Anoxybacillus ayderensis sp. nov. and *Anoxybacillus kestanbolensis* sp. nov.

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Two thermophilic bacilli were isolated from mud and water samples of the Ayder and Kestanbol hot springs in the provinces of Rize and Canakkale, respectively, in Turkey. Strains AB04^T and K4^T were sporulating, Gram-positive, rod-shaped bacteria. These isolates were moderately thermophilic (with an optimum temperature for growth of 50–55 °C), facultative anaerobes able to grow on a wide range of carbon sources including D-glucose, D-raffinose, D-sucrose, D-xylose, D-fructose, L-arabinose, maltose, D-mannose and D-mannitol. Analysis of the 16S rRNA gene sequences showed that these isolates resembled *Anoxybacillus flavithermus* DSM 2641^T and *Anoxybacillus gonensis* NCIMB 13933^T. DNA–DNA hybridization data revealed that thermophilic isolate AB04^T has only 51·2 % relatedness to *A. flavithermus*, 45·1 % relatedness to *Anoxybacillus pushchinoensis* and 68·6 % relatedness to *A. gonensis*. Thermophilic isolate K4^T showed only 60·4 % relatedness to *A. flavithermus*, 42·9 % relatedness to *A. pushchinoensis* and 38·5 % relatedness to *A. gonensis*. On the basis of the DNA–DNA hybridization data, isolates AB04^T and K4^T are not related to *A. flavithermus* DSM 2641^T, *A. pushchinoensis* DSM 12423^T or *A. gonensis* NCIMB 13933^T at the species level, but show relatedness to one another of 40·5 %. On the basis of the data presented, it is proposed that strains AB04^T (=NCIMB 13972^T=NCCB 100050^T) and K4^T (=NCIMB 13971^T=NCCB 100051^T) be designated as the type strains of *Anoxybacillus ayderensis* sp. nov. and *Anoxybacillus kestanbolensis* sp. nov., respectively.

The genus *Anoxybacillus* is separate from the genus *Bacillus*, and the type species is *Anoxybacillus pushchinoensis* DSM 12423^T (Pikuta *et al.*, 2000). Pikuta *et al.* (2000) first described the type species of the genus as an obligate anaerobe. Later, Pikuta *et al.* (2003) corrected the description of the species *A. pushchinoensis* from 'obligate anaerobe' to 'aerotolerant anaerobe' and also changed the description of the genus *Anoxybacillus* from one comprising obligate anaerobes to facultative anaerobes to one comprising aerotolerant anaerobes or facultative anaerobes. At the time of writing, the genus *Anoxybacillus* contained three species: *Anoxybacillus flavithermus*, *A. pushchinoensis* Pikuta *et al.* 2000 and *Anoxybacillus gonensis* Belduz *et al.* 2003.

The present paper describes the isolation, morphology, biochemical profile, 16S rRNA gene sequence and results of DNA–DNA hybridization with close relatives of two facultatively anaerobic, moderately thermophilic, facultatively alkaliphilic isolates that represent novel species of the genus *Anoxybacillus*. Strain AB04^T (pH range 6·0–11·0) is proposed as the type strain of *Anoxybacillus ayderensis* sp. nov. Strain K4^T (pH range 6·0–10·5) is proposed as the type strain of *Anoxybacillus kestanbolensis* sp. nov.

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The GenBank accession numbers for the 16S rRNA gene sequences of strains AB04^T and K4^T are AF001963 and AY248711.

Isolation of strains

Two strains of Gram-positive rods, strains AB04^T and K4^T, were isolated from mud and water samples of the Ayder and Kestanbol hot springs in the provinces of Rize and Canakkale, respectively, in Turkey. The water temperature of these hot springs is around 60–70 °C. After collection, mud and water samples were immediately used for enrichment in nutrient broth (NB) at 60–70 °C. One-day-old enrichment cultures were repeatedly subcultured in 10 ml NB and streaked on agar plates to obtain separate colonies. The purity of the isolates was assessed by using colony morphology and microscopy. After 48 h growth on nutrient agar medium, colonies of strain AB04^T were 1–2 mm in diameter, cream, regular in shape with round edges. Colonies of strain K4^T were the same except that they were 1–2·5 mm in diameter. Light microscopy revealed that cells of strains AB04^T and K4^T were motile and were, respectively, 0·55 × 4·60 μm and 0·65 × 4·75 μm in size.

