Bacillus arsenicus sp. nov., an arsenic-resistant bacterium isolated from a siderite concretion in West Bengal, India

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Strain Con a/3^T is a Gram-positive, motile, endospore-forming, rod-shaped and arsenic-resistant bacterium, which was isolated from a concretion of arsenic ore obtained from a bore-hole. The bacterium grew in the presence of 20 mM arsenate and 0.5 mM arsenite. Diaminopimelic acid was present in the cell wall peptidoglycan, MK-7 was the major menaquinone, and iso-C_{15:0}, anteiso-C_{15:0}, iso-C_{16:0} and C_{16:1}(δ^7 cis) were the major fatty acids. Based on its phenotypic, chemotaxonomic and phylogenetic characteristics, strain Con a/3^T was identified as a member of the genus *Bacillus*. It exhibited maximum similarity (97 %) at the 16S rRNA gene level with *Bacillus barbaricus* (DSM 14730^T); however, the DNA–DNA relatedness value with *B. barbaricus* was 60 %. Strain Con a/3^T also exhibited a number of phenotypic differences from *B. barbaricus* (DSM 14730^T). Strain Con a/3^T was therefore identified as representing a novel species of the genus *Bacillus*, for which the name *Bacillus arsenicus* sp. nov. is proposed. The type strain is Con a/3^T (=MTCC 4380^T=DSM 15822^T=JCM 12167^T).

Arsenic, a toxic element, exists in nature in two oxidation states, as arsenate [As(V)], and arsenite [As(III)]. Several bacteria (Cervantes *et al.*, 1994; Laverman *et al.*, 1995; Oremland *et al.*, 2001) belonging to the genera *Acidithiobacillus*, *Bacillus*, *Deinococcus*, *Desulfotobacterium* and *Pseudomonas* (de Vicente *et al.*, 1990; Sato & Kobayashi, 1998; Dopson *et al.*, 2001; Niggemyer *et al.*, 2001; Prithiviraj Singh *et al.*, 2001; Suresh *et al.*, 2004a, b) have been reported to be resistant to arsenic. In this study, we report the isolation of an arsenic-resistant novel species of *Bacillus* that was isolated from a bore well located in the Chakdah region of West Bengal, India. The bacterium, Con a/3^T, is resistant to arsenate and arsenite. Based on its phenotypic characteristics, chemotaxonomic properties and 16S rRNA gene sequence, the strain was identified as representing a novel species of the genus *Bacillus*, for which the name *Bacillus arsenicus* sp. nov. is proposed.

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Con a/3^T is AJ606700.

Scanning electron micrographs of the surface of a spheroidal concretion, a comparison of the lipid composition of *B. arsenicus* sp. nov. and *B. barbaricus*, and a neighbour-joining tree showing the phylogenetic relationships between *B. arsenicus* sp. nov. and other species of the genus *Bacillus* are available as supplementary material in IJSEM Online.

Source of the organism, media and growth conditions

Strain Con a/3^T was isolated from a concretion, which is an extra-appendage-like structure observed on arsenic ore, recovered from the sediment of a bore-hole (depth 45 m) located in the Chakdah region of West Bengal, India (23° 3' N, 88° 35' E). The medium used for isolation of the bacterium was arsenate medium [0.5 % (w/v) peptone, 0.5 % (w/v) NaCl, 0.2 % (w/v) yeast extract, 0.2 % (w/v) beef extract, 1.5 % (w/v) agar; pH 7.0], containing 5 % sodium arsenate. Nutrient agar without sodium arsenate was used for growth and maintenance of the strain. *Bacillus barbaricus* (DSM 14730^T) was obtained from the DSMZ, cultured on nutrient agar or the appropriate medium, and used for comparison of phenotypic and chemotaxonomic characteristics.

