Anoxybacillus gonensis sp. nov., a moderately thermophilic, xylose-utilizing, endospore-forming bacterium

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Seven closely related xylanolytic, thermophilic bacilli were isolated from mud and water samples from the Gonen and Diyadin hot springs, respectively located in the Turkish provinces of Balikesir and Agri. On the basis of morphology and biochemical characteristics, one of the isolates, designated strain G2^T, was studied further. Strain G2^T is a xylanolytic, sporulating, Gram-positive, rod-shaped bacterium. The isolate is a thermophilic (optimum temperature for growth, 55–60 °C), facultative anaerobe that grows on a wide range of carbon sources, including glucose, starch, xylose and mannitol. It expressed a high level of xylose isomerase activity on xylose and also on glucose. 16S rRNA gene sequence analysis showed that this isolate resembled *Anoxybacillus flavithermus* DSM 2641^T (>97 % similarity), but 16S–23S rDNA internally transcribed spacer polymorphism PCR showed variation between DSM 2641^T and isolate G2^T. However, it is also known that analysis of 16S rRNA gene sequences may be insufficient to distinguish between some species. In DNA–DNA hybridization, thermophilic isolate G2^T showed relatedness of 53·4 % to *A. flavithermus* and about 45·0 % to *Anoxybacillus pushchinoensis*, indicating that it is distinct at the species level. On the basis of the evidence presented, it is proposed that strain G2^T (=NCIMB 13933^T=NCCB 100040^T) be designated as the type strain of *Anoxybacillus gonensis* sp. nov.

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* (Guagliardi *et al.*, 1996).

In this study, we isolated some thermophilic bacilli from the Gonen and Diyadin hot springs, respectively located in the Turkish provinces of Balikesir and Agri. On the basis of preliminary experiments, a representative strain appeared to differ from other thermophilic bacilli with respect to the utilization of xylose; it was therefore characterized further. Xylose isomerase is an intracellular enzyme that catalyses the conversion of D-xylose to D-xylulose. Its practical significance stems from its ability to isomerize D-glucose to D-fructose. Therefore, this enzyme is often referred to as glucose isomerase and is widely used in industry for the production of high-fructose corn syrup.

The present paper describes the isolation, morphological, physiological and biochemical profiles and 16S rRNA sequence of this strain and the results of DNA–DNA hybridization with close relatives and proposes that it represents a novel species of the genus *Anoxybacillus* (Pikuta *et al.*, 2000), *Anoxybacillus gonensis* sp. nov.

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Abbreviation: ITS, internally transcribed spacer.

The GenBank accession number for the 16S rRNA gene sequence of *Anoxybacillus gonensis* G2^T is AY122325.

Isolation of strains

Seven Gram-positive rods were isolated from mud and water samples from the Gonen and Diyadin hot springs. The water temperature of these hot springs is around 70 °C. After collection, mud and water samples were used immediately for enrichment in nutrient broth at 60–70 °C. One-day-old enrichment cultures were repeatedly subcultured in 10 ml nutrient broth and streaked on agar plates to obtain separate colonies. The purity of the isolates was assessed by colony morphology and microscopy. After 48 h growth on nutrient agar medium, colonies of strain G2^T were small, cream, irregularly shaped with rough edges and 2–3 mm in diameter. Light microscopy revealed that cells of the strain were rod-shaped, Gram-positive and motile, measuring 0·75 × 5·0 μm.

Biochemical and nutritional characteristics

Utilization of organic compounds as sole carbon sources was tested in basal medium (5 ml) supplemented with 0·5 %

(w/v) of the following compounds, which had been separately sterilized as stock solutions: glucose, mannitol, mannose, sucrose, xylose, arabinose, lactose, raffinose, starch, glycogen and rhamnose. Incubation was carried out at 60 °C. The strain was nutritionally versatile and used a wide variety of carbohydrates when grown on basal medium. It grew on glucose, glycogen, raffinose, sucrose, xylose and mannitol (Table 1). Anaerobic growth was tested in anaerobic agar medium. Strain G2^T grew well aerobically, but was facultatively anaerobic.

The ranges of temperature (35–75 °C) and pH (5·5–10·5) for growth were determined in nutrient broth medium. Media were adjusted to the initial pH indicated with either 1 M NaOH or 1 M HCl. Strain G2^T grew well at 40–70 °C, with optimum growth at 55–60 °C, and grew well at pH 6·0–10·0, with optimum growth at pH 7·5–8·0. Catalase and oxidase were detected by the method of Cowan & Steel (1974). Strain G2^T is catalase- and oxidase-positive.