Biochemical and nutritional characteristics

The utilization of organic compounds as sole carbon sources was tested in basal medium (5 ml) supplemented with 0·5 % (w/v) concentrations of the following compounds (which had been separately sterilized as stock solutions): D-glucose, D-mannitol, D-mannose, D-sucrose, D-xylose, L-arabinose, D-fructose, lactose, D-raffinose, starch and L-rhamnose. Incubation was carried out at 60 °C. Strains AB04^T and K4^T were nutritionally versatile and used a wide variety of carbohydrates when grown on basal medium. Strain AB04^T grew on D-glucose, D-raffinose, D-sucrose, D-xylose, D-fructose, L-arabinose, maltose and D-mannose, while strain K4^T grew on D-mannitol, D-glucose, D-fructose, maltose, D-mannose, D-raffinose and D-sucrose (Table 1). Anaerobic growth was tested in anaerobic agar medium. Strains AB04^T and K4^T grew well aerobically but are facultatively anaerobic bacteria. Strain K4^T grew well on anaerobic agar medium without yeast extract but strain AB04^T grew on anaerobic agar medium only when supplemented with yeast extract.

The temperature range for growth (30–75 °C) and the pH range for growth (5·0–11·0) were determined in NB medium. Media were adjusted to the initial pH indicated with either 1 M NaOH or 1 M HCl. Strain AB04^T grew well at 30–70 °C, with optimum growth at 50 °C, and grew well at pH 6·0–11·0, with optimum growth at 7·5–8·5. Strain K4^T grew well at 40–70 °C, with optimum growth at 50–55 °C, and grew well at pH 6·0–10·5, with optimum growth at pH 7·5–8·5. Catalase and oxidase were detected by using the method of Cowan & Steel (1974); strains AB04^T and K4^T were catalase- and oxidase-positive.

Salt and antibiotic sensitivity

Four sets of NB were prepared containing NaCl at 1, 1·5, 2, 2·5, 3, 4, 5 and 7 %. The growth of the isolates at different salt concentrations was tested using NB as organic substrate and a control broth without any NaCl supplementation. Growth of strain AB04^T and growth of strain K4^T were inhibited in the presence of NaCl concentrations above 2·5 and 4·0 %, respectively, and in the presence of ampicillin (25 μg ml^{-1}), streptomycin sulphate (25 μg ml^{-1}), tetracycline (12·5 μg ml^{-1}), gentamicin (10 μg ml^{-1}) and kanamycin (10 μg ml^{-1}). The optimal NaCl concentrations for growth of AB04^T and K4^T were 1·5 and 2·5 %, respectively.

Spore formation

The formation of spores was tested for by using microscopic observation of both liquid cultures and single colonies of the isolates from agar plates at different incubation periods. Incubation periods of 1–2 days were required before spore formation became detectable on

Table 1. Physiological and biochemical properties of strains AB04^T and K4^T and *Anoxybacillus* type strains

Strains: 1, AB04^T; 2, K4^T; 3, *A. gonensis* NCIMB 13933^T; 4, *A. flavithermus* DSM 2641^T; 5, *A. pushchinoensis* DSM 12423^T. Cells of both novel taxa are sporulating rods, both novel taxa show anaerobic growth and oxidase activity and both novel taxa are positive for the utilization of starch, D-sucrose, D-glucose and D-fructose. ND, No data available; W, Weak growth.

Characteristic	1	2	3	4	5
DNA G+C content (mol%)	54	50	57	41·6	42
Temperature (°C) for growth:					
Range	30–70	40–70	40–70	30–72	37–66
Optimum	50	50–55	55–60	60–65	62
pH for growth:					
Range	6·0–11·0	6·0–10·5	6·0–10·0	5·5–9·0	8–10·5
Optimum	7·5–8·5	7·5–8·5	7·5–8·0	7·0	9·5–9·7
Maximum NaCl concentration for growth (%)	2·5	4·0	4·0	2·5	3·0
Carbon sources tested (in BM):					
D-Raffinose	+	+	+	−	ND
D-Xylose	+	−	+	−	ND
L-Arabinose	+	−	−	w	ND
D-Mannose	+	+	−	+	ND
L-Rhamnose	−	−	−	−	ND
D-Mannitol	+	+	+	+	ND
Lactose	−	−	−	−	ND
Nitrate reduction	−	+	+	−	+
Hydrolysis of gelatine	+	−	+	−	−

agar plates. Light microscopy revealed that strains AB04^T and K4^T were sporulating bacilli. Cells of strains AB04^T and K4^T formed terminal, spherical endospores.