Electron microscopy of the concretion and electron probe micro analyser (EPMA) study

Scanning electron microscopy was used to study the external and internal morphologies of the concretions and also to visualize the bacteria. For these purposes, the concretion was surface-sterilized with alcohol, crushed with pincers to reveal the interior, mounted on stubs, coated with gold, and observed immediately using a model 5-440, LEO electron microscope; a working distance of 19 mm and

an accelerating voltage of 20 kVA, 300 pA were used. Under the scanning electron microscope, the concretion appeared as a spherical, pimpled structure with rhombohedral crystals on the surface (see Supplementary Figure A(a) in IJSEM Online), whereas the internal cavity had a ruffled surface associated with rod-like structures, which were probably bacteria (see Supplementary Figure A(b) in IJSEM Online). Studies on the chemical composition of the concretion using an EPMA (CAMECA SX 51 instrument at the Geological Survey of India Laboratory, Faridabad, India) indicated that the mineral associated with the concretion was siderite, made up of FeCO_3 (25.75–49.58 %), MnCO_3 (0.24–16.75 %), CaCO_3 (2.01–12.23 %) and MgCO_3 (0.15–0.74 %).

Morphology, motility, and biochemical and chemotaxonomic characteristics

Strain Con a/3^T was also observed under a phase-contrast microscope (DIAPLAN; Leitz) using an oil-immersion objective ($\times 100$) to ascertain the shape and motility. The physiological tests were done as described by Claus & Berkeley (1986). For the various biochemical tests listed in Table 1 and used in the description of the species, the culture was grown at 30 °C in Luria–Bertani broth medium [1.0 % (w/v) tryptone, 0.5 % (w/v) yeast extract, 2.5 % (w/v) NaCl; pH 7.2], and the tests were performed as described by Lanyi (1987) and Smibert & Krieg (1994). To check for the level of resistance to arsenic, strain Con a/3^T was grown at 25 °C in nutrient broth [1.0 % (w/v) peptone, 0.5 % (w/v) yeast extract, 0.5 % (w/v) NaCl] with 1.0 % (w/v) glucose, and containing either 5, 10 or 20 mM arsenate (Na_2HAsO_4) or 0.25 or 0.5 mM arsenite (As_2O_3). Growth was monitored spectrophotometrically (at OD₆₀₀) for a period of 72 h. In addition, the ability of strain Con a/3^T to utilize a carbon compound as the sole carbon source was investigated by supplementing the minimal medium [1.05 % (w/v) K_2HPO_4 , 0.45 % (w/v) KH_2PO_4 , 0.1 % (w/v) $(\text{NH}_4)_2\text{SO}_4$, 1.5 % (w/v) agar] with 0.5 % (w/v) filter-sterilized carbon compound. Nutrient agar was used to check the sensitivity of strain Con a/3^T to various antibiotics using antibiotic discs supplied by Hi-Media Pvt. (Mumbai, India).

Fatty acid methyl esters (Sato & Murata, 1988) were extracted from cells grown in nutrient broth at 30 °C and analysed as described by Reddy *et al.* (2002). Isoprenoid quinones were extracted according to the method described by Collins *et al.* (1977), separated by HPLC and identified as described by Reddy *et al.* (2003). Peptidoglycan was prepared and analysed according to the method described by Komagata & Suzuki (1987). Isolation of DNA, determination of the mol% G+C content of the DNA and analysis of the phospholipids were performed as described previously (Shivaji *et al.*, 1989). DNA–DNA hybridization was performed by using the membrane filter method (Tourova & Antonov, 1987), as described previously (Shivaji *et al.*, 1992).

Table 1. Phenotypic characteristics that differentiate *B. arsenicus* sp. nov. (Con a/3^T) from related *Bacillus* species

Species: 1, *B. arsenicus* sp. nov. (Con a/3^T); 2, *B. barbaricus* (DSM 14730^T); 3, *B. indicus* (Sd/3^T); 4, *B. arseniciselenatis* (DSM 15340^T) (data from Switzer Blum *et al.*, 1998); 5, *B. selenitireducens* (DSM 15326^T) (Switzer Blum *et al.*, 1998). +, Positive; –, negative; NA, not available; ND, not determined.