Salt and antibiotic sensitivity

Four replicate sets of nutrient broth were prepared containing 1, 2, 3, 4, 5 or 7 % NaCl. Growth of the isolate at different salt concentrations was tested using nutrient broth as the organic substrate and using a control broth without any NaCl supplementation. Growth was inhibited in the presence of NaCl concentrations above 4 % and in the presence of chloramphenicol (25 μg ml^{-1}), ampicillin (25 μg ml^{-1}), streptomycin sulphate (25 μg ml^{-1}) and tetracycline (12·5 μg ml^{-1}). The optimal NaCl concentration for growth was 2 %.

Spore formation

The formation of spores was tested by microscopic observation of both liquid cultures and single colonies of the isolates from agar plates after different incubation periods. Incubation for 1–2 days was required before spore formation became detectable on agar plates. Light microscopy revealed that strain G2^T was a sporulating bacillus. It formed terminal spherical endospores.

16S rRNA gene sequence analysis

The 16S rRNA gene was selectively amplified from purified genomic DNA by using oligonucleotide primers designed to anneal to conserved positions in the 3′ and 5′ regions of bacterial 16S rRNA genes. The forward primer, UNI16S-L (5′-ATTCTAGAGTTTGATCATGGCTTCA-3′), corresponded to positions 11–26 of the *Escherichia coli* 16S rRNA, while the reverse primer, UNI16S-R (5′-ATGGTACCGTGTGACGGGCGGTGTTGTA-3′), corresponded to the complement of positions 1411–1393 of *E. coli* 16S rRNA (Brosius *et al.*, 1978). PCR conditions were according to Beffa *et al.* (1996). The PCR product was cloned into pGEM-T and then the 16S rRNA gene sequence was determined with an Applied Biosystems model 373A DNA sequencer, using the ABI PRISM cycle-sequencing kit. A sequence consisting of about 1400 nt of the 16S rRNA gene of strain

Table 1. Physiological and biochemical properties of strains of *Anoxybacillus gonensis* sp. nov. and *Anoxybacillus flavithermus* DSM 2641^T

Cells of all strains are sporulating rods. All strains show anaerobic growth and oxidase activity, are negative for utilization of lactose and rhamnose and positive for utilization of starch, sucrose, glucose and mannitol. ND, No data; w, weak growth.

Characteristic	*Anoxybacillus gonensis* sp. nov.							*Anoxybacillus flavithermus* DSM 2641^T
	A2	A4	A5	A6	A7	A9	G2^T	
G+C content (mol%)	ND	ND	ND	ND	ND	ND	57	61
Temperature for growth (°C):								
Range	37–70	37–70	37–70	37–70	37–70	37–70	40–70	30–72
Optimum	55–60	55–60	55–60	55–60	55–60	55–60	55–60	60–65
pH for growth:								
Range	5·5–9·5	5·5–9·5	5·5–10·0	5·5–9·5	5·5–9·5	5·5–9·5	6·0–10·0	5·5–9·0
Optimum	7·0	7·0	7·0	7·0	7·0	7·0	7·5–8·0	7·0
Tolerance of NaCl (%)	4·0	4·0	4·0	4·0	3·5	4·0	4·0	2·5
Carbon sources (basal medium):								
Glycogen	+	+	+	+	+	+	+	−
Raffinose	+	+	+	+	−	+	+	−
Xylose	+	+	+	+	−	+	+	−
Arabinose	−	−	−	−	−	−	−	w
Mannose	−	−	−	−	+	−	−	+
Fructose	+	+	+	+	+	+	+	ND
Nitrate reduction	+	+	+	+	+	+	+	−
Hydrolysis of gelatin	+	−	−	+	−	−	+	−

$G2^T$ was determined. The sequence was compared with the 16S rDNA sequences of some representatives of the *Bacillus* group by using PHYLIP version 3.5 (Felsenstein, 1989). Phylogenetic analysis revealed a clustering with *Anoxybacillus flavithermus* DSM 2641^T (97 % sequence similarity). These sequences differed by 7–16 % from sequences of species of the genus *Bacillus* and can therefore be distinguished as a separate genus. The sequence of strain $G2^T$ showed 96, 93 and 86 % similarity, respectively, to sequences from *Anoxybacillus pushchinoensis* DSM 12423^T, *Saccharococcus caldoxylosilyticus* DSM 12041^T and *Alicyclobacillus acidocaldarius*.