SDS-PAGE analysis

Extraction of proteins from growing cells, measurement of protein concentrations in the extracts, electrophoresis and staining of proteins bands were performed as described previously (Belduz *et al.*, 2003). The electrophoretic patterns of the soluble cellular proteins, as determined by the PAGE method (Fig. 1), showed that AB04^T and K4^T are not similar to *A. flavithermus* DSM 2641^T, *A. pushchinoensis* DSM 12423^T, *A. gonensis* NCIMB 13933^T or each other (Fig. 1).

16S rRNA gene sequence analysis

The 16S rRNA genes were selectively amplified and cloned into the pGEM-T vector system and then the gene sequences were determined and compared with the 16S rRNA gene sequences of some representatives of the *Bacillus* group, as described previously (Belduz *et al.*, 2003). On the basis of 16S rRNA gene sequence analysis, AB04^T has more than 98 % similarity to the sequences of *A. gonensis* NCIMB 13933^T and *A. flavithermus* DSM 2641^T, and has 97 % similarity to *A. pushchinoensis* DSM 12423^T. The 16S rRNA gene sequence of K4^T exhibits 97 % similarity to that of *A. flavithermus* DSM 2641^T and more than 96 % sequence similarity to *A. gonensis* NCIMB 13933^T and *A. pushchinoensis* DSM 12423^T.

16S rRNA gene sequences obtained from the GenBank database were aligned and a neighbour-joining phylogenetic tree was constructed by using DNADIST and NEIGHBOR programs implemented as part of the PHYLIP package (Felsenstein, 1993). Phylogenetic analysis revealed a clustering of K4^T and AB04^T in the same branch with other *Anoxybacillus* species (Fig. 2).

G+C content and DNA–DNA hybridization analyses

Extraction, purification and determination of the G+C content of DNA were performed as described previously (Belduz *et al.*, 2003). The G+C contents of strains AB04^T and K4^T are 54 and 50 mol%, respectively, which are lower than those of *A. flavithermus* DSM 2641^T and *A. gonensis* NCIMB 13933^T.

On the basis of 16S rRNA gene sequence analysis, isolates AB04^{T}and K4^T showed $\geqslant$97 % similarity to other *Anoxybacillus* species; therefore, a DNA–DNA hybridization study was performed among AB04^T, K4^T, *Anoxybacillus flavithermus* DSM 2641^T, *Anoxybacillus gonensis* NCIMB 13933^T and *Anoxybacillus pushchinoensis* DSM 12423^T. Isolation of genomic DNA for DNA–DNA hybridization and determination of DNA–DNA hybridization were performed as described previously (Belduz *et al.*, 2003). DNA–DNA hybridization performed between AB04^T and *A. gonensis* NCIMB 13933^T revealed 68·6 % relatedness. However, strain AB04^T differs from *A. gonensis* NCIMB 13933^T in its growth temperature range and optimum, pH range and optimum, NaCl tolerance, reduction of nitrate to nitrite and utilization of some sugars as carbon source. Thermophilic

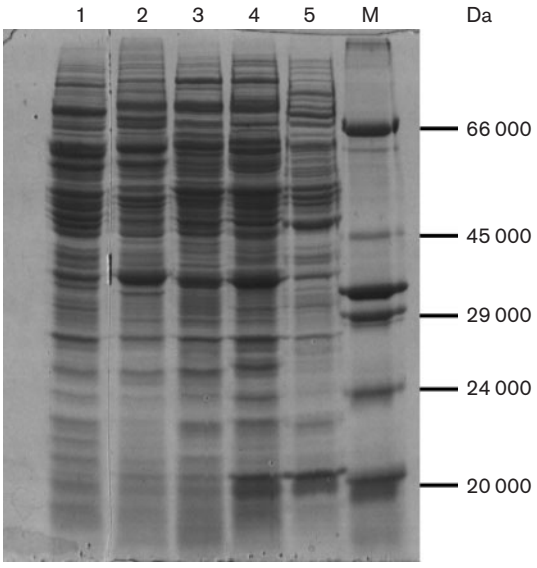

Fig. 1. SDS-PAGE whole-cell protein profiles of *A. gonensis* NCIMB 13933^T (lane 1), *A. kestanbolensis* sp. nov. K4^T (2), *A. ayderensis* sp. nov. AB04^T (3), *A. flavithermus* DSM 2641^T (4) and *A. pushchinoensis* DSM 12423^T (5). Lane M, markers.

