Anoxybacillus voinovskiensis sp. nov., a moderately thermophilic bacterium from a hot spring in Kamchatka

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A novel moderately thermophilic bacterium, strain TH13^T, was isolated from a hot spring in Kamchatka. It was found to be a Gram-positive, facultative aerobe; the straight, non-motile rods grew at 30–64 °C (optimum 54 °C). The isolate was positive for catalase and oxidase tests and reduced nitrate to nitrite, but was negative for H_2S production and growth in more than 3 % NaCl (w/v). The isolate grew at pH 7–8, but not at pH values higher than 9. The DNA G+C content was 43·9 mol%. Phylogenetic analysis based on 16S rRNA gene sequencing indicated that strain TH13^T was a member of the genus *Anoxybacillus*. DNA–DNA hybridization revealed a low relatedness (less than 30·2 %) between the isolate and its close phylogenetic neighbours *Anoxybacillus pushchinoensis* and *Anoxybacillus flavithermus*. On the basis of phenotypic characteristics, phylogenetic data and DNA–DNA hybridization data, it was concluded that the isolate merited classification as a novel species, for which the name *Anoxybacillus voinovskiensis* sp. nov. is proposed. The type strain of this species is TH13^T (=NCIMB 13956^T=JCM 12111^T).

There have been several reports in the last few years on the microflora of hot-spring biomats (Yamamoto *et al.*, 1998; Hiraishi *et al.*, 1999; Nübel *et al.*, 2002). Research on hot-spring biomats represents a rather appropriate case study for determining microbial ecosystems in environments with extreme temperatures. It has been reported that the isolation of micro-organisms from such biomats is difficult (Hiraishi *et al.*, 1999). Therefore, several non-culture methods have been applied to the analysis of microbial flora in hot-spring biomats (Yamamoto *et al.*, 1998; Hiraishi *et al.*, 1999). Nevertheless, there have been several attempts at isolating micro-organisms from hot-spring biomats: in the course of such studies, a number of micro-organisms have been isolated and identified (Pierson *et al.*, 1985; Hanada *et al.*, 1995, 2002).

In 1978, drilling opened up the Voinovskie Hot Springs in Kamchatka, Russia. Prior to this, no hot springs had been found in this area. Natural springs are located 300–400 m upstream of the Mutnovskaya River on both banks. In Voinovskie Hot Springs, hair-like microbial vegetation, called 'Veronica's hair', was observed. We began to investigate the bacterial flora of this vegetation. By using light microscopic observation of a sample obtained from the hot spring, we observed that the relatively larger cells appeared dominant.

In this study, a relatively large, moderately thermophilic micro-organism among our isolates was isolated from a biomat sample obtained from the Voinovskie Hot Springs. Phenotypic characterization, phylogenetic analysis based on 16S rRNA gene sequences and DNA–DNA hybridization

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A more complete version of the phylogenetic tree presented in Fig. 1 is available as supplementary material in IJSEM Online.

with close relatives showed that the isolate merited classification as a novel *Anoxybacillus* species.

Water samples collected from Voinovskie Hot Springs in Kamchatka, Russia, were plated on TH agar medium (pH 7·5) consisting of the following (per litre distilled water): 5 g peptone (Kyokuto), 3 g yeast extract (Kyokuto), 5 g NaCl, 15 g agar, 3·5 mg EDTA, 3 mg $ZnSO_4.7H_2O$, 10 mg $FeSO_4.7H_2O$, 2 mg $MnSO_4.nH_2O$, 1 mg $CuSO_4.5H_2O$, 2 mg $Co(NO_3)_2.6H_2O$ and 1 mg H_3BO_3. The cultures were then incubated aerobically at 70 °C for 24 h. This isolate was picked and subcultured five times for purification and maintained on TH agar medium at 70 °C. In addition to the isolate, *Anoxybacillus flavithermus* NBRC 15317^T (=IFO 15317^T) and *Anoxybacillus pushchinoensis* DSM 12423^T were used as reference strains for DNA–DNA hybridization experiments. These microorganisms were cultivated in TH broth in a rotary shaker at 105 r.p.m. and 50 °C.

For phenotypic characterization, TH medium was used as the basal medium. The isolate was incubated at 50 °C for 1 week and all the experiments were performed three times. Acid production from carbohydrates was determined by the method of Hugh & Leifson (1953). Growth experiments at pH 7–10 were performed using TH medium containing 100 mM NaH_2PO_4/Na_2HPO_4 buffer (pH 7–8) or 100 mM $NaHCO_3/Na_2CO_3$ buffer (pH 9–10). Anaerobic growth was tested on a TH plate by substituting air with argon gas. Other physiological and biochemical characteristics were examined according to Kawasaki *et al.* (2002) and as described in Barrow & Feltham (1993).