PCR amplification of intergenic 16S–23S rDNA sequences

Primers FGPS1490-72 (5′-TGCGGCTGGATCCCCTCCTT-3′; positions 1521–1541 of the *E. coli* 16S rRNA gene sequence) and FGPL132′-38 (5′-CCGGGTTTCCCCATTCGG-3′; positions 114–132 of the *E. coli* 23S rRNA gene sequence) were used for amplification of intergenic 16S–23S rDNA sequences. PCR conditions were according to Riffard *et al.* (1998). As shown in Fig. 1, all seven novel strains showed a faint band of about 300 bp, but only A2, A6 and $G2^T$ had the same pattern, with A4, A5, A7 and A9 showing a different banding pattern. However, the internally transcribed spacer (ITS) patterns of all the novel strains were different from those of *Anoxybacillus flavithermus* DSM 2641^T and *S. caldoxylosilyticus* DSM 12041^T; therefore, $G2^T$ is different from these strains.

G+C content analysis and DNA–DNA hybridization studies

After extraction and purification of the DNA (Johnson, 1985), the G+C content was determined from the denaturation temperature in 0·5× SSC. Denaturation profiles were followed at 260 nm using a thermoprogrammable spectrophotometer (Jenway 6105 UV/Vis spectrophotometer) in accordance with the principles of Mandel & Marmur (1968). The G+C content of this strain is 57 mol%, which is lower than that of *Anoxybacillus flavithermus* DSM 2641^T.

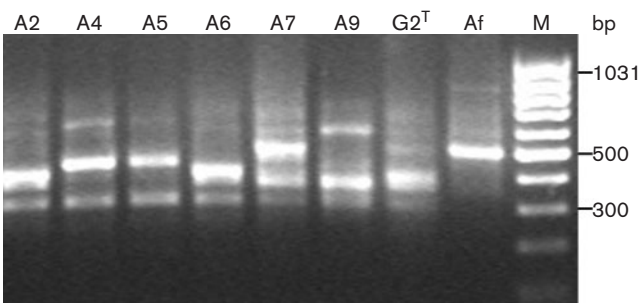


Fig. 1. ITS-PCR analysis of strains $G2^T$, A2, A4, A5, A6, A7, A9 and *Anoxybacillus flavithermus* DSM 2641^T (Af). Lane M, 100 bp ladder.

Table 2. DNA–DNA relatedness (%)

Strain	$G2^T$	A4	A7
Anoxybacillus pushchinoensis DSM 12423^T	45·0	ND	ND
Strain A4	82·3	–	88·2
Strain A7	77·9	88·2	–
Anoxybacillus flavithermus DSM 2641^T	53·4	45·4	52·3

DNA was isolated by chromatography on hydroxyapatite. DNA–DNA hybridization was determined at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany, as described by De Ley *et al.* (1970), with the modifications described by Huß *et al.* (1983) and Escara & Hutton (1980). A Gilford System model 2600 spectrophotometer equipped with a Gilford model 2527-R thermoprogrammer and plotter was used. Renaturation rates were computed with the TRANSFER.BAS program (Ahmad *et al.*, 2000). DNA–DNA hybridization studies were performed among $G2^T$, A4, A7 and *Anoxybacillus flavithermus* DSM 2641^T and between $G2^T$ and *Anoxybacillus pushchinoensis* DSM 12423^T (Table 2).

SDS-PAGE analysis

Extracts from cells growing actively on nutrient broth medium were obtained according to the method of Belduz *et al.* (1993). The protein concentration in the extracts was measured according to the method of Bradford (1976) and 40 μg crude extract was loaded per lane. Electrophoresis on 12 % SDS-PAGE was carried out as described by Laemmli (1970). Proteins were stained in a solution that contained Coomassie brilliant blue R-250 (0·125 %), methanol (50 %) and acetic acid (10 %) for 2–4 h and then visualized by destaining in a solution of 5 % methanol/7 % acetic acid. The electrophoretic patterns of soluble cellular proteins, as determined by SDS-PAGE (Fig. 2), showed that $G2^T$ is not similar to *Anoxybacillus flavithermus* DSM 2641^T, *Anoxybacillus pushchinoensis* DSM 12423^T or *S. caldoxylosilyticus* DSM 12041^T, and $G2^T$ therefore does not belong to any of these species. The other six novel strains were examined; SDS-PAGE analysis showed the similarity of these strains to $G2^T$ (Fig. 2).