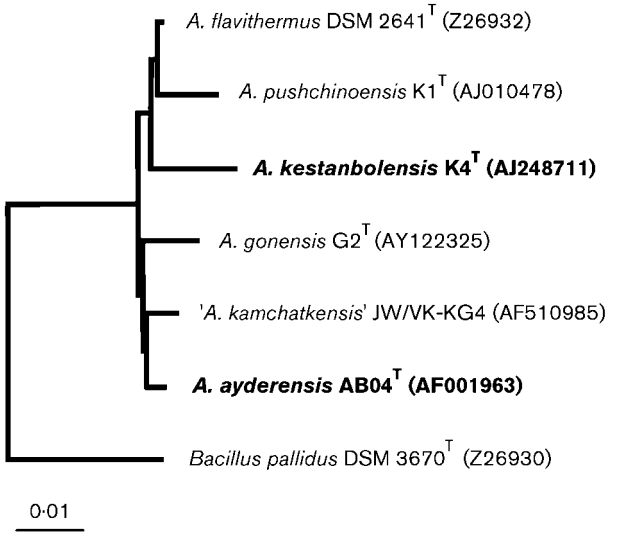

Fig. 2. Phylogenetic relationships of *Anoxybacillus* species, showing the positions of the novel isolates. Neighbour-joining tree based on 16S rRNA gene sequences. Bar, 1 substitution per 100 nucleotide positions.

isolate K4^T showed similarity to *A. flavithermus* DSM 2641^T, but DNA–DNA hybridization performed between K4^T and *A. flavithermus* DSM 2641^T showed only 60·4 % relatedness.

As the novel isolates were found to be closely related genetically to *A. gonensis* NCIMB 13933^T and *A. flavithermus* DSM 2641^T, we decided that they belong to the genus *Anoxybacillus*. The genus contains another species, *A. pushchinoensis*. In this study, DNA–DNA hybridization revealed 45·1 % DNA–DNA relatedness between AB04^T and *A. pushchinoensis* and 42·9 % DNA–DNA relatedness between K4^T and *A. pushchinoensis*. On the basis of DNA–DNA hybridization, strains AB04^T and K4^T show 40·5 % relatedness (Table 2). Wayne *et al.* (1987) suggested that relatedness levels below 70 % indicate that strains belong to different species.

Cellular fatty acids

Cultivation, harvesting, preparation and analysis of cellular fatty acid methyl esters (FAMEs) from whole-cell fatty acids from strain AB04^T, strain K4^T, *A. flavithermus* DSM 2641^T and *A. gonensis* NCIMB 13933^T were performed using the Sherlock Microbial Identification System, version 4.0, according to the instructions of the manufacturer (MIDI). FAME profiles of strain AB04^T, strain K4^T, *A. flavithermus* DSM 2641^T and *A. gonensis* NCIMB 13933^T were identified by comparing the commercial M17H10 database with the MIS software package (version 3.8; Microbial ID) (Table 3). The FAME profiles of AB04^{T}and K4^T show that the main fatty acid is $C_{15:0}$ iso (48·17 and 68·62 %, respectively); $C_{15:0}$ iso is also the main fatty acid in *A. gonensis*, *A. pushchinoensis* and *A. flavithermus*. AB04^T, K4^T, *A. flavithermus* DSM 2641^T and *A. gonensis* NCIMB 13933^T contain $C_{17:0}$ iso as a major fatty acid, but *A. pushchinoensis* DSM 12423^T differs by having $C_{16:0}$ as a major component. This indicates that there are similarities among the FAME profiles of *Anoxybacillus* species, including strains AB04^T and K4^T, which also share approximately $\geqslant$97 % 16S rRNA sequence similarity.

On the basis of these data, we suggest that thermophilic isolates AB04^T and K4^T are not related to *A. gonensis* NCIMB 13933^T, *A. flavithermus* DSM 2641^T or *A. pushchinoensis* DSM 12423^T at the species level, and we propose that they be placed in the genus *Anoxybacillus* as *Anoxybacillus ayderensis* sp. nov. (strain AB04^T) and *Anoxybacillus kestanbolensis* sp. nov. (strain K4^T).

Table 2. DNA–DNA relatedness (%)

Strain	K4^T	AB04^T
AB04^T	40·5	–
A. gonensis NCIMB 13933^T	38·5	68·6
A. flavithermus DSM 2641^T	60·4	51·2
A. pushchinoensis DSM 12423^T	42·9	45·1

Table 3. Fatty acid profiles of strains AB04^T and K4^T and *Anoxybacillus* type strains

Strains: 1, AB04^T; 2, K4^T; 3, *A. gonensis* G2^T; 4, *A. flavithermus* DSM 2641^T; 5, *A. pushchinoensis* DSM 12423^T. Values are percentages of total fatty acids. Data for *A. pushchinoensis* were taken from Pikuta *et al.* (2000).