For the morphological observation of negatively stained cells under a transmission electron microscope and of platinum- and palladium-coated cells under a scanning electron microscope, cells were grown on a TH agar slant for 1 day. The transmission electron microscope and scanning electron microscope preparations and observations were performed as described previously (Yumoto *et al.*, 2000).

Analyses of whole-cell fatty acids and isoprenoid quinones were performed as described previously (Yumoto *et al.*, 2001, 2002).

Bacterial DNA was prepared according to Marmur (1961). DNA base composition was determined according to Tamaoka & Komagata (1984). The level of DNA–DNA hybridization was determined fluorometrically according to Ezaki *et al.* (1989), using photobiotin-labelled DNA probes and black microplates.

16S rRNA gene sequence analysis was performed as reported previously (Yumoto *et al.*, 2002). Multiple alignments of the sequence were performed and the nucleotide substitution rate (K_{nuc}) was calculated. A phylogenetic tree was constructed by the neighbour-joining method (Kimura, 1980; Saitou & Nei, 1987) using the CLUSTAL W program (Thompson *et al.*, 1994). Sequence similarity was calculated using the GENETYX computer program (Software Development).

The morphological, physiological and biochemical characteristics of the isolate are given in the species description. The isolate was revealed to be Gram-positive and moderately thermophilic (optimum temperature 54 °C). Electron microscopic observation showed that cells were non-flagellated, straight rods (0·4–0·6 × 1·5–5·0 μm). The cell surface was smooth when observed using scanning electron microscopy. No pronounced structures were observed under transmission electron microscopy of thin sections.

GLC analysis of the fatty acids of strain TH13^T revealed that the major components were $isoC_{14:0}$ (1·3 %), $C_{14:0}$ (1·3 %), $isoC_{15:0}$ (54·7 %), $anteisoC_{15:0}$ (8·0 %), $isoC_{16:0}$ (7·1 %), $C_{16:0}$ (1·9 %), $isoC_{17:0}$ (3·9 %), $anteisoC_{17:1}$ (7·1 %) and $C_{17:1}$ (2·6 %).

The 16S rRNA gene sequence of strain TH13^T was analysed to determine its phylogenetic position. The sequence of 1506 bases of the 16S rRNA gene of strain TH13^T was compared with those of two other *Anoxybacillus* spp. and other related taxa. The phylogenetic tree constructed using the neighbour-joining method (Fig. 1) and the 16S rRNA gene sequence similarity showed that strain TH13^T is a member of the genus *Anoxybacillus*. The highest similarity values were observed with *A. flavithermus* DSM 2641^T (95·7 %), *Anoxybacillus gonensis* NCIMB 13933^T (94·8 %) and *A. pushchinoensis* DSM 12423^T (94·5 %). These results demonstrate that strain TH13^T is apparently distinct from other *Anoxybacillus* spp.

The DNA G+C content of strain TH13^T was 43·9 mol%, which is similar to that of species phylogenetically related to this strain.

According to the results of the 16S rRNA gene sequence analysis, strain TH13^T is closely related to *A. flavithermus* DSM 2641^T and *A. pushchinoensis* DSM 12423^T. Therefore, DNA–DNA hybridization between strain TH13^T and closely related strains, given above, was estimated. DNA–DNA relatedness data indicated that the isolate is distinct from *A. flavithermus* NBRC 15317^T (20·6 % similarity) and *A. pushchinoensis* DSM 12423^T (30·2 % similarity).

Strain TH13^T differed from other relatively closely related species in terms of the following phenotypic characteristics: catalase production, hydrolysis of casein and starch, reduction of NO_3^- to NO_2^-, pH range for growth, and utilization of substrates (Table 1).

On the basis of the above results, the isolate was designated as a new species for which the name *Anoxybacillus voinovskiensis* sp. nov. is proposed; the type strain is TH13^T. A description of the new species is given below.

