Variovorax boronicumulans sp. nov., a boron-accumulating bacterium isolated from soil

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A non-spore-forming, Gram-negative, motile, rod-shaped, boron-accumulating bacterium isolated from soil was characterized by polyphasic taxonomy. The strain, designated BAM-48^T, was positive for catalase and oxidase activities and grew at 4–37 °C, 0–1% NaCl and pH 5–9. Comparative analysis of the 16S rRNA gene sequence demonstrated that the novel isolate BAM-48^T was closely related to *Variovorax paradoxus* IAM 12373^T (99.3% sequence similarity), *Variovorax soli* GH9-3^T (98.3%) and *Variovorax dokdonensis* DS-43^T (97.0%). DNA–DNA hybridization values between strain BAM-48^T and *V. paradoxus* NBRC 15149^T, *V. soli* KACC 11579^T and *V. dokdonensis* KCTC 12544^T were only 49.1, 25.4 and 24.6%, respectively. The major respiratory quinone was ubiquinone 8 (Q-8). The DNA G + C content was 71.2 mol%. Strain BAM-48^T contained C_{16:0} (36.1%), C_{16:1ω7c} (21.4%) and C_{17:0} cyclo (19.0%) as the major fatty acids. Based on phenotypic, chemotaxonomic and phylogenetic data, strain BAM-48^T should be classified as a member of a novel species in the genus *Variovorax*, for which the name *Variovorax boronicumulans* sp. nov. is proposed. The type strain is BAM-48^T (=NBRC 103145^T =KCTC 22010^T).

Although boron is known to be an essential micronutrient for plants (Warington, 1923) and possibly for animals (Rowe *et al.*, 1998; Rowe & Eckhert, 1999), its essentiality to bacteria has been reported only recently (in the case of *Bacillus boroniphilus*; Ahmed *et al.*, 2007a). In bacteria, boron also has a biological function in quorum sensing as a structural component of a receptor complex (Chen *et al.*, 2002). On the other hand, boron is toxic to living cells when present above a certain threshold. Because of its toxicity, boron has long been used to kill *Candida* and *Saccharomyces*, the causal organisms of recurrent vulvovaginal candidiasis (Swate & Weed, 1974). However, recently, *Bacillus boroniphilus*, *Gracilibacillus boracitolerans*, *Chimaericella boritolerans* and *Lysinibacillus boronitolerans* have been reported to tolerate more than 450, 450, 300 and 150 mM boric acid, respectively (Ahmed *et al.*, 2007a, b, c, d).

The genus *Variovorax* was proposed by reclassification of *Alcaligenes paradoxus* as *Variovorax paradoxus* (Willems *et al.*, 1991). The type species of this genus is *Variovorax paradoxus* and, only recently, two other members have been characterized as *Variovorax dokdonensis* (Yoon *et al.*, 2006a) and *Variovorax soli* (Kim *et al.*, 2006).

In the course of screening for boron-accumulating bacteria, strain BAM-48^T was isolated from soil sampled from an experimental field of the University of Tokyo (Yayoi campus), Tokyo, Japan. The soil samples (5 g) were incubated in sterilized PBS (pH 7.0) at 30 °C for several days, after which the supernatant was streaked on tryptic soya agar (TSA; Difco) and incubated at 25 °C. Purified cultures of the isolates recovered were maintained on TSA medium and also maintained as glycerol (35%, w/v) stocks at –80 °C. Based upon the taxonomic characterization, we propose that strain BAM-48^T represents a novel species of the genus *Variovorax*.

Intracellular boron concentrations were studied as follows. Individual colonies of the isolate grown in 1.5 ml tryptic soya broth (TSB; Difco) (pH 7.2) containing 100 µM boric acid at 25 °C for 7 days were harvested by centrifugation

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain BAM-48^T is AB300597.

Details of boron uptake by strain BAM-48^T and related type strains are available as supplementary material with the online version of this paper.

and analysed for their ability to accumulate boric acid by the Azomethine-H staining method (Gupta & Stewart, 1975). To determine the intracellular boron concentration more precisely, cells of candidate boron-accumulating strains, grown at 25 °C for 2 days in 5 ml TSB containing 100 µM boric acid, were harvested and washed with TSB. The pellet was boiled in MilliQ water and the supernatant was analysed for boron content by inductively coupled plasma-mass spectrometer (ICP-MS) (model SII SPQ-8000A; Seiko Instruments) after digestion with boron-free nitric acid (60–61 %, w/w) in Teflon tubes. The weight of the freeze-dried pellet was also measured to calculate the intracellular boron concentration. Strain BAM-48^T contained 728 ± 88.3 nmol boric acid (g dry weight)⁻¹, which was higher than closely related species belonging to *Variovorax* (Supplementary Table S1, available in IJSEM Online). The ability to accumulate boric acid distinguished strain BAM-48^T from closely related species and BAM-48^T was therefore subjected to polyphasic characterization.