Fatty acid	1	2	3	4	5
$C_{12:0}$	–	–	–	–	6·9
$C_{14:0}$ iso	–	0·88	1·25	–	–
$C_{14:0}$	1·02	1·29	1·18	1·96	7·3
$C_{15:0}$ iso	48·17	68·62	65·19	54·85	38·7
$C_{15:0}$ anteiso	3·58	3·56	2·64	4·02	2·0
$C_{15:0}$	0·83	1·11	1·12	1·18	0·9
$C_{16:0}$ iso	7·47	6·37	5·99	2·97	0·3
$C_{16:1}$	–	–	–	–	2·6
$C_{16:0}$	9·10	3·47	2·38	11·13	14·5
$C_{16:0}$ 10-methyl	–	–	–	–	0·9
$C_{17:1}\omega 5c$	–	0·59	2·63	–	–
$C_{15:0}$ iso OH	–	–	–	–	0·3
$C_{17:0}$ anteiso A	–	–	0·82	–	–
$C_{17:0}$ iso	20·62	9·54	11·96	17·74	0·8
$C_{17:0}$	–	–	–	–	0·5
$C_{17:0}$ anteiso	9·22	3·69	3·29	6·15	0·1
$C_{18:2}$	–	–	–	–	2·2
$C_{18:1\delta 9}$	–	–	–	–	4·3
$C_{18:1\delta 1}$	–	–	–	–	1·0
$C_{18:0}$	–	–	–	–	10·4
$C_{20:0}$	–	–	–	–	0·6

Description of *Anoxybacillus ayderensis* sp. nov.

Anoxybacillus ayderensis (ay.de.ren′sis. N.L. masc. adj. *ayderensis* pertaining to Ayder, a hot spring in the province of Rize, Turkey, where the type strain was isolated).

Cells are Gram-positive, motile, spore-forming rods, 0·55 × 4·60 μm in size. Terminal, spherical endospores are formed. Colonies are 1–2 mm in diameter, cream, regular in shape with round edges. Catalase- and oxidase-positive. Starch and gelatin are hydrolysed. D-Glucose, D-raffinose, D-sucrose, D-xylose, D-fructose, L-arabinose, maltose and D-mannose are utilized. Nitrate is reduced to nitrite. Urease, indole and H_2S are not produced. Growth occurs in the absence of NaCl; optimum growth at 1·5 % NaCl. No growth at concentrations above 2·5 % NaCl. The pH range for growth is 6·0–11·0; optimum pH is 7·5–8·5. Growth is inhibited in the presence of ampicillin (25 μg ml^{-1}), streptomycin sulphate (25 μg ml^{-1}), tetracycline (12·5 μg ml^{-1}), gentamicin (10 μg ml^{-1}) and kanamycin (10 μg ml^{-1}). The temperature range for growth is 30–70 °C; optimum growth at 50 °C. Facultative anaerobe. DNA G+C content is 54 mol% (by melting temperature).

The type strain, AB04^T (=NCIMB 13972^T=NCCB 100050^T), was isolated from Ayder Hot Spring, Turkey.

Description of *Anoxybacillus kestanbolensis* sp. nov.

Anoxybacillus kestanbolensis (kes.tan.bo.len′sis. N.L. masc. adj. *kestanbolensis* pertaining to Kestanbol, a hot spring in the province of Canakkale, Turkey, where the type strain was isolated).

Cells are Gram-positive, motile, spore-forming rods, 0·65 × 4·75 μm in size. Terminal, spherical endospores are formed. Colonies are 1–2·5 mm in diameter, cream, regular in shape with round edges. Catalase- and oxidase-positive. Starch is hydrolysed but gelatin is not. D-Mannitol, D-glucose, D-fructose, maltose, D-mannose, D-raffinose and D-sucrose are utilized. Nitrate is reduced to nitrite. Urease, indole and H_2S are not produced. Growth occurs in the absence of NaCl; optimum growth at 2·5 % NaCl. No growth at concentrations above 4 % NaCl. The pH range for growth is 6·0–10·5; optimum pH is 7·5–8·5. Growth is inhibited in the presence of ampicillin (25 μg ml^{-1}), streptomycin sulphate (25 μg ml^{-1}), tetracycline (12·5 μg ml^{-1}), gentamicin (10 μg ml^{-1}) and kanamycin (10 μg ml^{-1}). The temperature range for growth is 40–70 °C; optimum at 50–55 °C. Facultative anaerobe. DNA G+C content is 50 mol% (by melting temperature).

The type strain, K4^T (=NCIMB 13971^T=NCCB 100051^T), was isolated from Kestanbol Hot Spring, Turkey.

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