Cellular fatty acids

Cultivation, harvesting, preparation and analysis of cellular fatty acid methyl esters from whole-cell fatty acids from strains $G2^T$, A2, A4, A6, A7, A9 and *Anoxybacillus flavithermus* DSM 2641^T were performed according to the method described in the Sherlock Microbial Identification System manual (version 4.0; MIDI). The fatty acid methyl esters of strain $G2^T$ and the other six strains and *Anoxybacillus flavithermus* DSM 2641^T were identified by comparing the commercial M17H10 database using the MIS software package, version 3.8 (Microbial ID). The cellular

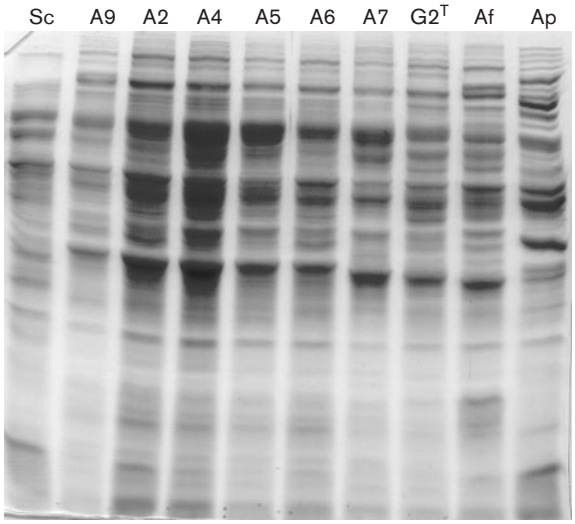


Fig. 2. SDS-PAGE whole-cell protein profiles of strains A2, A4, A5, A6, A7, A9 (strains of *Anoxybacillus gonensis* sp. nov. isolated from Diyadin hot spring), *Anoxybacillus gonensis* G2^T, *Anoxybacillus flavithermus* DSM 2641^T (lane Af); *Anoxybacillus pushchinoensis* DSM 12423^T (Ap) and *Saccharococcus caldoxylosilyticus* DSM 12041^T (Sc).

fatty acid profiles of the seven novel strains and *Anoxybacillus flavithermus* DSM 2641^T are shown in Table 3; the fatty acid profiles of the novel strains were very similar, with $C_{15:0}$ iso as the main fatty acid (62–68 %). Strain G2^T also resembled *Alicyclobacillus acidocaldarius* on the basis of fatty acid profiles, but the morphological and biochemical properties and 16S rRNA gene sequence of G2^T do not resemble those of this bacterium.

Enzyme assays

Activity of xylose isomerase was determined in extracts of G2^T. For this experiment, cells were cultivated in Luria–Bertani medium containing either 0·5 % xylose or 0·5 % glucose in a 500 ml flask at 60 °C. Cells were lysed by adding 5 ml 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 %) per g (wet weight) cells. The mixture was stirred gently for 2–3 h at room temperature. The lysate was centrifuged (20 000 ***g*** for 20 min) and the pellet was used for the enzyme assay. The protein content of the lysate was measured according to the method of Bradford (1976). Xylose isomerase activity was then measured as described by Lee *et al.* (1990). The enzyme activity was determined as 0·78 and 0·5 U (mg protein)$^{-1}$ at 60 °C on xylose and glucose, respectively, from crude cell lysate.

Initial studies indicated that G2^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 distinguished from other *Bacillus* species described. Despite the morphological observations that clearly class this bacterium as a *Bacillus*, its closest relative, on the basis of

Table 3. Fatty acids of strains of *Anoxybacillus gonensis* sp. nov.

Values are percentages of total fatty acids.

Fatty acid	*Anoxybacillus gonensis* sp. nov.							*Anoxybacillus flavithermus* DSM 2641^T
	A2	A4	A5	A6	A7	A9	G2^T	
$C_{13:0}$ iso	–	0·16	–	0·29	0·21	0·26	–	–
$C_{14:0}$ iso	0·72	1·09	1·03	0·41	0·38	0·53	1·25	–
$C_{14:0}$	1·10	0·93	0·99	1·16	0·69	1·19	1·18	1·96
$C_{14:0}$ iso 3-OH	–	0·32	0·44	–	0·32	–	–	54·85
$C_{15:0}$ iso	62·13	64·55	66·36	68·06	64·76	63·07	65·19	4·02
$C_{15:0}$ anteiso	2·50	2·76	2·86	2·66	2·17	2·14	2·64	1·18
$C_{15:0}$ $\omega5c$	–	0·41	–	–	–	–	–	–
$C_{15:1}$ $\omega5c$	–	–	0·25	–	–	–	–	–
$C_{15:0}$	0·90	1·65	1·45	0·75	1·06	0·96	1·12	2·97
$C_{16:0}$ iso	5·46	5·54	4·80	2·94	3·60	2·34	5·99	11·13
$C_{16:0}$	3·86	1·89	1·98	3·10	2·94	2·98	2·38	–
$C_{17:1}$ $\omega5c$	2·04	3·87	4·28	1·87	2·03	1·42	2·63	–
$C_{17:0}$ anteiso A	–	0·93	–	–	–	–	0·82	17·74
$C_{17:1}$ anteiso A	–	–	1·03	0·39	0·47	0·44	–	–
$C_{17:0}$ iso	15·67	10·57	9·16	13·56	16·41	10·02	11·96	6·15
$C_{17:0}$ anteiso	4·10	2·76	2·47	3·36	3·56	2·47	3·29	–
$C_{18:0}$	–	–	–	–	–	0·64	–	–
$C_{19:0}$ 10-methyl	–	–	–	–	–	10·66	–	–