The morphology, sporangia and motility of the cells were examined under phase-contrast microscopy as described previously (Ahmed *et al.*, 2007a). Gram-staining, performed according to Hucker's modified method, showed that cells of strain BAM-48^T were Gram-negative. The temperature range for growth was determined by incubation for 2 weeks at various temperatures (4, 10, 17, 20, 25, 28, 30, 37, 45 and 50 °C) on TSA. The pH range for growth was determined in nutrient broth (Difco) adjusted to various pH values (pH 4.0–10.0 at intervals of 1.0 pH unit) using Na₂CO₃ or HCl prior to sterilization (Yoon *et al.*, 2006a). Growth under anaerobic conditions was determined on TSA after 2 weeks incubation in an AnaeroPack (Mitsubishi Gas). Cells of strain BAM-48^T were positive for catalase and oxidase activities, determined as described previously (Yoon *et al.*, 2006b). API 20E, 20NE and API ZYM strips (bioMérieux) were used to examine physiological and biochemical characteristics according to the manufacturer's protocols. Strain BAM-48^T showed many physiological characteristics that differentiated it from closely related species (Table 1); additional features are included in the species description.

A nearly complete 16S rRNA gene sequence of the strain was amplified by PCR and the purified PCR product was sequenced as described previously by Katsivela *et al.* (1999) and Ahmed *et al.* (2007a). The 16S rRNA gene sequence of strain BAM-48^T was compared with sequences obtained from GenBank. The sequences were aligned with CLUSTAL_X software (version 1.8msw; Thompson *et al.*, 1997) and evolutionary distances and *K*_{nuc} values (Kimura, 1980) were calculated. Alignment gaps and ambiguous bases were not taken into consideration in the comparison. Phylogenetic trees were constructed using the neighbour-joining and maximum-parsimony methods by MEGA version 3.1 (Kumar *et al.*, 2004). The stability of relationships was assessed by bootstrap analysis by performing 1000 resamplings for the tree topology.

Table 1. Characteristics that differentiate strain BAM-48^T (*Variovorax boronicumulans* sp. nov.) from the most closely related species

Strains: 1, *V. boronicumulans* sp. nov. BAM-48^T; 2, *V. soli* GH9-3^T; 3, *V. paradoxus* DSM 30034^T; 4, *V. dokdonensis* DS-43^T. Data for reference strains were taken from Kim *et al.* (2006). All type strains produced positive results for alkaline phosphatase, esterase lipase (C8) and leucine arylamidase and negative results for indole production, glucose fermentation, arginine dihydrolase, aesculin and gelatin hydrolysis, β-galactosidase, lipase (C14), cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. None of the strains assimilated N-acetyl-β-glucosamine, inositol or L-rhamnose. +, Positive; –, negative; w+, weakly positive.

Characteristic	1	2	3	4
DNA G + C content (mol%)	71.2	67.1	67.0	66.0
Nitrate reduction	–	+	+	–
Urease	–	–	–	+
Valine arylamidase	–	–	w+	–
Acid phosphatase	w+	+	–	+
α-Glucosidase	–	–	+	w+
Esterase (C4)	–	+	+	+
Naphthol-AS-BI-phosphohydrolase	–	+	+	+
Assimilation of:				
D-Mannose	–	–	+	–
D-Mannitol	w+	+	+	–
Phenylacetic acid	–	+	–	–
D-Sorbitol	–	+	+	–
Potassium gluconate	–	+	+	+
Adipic acid	–	+	+	+
Capric acid	+	–	–	–

The 16S rRNA gene sequence (1416 nt) of strain BAM-48^T showed the highest similarity to the sequences of *V. paradoxus* IAM 12373^T (99.3 %), *V. soli* GH9-3^T (98.3 %), *V. dokdonensis* DS-43^T (97.0 %), *Xylophilus ampelinus* DSM 7250^T (97.0 %) and *Curvibacter gracilis* IAM 15033^T (96.3 %), but low sequence similarity (<96 %) to members of other genera. The phylogenetic analysis (Fig. 1) demonstrated that strain BAM-48^T was clustered in the genus *Variovorax* with 91 % bootstrap support and occupied a position with *V. paradoxus* (99 % bootstrap value) in the neighbour-joining tree. The topology of the phylogenetic tree constructed using the maximum-parsimony algorithm (not shown) was similar to that of the neighbour-joining tree, especially for the clade comprising the novel strain (79 % bootstrap value), indicating that this strain is a member of the genus *Variovorax*.

For the whole-cell fatty acid analysis, cells were grown on R2A medium (Reasoner & Geldreich, 1985) for 2 days at 30 °C and the cellular fatty acid profile was determined using the GC-based Microbial Identification system (MIDI) according to the manufacturer's instructions. Since summed feature 3 represented a significant proportion of the total fatty acids in the profile, it was thought

Fig. 1. Neighbour-joining phylogenetic tree of strain BAM-48^T, other members of the genus *Variovorax* and representatives of some other taxa based on 16S rRNA gene sequence comparisons. Bootstrap percentages above 70% (from 1000 replicates) are shown at nodes. The sequence of *Alcaligenes faecalis* IAM 12369^T was used as the outgroup. Bar, 1% sequence divergence.