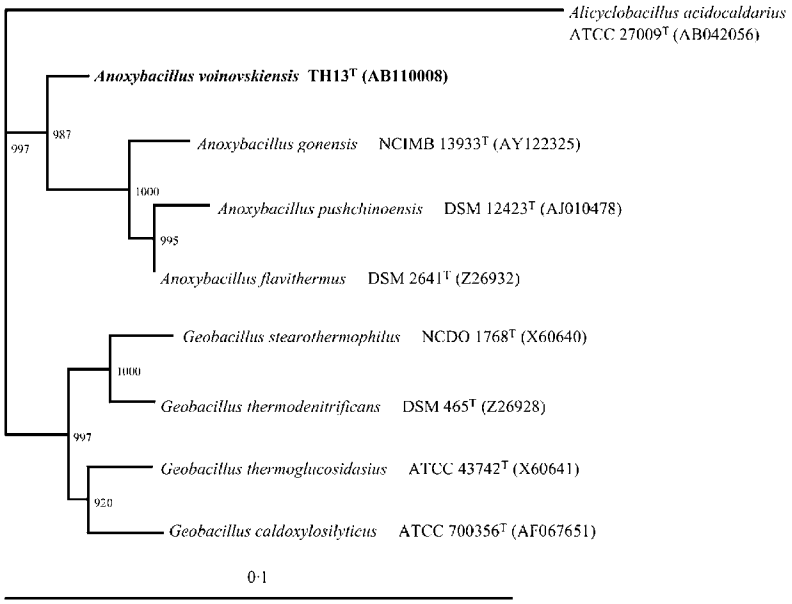

Fig. 1. Phylogenetic tree constructed on the basis of 16S rRNA gene sequence data of *A. voinovskiensis* TH13^T and other related organisms, using the neighbour-joining method. Bootstrap analysis shows the robustness of the branching. Only the nearest neighbours are shown here; a more complete tree is available as supplementary material in IJSEM Online. Bar, 0·01 K_{nuc} unit.

Description of *Anoxybacillus voinovskiensis* sp. nov.

Anoxybacillus voinovskiensis (vo.in.ov.ski.en′sis. N.L. adj. *voinovskiensis* from Voinovskie, referring to the Voinovskie Hot Springs, the place of isolation).

Cells are Gram-positive, non-flagellated, straight rods (0·4–0·6 × 1·5–5·0 μm). It is facultatively aerobic. Colonies are circular and pale cream in colour. Catalase and oxidase reactions are positive. Negative for H_2S production and hydrolysis of casein, gelatin, starch, DNA, Tween 20 and Tween 80. Hydrolyses Tween 40 and Tween 60. Growth occurs at pH 7–8, but not at pH 9–10. Growth occurs at 30–64 °C with the optimum temperature at 54 °C. Grows in the absence of NaCl, but not at concentrations higher than 3 % (w/v). Nitrate is reduced to nitrite. Acid is produced from D-glucose, D-xylose, D-arabinose, D-fructose, maltose, D-mannose, sucrose, sorbitol and cellobiose in aerobic conditions. No acid is produced from D-galactose, raffinose, melibiose, inositol, mannitol, trehalose,

Table 1. Characteristics of *A. voinovskiensis* and related species

+, Positive; −, negative; NA, not available. Data for *A. flavithermus* from Heinen *et al.* (1982); for *A. pushchinoensis* from Pikuta *et al.* (2000); for *A. gonensis* from Belduz *et al.* (2003).

Characteristic	*A. voinovskiensis* TH13^T	*A. flavithermus* DSM 2641^T	*A. pushchinoensis* DSM 12423^T	*A. gonensis* NCIMB 13933^T
Size (μm)	0·4–0·6 × 1·5–5·0	0·85 × 2·3–7·1	0·5–0·6 × 3·0–5·0	0·75 × 5·0
Motility	−	−	+	+
Catalase	+	+	−	+
Reduction of NO_3^- to NO_2^-	+	+	+	−
Hydrolysis of:				
Casein	−	+	−	NA
Gelatin	−	−	−	+
Starch	−	+	−	+
Growth temperature range (°C)	30–64	30–72	31–66	40–70
Optimum growth temperature (°C)	54	60–65	62	55–60
Growth pH range	7·0–8·0	5·5–9·0	9·5–9·7	6·0–10·0
O_2 metabolism	Facultative aerobe	Facultative aerobe	Anaerobe	Facultative aerobe
Utilization of:				
D-Glucose	+	−	+	+
Sucrose	+	−	+	+
DNA G+C content (mol%)	43·9	61·0	42·2	57

L-rhamnose or lactose in aerobic conditions. The DNA G+C content is 43·9 mol%, as determined by HPLC. Strain TH13^T was isolated from a water sample collected from a hot spring in Kamchatka.

The type strain is TH13^T (=NCIMB 13956^T=JCM 12111^T).

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References

Barrow, G. I. & Feltham, R. K. A. (editors) (1993). *Cowan and Steel's Manual for the Identification of Medical Bacteria*, 3rd edn. Cambridge: Cambridge University Press.