16S rRNA sequence analysis, is *Anoxybacillus flavithermus* DSM 2641^T.

Stackebrandt & Goebel (1994) reached the conclusion that strains belonging to the same genus that exhibit less than 97 % 16S rRNA gene sequence similarity should be considered members of different species. However, it is also known that analysis of 16S rRNA sequences may be insufficient to distinguish between some species (Vandamme *et al.*, 1996). In this study, we determined the 16S rRNA gene sequence of G2^T and found more than 97 % similarity to that of *Anoxybacillus flavithermus*. However, we also determined that some physiological, morphological and biochemical characteristics of our isolate differ from those of *Anoxybacillus flavithermus* DSM 2641^T. In addition, Daffonchio *et al.* (1998) showed that the 16S–23S ITS of *Bacillus cereus* are well conserved in terms of length; in contrast, bacilli such as *Bacillus licheniformis* and *Bacillus subtilis* have at least two different ITS fingerprints. In this study, we showed that G2^T has a different ITS fingerprint from *Anoxybacillus flavithermus* DSM 2641^T and *S. caldoxylosilyticus* DSM 12041^T. As a result of the ITS study, we suggest that our isolate is different from *Anoxybacillus flavithermus*.

On the basis of 16S rRNA sequence analysis, these thermophilic isolates resemble *Anoxybacillus flavithermus*, but a DNA–DNA hybridization study performed between G2^T and *Anoxybacillus flavithermus* showed that this isolate is only 53·4 % similar to *Anoxybacillus flavithermus*. Since the novel isolate was found to be closely related genetically to *Anoxybacillus flavithermus*, we conclude that our novel isolates belong to the genus *Anoxybacillus*. The genus has one other species, *Anoxybacillus pushchinoensis*. In this study, we found 45 % similarity between G2^T and *Anoxybacillus pushchinoensis* on the basis of DNA–DNA hybridization. Wayne *et al.* (1987) suggested that strains of a species show more than 70 % DNA–DNA relatedness, indicating that strain G2^T and *Anoxybacillus pushchinoensis* represent different species.

On the basis of these data, we suggest that our thermophilic isolate (G2^T) is not related to either *Anoxybacillus flavithermus* DSM 2641^T or *Anoxybacillus pushchinoensis* DSM 12423^T at the species level (in view of threshold value of 70 % recommended by Wayne *et al.*, 1987), and we propose that strain G2^T should be placed in the genus *Anoxybacillus* as the type strain a novel species, *Anoxybacillus gonensis* sp. nov.

On the basis of their morphological, physiological, biochemical and fatty acid profiles and 16S rRNA sequences, the other six novel isolates are strains of *Anoxybacillus gonensis*. Although there are some differences between A4, A5, A7 and G2^T in terms of ITS patterns and total protein profiles, DNA–DNA hybridization also indicated that all seven novel isolates are strains of *Anoxybacillus gonensis* (Table 2).

Description of *Anoxybacillus gonensis* sp. nov.

Anoxybacillus gonensis (gon.en′sis. N.L. masc. adj. *gonensis* pertaining to Gonen, a hot spring in the province of Balikesir, Turkey, where the type strain was isolated).

Cells are rod-shaped, Gram-positive, motile and spore-forming, 0·75 × 5·0 μm. Forms terminal spherical endospores. Colonies are rough and cream in colour. Weakly catalase-positive. Oxidase-positive. Starch and gelatin are hydrolysed. Glucose, glycogen, raffinose, sucrose, xylose, fructose and mannitol are utilized. Nitrate is not reduced to nitrite. Urease, indole and H_2S are not produced. Grows in 4 % NaCl broth. The pH range for growth is 6·0–10·0 (optimum pH 7·5–8·0). The temperature range for growth is 40–70 °C (optimum 55–60 °C). Facultative anaerobe. The G + C content of the DNA is 57 mol% (by melting temperature).

The type strain, G2^T (= NCIMB 13933^T = NCCB 100040^T), was isolated from Gonen hot spring, Turkey.

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