worthwhile to attempt to identify this compound. Fatty acids were therefore separated by TLC, and summed feature 3 was identified as $C_{16:1\omega7c}$ on the MIDI system. The major fatty acids of strain BAM-48^T were $C_{16:0}$ (36.1%), $C_{16:1\omega7c}$ (21.4%) and $C_{17:0}$ cyclo (19.0%). This profile is similar to those of other members of the genus *Variovorax* (Table 2), supporting the affiliation of the novel strain to this genus. Ubiquinone-8 (Q-8) was determined as the predominant quinone system in the novel strain

Table 2. Cellular fatty acid compositions of strain BAM-48^T (*V. boronicumulans* sp. nov.) and other species of genus *Variovorax*

Strains: 1, *V. boronicumulans* sp. nov. BAM-48^T; 2, *V. soli* GH9-3^T; 3, *V. paradoxus* DSM 30034^T; 4, *V. dokdonensis* DS-43^T. Data for reference strains were taken from Kim *et al.* (2006). Values are percentages of total fatty acids; only fatty acids that account for more than 1% in at least one strain are included. Summed features are groups of two or three fatty acids that cannot be separated by GLC using MIDI system; summed feature 3 comprises $C_{15:0}$ iso 2-OH and/or $C_{16:1\omega7c}$.

Fatty acid	1	2	3	4
$C_{10:0}$ 3-OH	2.9	5.1	3.7	3.2
$C_{12:0}$	3.5	3.2	3.7	4.0
$C_{12:0}$ 3-OH	—	—	—	3.3
$C_{14:0}$	1.1	—	—	1.0
$C_{14:0}$ 2-OH	2.2	—	2.7	—
$C_{15:0}$	—	—	—	1.8
$C_{16:0}$	36.1	33.2	29.8	25.2
$C_{17:0}$ cyclo	19.0	15.9	17.1	23.6
$C_{17:0}$	—	1.0	1.2	1.6
$C_{18:1\omega7c}$	11.3	9.0	13.1	13.1
Summed feature 3	21.4*	29.3	23.2	21.0

*Identified by TLC as $C_{16:1\omega7c}$.

using the method described previously (Xie & Yokota, 2003). These chemotaxonomic properties provided further evidence to confirm the phylogenetic results.

The genomic DNA of the strain was prepared according to a standard protocol. DNA G+C content was determined by the HPLC method of Mesbah *et al.* (1989). The DNA G+C content of strain BAM-48^T was 71.2 mol%. DNA–DNA hybridizations were performed at 53 °C using photobiotin-labelled DNA probes and microplates, as described by Ezaki *et al.* (1989). DNA–DNA hybridization values between strain BAM-48^T and *V. paradoxus* NBRC 15149^T, *V. dokdonensis* KCTC 12544^T and *V. soli* KACC 11579^T were 49.1, 24.6 and 25.4%, respectively. These results indicate that strain BAM-48^T represents a separate species of the genus *Variovorax* (Stackebrandt & Goebel, 1994).

On the basis of these morphological, physiological, biochemical, phylogenetic and genotypic results, we suggest that strain BAM-48^T belongs to the genus *Variovorax* and represents a novel species, for which we propose the name *Variovorax boronicumulans* sp. nov.

Description of *Variovorax boronicumulans* sp. nov.

Variovorax boronicumulans (N.L. n. *boron*, -onis boron; L. part. adj. *cumulans* accumulating; N.L. part. adj. *boronicumulans* boron-accumulating).

Cells are Gram-negative, motile, non-spore-forming and rod-shaped (0.5–0.7 µm long and 1.0–2.0 µm in diameter). Colonies are yellow-coloured and circular with entire margins after 2 days on TSA plates at 30 °C. The temperature range for growth is 4–37 °C, with optimum growth at 30 °C; no growth at ≥45 °C and little growth at 4 °C after 2 weeks of incubation. Growth is observed at pH 5–9, with optimum growth at pH 7; no growth at pH 4 or 10. NaCl is tolerated up to 1% (w/v). Grows on TSA

(Difco) and nutrient agar (Difco). Positive for catalase and oxidase activities, citrate utilization and the Voges–Proskauer test and weakly positive for assimilation of L-arabinose, mannitol, malic acid and capric acid. Negative for nitrate reduction, β -galactosidase, arginine dihydrolase, lysine and ornithine decarboxylases, H₂S production, urease, tryptophan deaminase, assimilation of tryptophan, gelatin hydrolysis and utilization of inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin, D-glucose, D-mannose, N-acetyl-D-glucosamine, maltose, potassium gluconate, adipic acid and phenylacetic acid. Strong enzyme activity is observed for alkaline phosphatase, esterase lipase (C8) and leucine arylamidase and weak activity is observed for acid phosphatase; the other reactions of the API ZYM strip are negative. Major cellular fatty acids are C_{16:0} (36.1 %), C_{16:1 ω 7c} (21.4 %) and C_{17:0} cyclo (19.0 %). The G+C content of the type strain is 71.2 mol%.

The type strain, strain BAM-48^T (=NBRC 103145^T =KCTC 22010^T), was isolated from soil collected in the experimental area of the University of Tokyo (Yayoi campus), Tokyo, Japan.

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