affect the relationship of strain S1812^T with its nearest relatives.

Extracts of the cells were tested for various enzyme activities associated with xylose utilization. Xylose isomerase and xylulokinase activities were high in cells grown either on xylose or on glucose, indicating perhaps that the *XylA-XylB* operon was constitutive rather than inducible. Transketolase and transaldolase were induced only in the presence of xylose (Table 3).

Alcohol dehydrogenase activity (specific activity up to 20 units mg⁻¹ at 60 °C), was found to be present only in anaerobically grown cells. It was not detected at a sensitivity of 0.5 units mg⁻¹ in extracts of fully aerobic cells. This alcohol dehydrogenase has been isolated (J.

Table 2. DNA–DNA hybridization

Similarities (%) are given.

	<i>Saccharococcus caldxylosilyticus</i> strain S1812 ^T	<i>Bacillus stearothermophilus</i> DSM 22	' <i>Bacillus thermodenitrificans</i> ' DSM 465
<i>B. stearothermophilus</i> DSM 22	33.4		
' <i>B. thermodenitrificans</i> ' DSM 465	37.0	48.3	
<i>Saccharococcus thermophilus</i> DSM 4749	67.0	45.2	44.0

Fig. 3. Dendrogram indicating the position of strain S1812^T amongst the representatives of the members of the low-G+C Gram-positive *Bacillus* groups (cluster 5 according to Ash *et al.*, 1991). The dendrogram was derived from the evolutionary distance matrix as described in Methods. Bar, 1 substitution per 100 nucleotides.

Scoble, personal communication) and found to have a versatile substrate specificity and to be similar in characteristics to the *B. stearothermophilus* enzyme described by Guagliardi *et al.* (1996). However, the latter was reportedly isolated from aerobically grown cells.

DISCUSSION

Initial studies indicated that S1812^T was a member of *Bacillus* cluster 5 as defined by Ash *et al.* (1991). On the basis of genotypic and phenotypic properties, the isolate can be clearly distinguished from other described *Bacillus* species. Despite the morphological observations that clearly class this bacterium as a *Bacillus*, its closest relative based on 16S rRNA sequence analysis and on DNA–DNA hybridization is *S. thermophilus*. Phylogenetic analysis of *S. thermophilus* (Nystrand, 1984; Rainey & Stackebrandt, 1993) and other non-spore-forming rods such as *Filibacter limicola* (Clausen *et al.*, 1985) and *Caryophanon latum* (Stackebrandt *et al.*, 1987) has suggested that these Gram-positive bacteria are closely related to *Bacillus* species and can be considered as offshoots. The 16S

rRNA sequence showed that S1812^T shared a common ancestry with members of the *Bacillus* cluster group 5, as defined by Ash *et al.* (1991), as well as an even closer relationship with *S. thermophilus*. Furthermore, DNA–DNA hybridization has shown its relatively distant relationship to *B. stearothermophilus* (33.4%) and '*B. thermodenitrificans*' (37.0%) and its closeness to *S. thermophilus* (76.0%).

It is not unusual that phylogeny does not reflect phenotypic properties. It has been reported that *Deinobacter*, a rod-shaped bacterium, is more phylogenetically related to *Deinococcus*, a coccus-shaped bacterium, and hence was transferred as a member of the *Deinococcus* (Oyaizu *et al.*, 1987; Rainey *et al.*, 1997). The similarity of S1812^T to *S. thermophilus* is at the borderline of species level, but the cell morphology is completely different. In addition, several phenotypic characteristics differentiate the two strains (Table 1).

The isolation of this xylolytic thermophile was initiated as part of a study into thermophilic ethanol fermentations. Although the strain does possess an alcohol dehydrogenase, and so presumably makes alcohol under certain conditions, this enzyme is entirely absent

Table 3. Metabolic enzymes of xylose utilization in *S. caldxylosilyticus* S1812^T cells

Cells were grown in LB medium for 24 h at 60 °C with 1 % xylose or glucose. Enzyme activity was determined from crude cell lysate and is shown as units (mg protein)⁻¹ at 60 °C. Figures for transaldolase and transketolase are indicative only, as several side reactions occur during the incubations.

Carbon source	Xylose isomerase	Xylulokinase	Transketolase	Transaldolase
Xylose	0.8	3.1	1.0	0.8
Glucose	0.6	3.0	0.2	0.1

when the cells are grown aerobically. Unusually, the first two enzymes of xylose utilization, xylose isomerase and xylulokinase, were present at high levels even when the cells were grown on glucose. On the other hand, transketolase and transaldolase were strongly induced by growth on xylose. It appears that this organism has potential as a high-temperature producer of ethanol from lignocellulose hydrolysates when fermented anaerobically if additional alcohol dehydrogenase and pyruvate decarboxylase enzymes can be introduced genetically.

Based on the evidence presented, it is proposed to designate strain S1812^T *Saccharococcus caldxylosilyticus*.

Description of *Saccharococcus caldxylosilyticus* sp. nov.

Genus *Saccharococcus*: the description of the genus is essentially as described by Nystrand (1984) but is amended to now include phylogenetic relatives which possess rod-shaped morphologies.

Saccharococcus caldxylosilyticus (cal.do.xy.lo.si.ly'ti.cus. L. adj. *caldus* hot; M.L. neut. N. *xylosum* xylose; M.L. adj. *lyticus* dissolving, degrading; *caldxylosilyticus* hot and xylose-degrading).

Gram-positive rods, 0.8–0.9 by 2.3 µm, occurs singly, in pairs or chains. Central to terminal endospore ellipsoidal; sporangium slightly swollen. Colonies flat to convex, round or lobed, smooth, opaque. Growth between 43 and 75 °C; optimal growth at 65 °C, pH 6.8–7.2. Generation time under optimum conditions 24–30 min. Facultative anaerobe. DNA G+C content of 44.4 mol%. Habitat: soil. Type strain is *Saccharococcus caldxylosilyticus* S1812^T and has been deposited in the American Type Culture Collection (ATCC), Manassas, VA 20852, USA under number ATCC 700356^T.

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