Belduz, A. O., Dulger, S. & Demirbag, Z. (2003). *Anoxybacillus gonensis* sp. nov., a moderately thermophilic, xylose-utilizing, endospore-forming bacterium. *Int J Syst Evol Microbiol* **53**, 1315–1320.

Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in micro-dilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.

Hanada, S., Hiraishi, A., Shimada, K. & Matsuura, K. (1995). *Chloroflexus aggregans* sp. nov., a filamentous phototrophic bacterium which forms dense cell aggregates by active gliding movement. *Int J Syst Bacteriol* **45**, 676–681.

Hanada, S., Takaichi, S., Matsuura, K. & Nakamura, K. (2002). *Roseiflexus castenholzii* gen. nov., sp. nov., a thermophilic, filamentous, photosynthetic bacterium that lacks chlorosomes. *Int J Syst Evol Microbiol* **52**, 187–193.

Heinen, W., Lauwers, A. M. & Mulders, J. W. M. (1982). *Bacillus flavothermus*, a newly isolated facultative thermophile. *Antonie van Leeuwenhoek* **48**, 265–272.

Hiraishi, A., Umezawa, T., Yamamoto, H., Kato, K. & Yonosuke, M. (1999). Changes in quinone profiles of hot spring microbial mats with a thermal gradient. *Appl Environ Microbiol* **65**, 198–205.

Hugh, R. & Leifson, E. (1953). The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various Gram-negative bacteria. *J Bacteriol* **66**, 24–26.

Kawasaki, K., Nogi, Y., Hishinuma, M., Nodasaka, Y., Matsuyama, H. & Yumoto, I. (2002). *Psychromonas marina* sp. nov., a novel halophilic, facultatively psychrophilic bacterium isolated from the coast of the Okhotsk Sea. *Int J Syst Evol Microbiol* **52**, 1455–1459.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.

Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from micro-organisms. *J Mol Biol* **3**, 208–218.

Nübel, U., Bateson, M. M., Vandieken, V., Wieland, A., Kühl, M. & Ward, D. M. (2002). Microscopic examination of distribution and phenotypic properties of phylogenetically diverse *Chloroflexaceae*-related bacteria in hot spring microbial mat. *Appl Environ Microbiol* **68**, 4593–4603.

Pierson, B. K., Giovannoni, S. J., Stahl, D. A. & Castenholz, R. W. (1985). *Heliothrix oregonensis*, gen. nov., a phototrophic filamentous gliding bacterium containing bacteriochlorophyll *a*. *Arch Microbiol* **142**, 164–167.

Pikuta, E., Lysenko, A., Chuvilskaya, N., Mendrock, U., Hippe, H., Suzina, N., Nikitin, D., Osipov, G. & Laurinavichius, K. (2000). *Anoxybacillus pushchinensis* gen. nov., sp. nov., a novel anaerobic bacterium from manure, and description of *Anoxybacillus flavithermus* comb. nov. *Int J Syst Evol Microbiol* **50**, 2109–2117.

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

Tamaoka, J. & Komagata, K. (1984). Determination of DNA base composition by reversed-phase high-performance liquid chromatography. *FEMS Microbiol Lett* **25**, 125–128.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.

Yamamoto, H., Hiraishi, A., Kato, K., Chiura, H. X., Maki, Y. & Shimizu, A. (1998). Phylogenetic evidence for the existence of novel thermophilic bacteria in hot spring sulfur-turf microbial mats in Japan. *Appl Environ Microbiol* **64**, 1680–1687.

Yumoto, I., Yamazaki, K., Hishinuma, M., Nodasaka, Y., Inoue, N. & Kawasaki, K. (2000). Identification of facultatively alkaliphilic *Bacillus* sp. strain YN-2000 and its fatty acid composition and cell-surface aspects depending on culture pH. *Extremophiles* **4**, 285–290.

Yumoto, I., Yamazaki, K., Hishinuma, M., Nodasaka, Y., Suemori, A., Nakajima, K., Inoue, N. & Kawasaki, K. (2001). *Pseudomonas alcaliphila* sp. nov., a novel facultatively psychrophilic alkaliphile isolated from seawater. *Int J Syst Evol Microbiol* **51**, 349–355.

Yumoto, I., Nakamura, A., Iwata, H., Kojima, K., Kusumoto, K., Nodasaka, Y. & Matsuyama, H. (2002). *Dietzia psychralcaliphila* sp. nov., a novel, facultatively psychrophilic alkaliphile that grows on hydrocarbons. *Int J Syst Evol Microbiol* **52**, 85–90.