Characteristic	1	2	3	4	5
Colony colour	Cream	Brownish	Yellow–orange	ND	Red
NaCl tolerance (%)					
2	–	+	+	+	+
5	–	–	–	+	+
Resistance to As(V)					
5 mM	+	–	+	+	+
10 mM	+	–	+	+	+
20 mM	+	–	+	–	NA
Resistance to As(III)					
0.3 mM	+	–	+	+	+
Oxidase	+	–	–	NA	NA
DL-Lactate	+	–	+	NA	NA
i-Inositol	+	–	+	NA	NA
Inulin	+	–	NA	NA	NA
D-Melibiose	+	–	+	NA	NA
Pyruvate	–	+	–	–	+
Raffinose	+	–	+	NA	NA
Sorbitol	+	–	NA	NA	NA
D-Ribose	–	+	+	NA	NA
Sucrose	+	–	+	NA	NA
D-Mannose	–	+	+	NA	NA
L-Malate	–	+	–	NA	NA
L-Alanine	–	+	–	NA	NA
L-Phenylalanine	–	+	–	NA	NA
L-Proline	–	+	–	NA	NA
L-Serine	–	+	–	–	–
D-Mannitol	+	–	+	NA	NA
Sucrose	+	–	–	NA	NA

To ascertain whether bacteria were present within the cavity of the concretion, about 10 mg of the concretion sample was separated from the ore surface by using forceps, surface-sterilized and crushed. The sample was then suspended in 1 ml of sterile distilled water, and serially diluted and plated on nutrient agar containing 5 % sodium arsenate. Colonies were visible after 3 days; the colony count was 2.0×10^3 (g concretion)^{–1}. The colonies were all cream in colour, circular, raised, smooth, convex and 1–2 mm in diameter (Table 1). One of the colonies was purified and the strain was labelled as Con a/3. The bacterium was rod-shaped, as confirmed by both electron microscopy and phase-contrast microscopy.

The phenotypic and chemotaxonomic characteristics of strain Con a/3^T are given in the species description (see below). Strain Con a/3^T is a Gram-positive, motile,

Table 2. Fatty acid content (%) of *B. arsenicus* Con a/3^T and *B. barbaricus* DSM 14730^T

Strains: 1, *B. arsenicus* sp. nov. (Con a/3^T); 2, *B. barbaricus* (DSM 14730^T). Cells of strains Con a/3^T and DSM 14730^T were grown to late exponential phase in nutrient broth at 30 °C and the cell pellets were used for fatty acid analysis. Results are the mean of two separate experiments carried out under identical conditions. The variation in values was less than 2%.

Fatty acid methyl ester	1	2
C _{13:0}	5.8	0
iso-C _{14:0}	3.4	4.4
C _{14:0}	1.1	0.6
iso-C _{15:0}	10.6	19.1
anteiso-C _{15:0}	10.5	27.6
C _{15:0}	1.6	1.3
iso-C _{15:1}	4.2	0.9
iso-C _{16:0}	19.0	5.3
C _{16:0}	9.6	6.5
C _{16:1} (δ 7 <i>cis</i>)	25.3	6.8
C _{16:1} (δ 11 <i>cis</i>)	4.2	9.2
iso-C _{17:0}	1.1	4.1
anteiso-C _{17:0}	0	2.3

endospore-forming, rod-shaped organism. Diaminopimelic acid is the characteristic peptidoglycan diamino acid, MK-7 is the major respiratory quinone, and iso-C_{15:0} (10.6%), anteiso-C_{15:0} (10.5%), iso-C_{16:0} (19.0%), C_{16:0} (9.6%) and C_{16:1}(δ 7*cis*) (25.3%) are the prominent fatty acids (Table 2). The lipids present are phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine and, in addition, two unidentified aminolipids, one unidentified aminophospholipid and one unknown phospholipid. *B. barbaricus* (DSM 14730^T) has a similar lipid composition except that, instead of two unknown aminolipids, it has one aminolipid and one lipid (see Supplementary Figure B in IJSEM Online). The DNA G+C content of strain Con a/3^T was 35.0 mol%, thus conforming to the characteristics of the genus *Bacillus* (Kämpfer, 1994).

Phylogenetic analysis

The 16S rRNA gene was amplified as described previously (Shivaji *et al.*, 2000), and the PCR product was purified using the QIA quick PCR purification kit (Qiagen). Sequencing was performed by using an ABI PRISM model 3700 automatic DNA sequencer and the Big Dye Terminator cycle sequencing kit (both from Applied Biosystems). The almost complete 16S rRNA gene sequence (1505 bp) was aligned with closely related sequences retrieved from EMBL by using CLUSTAL W (Thompson *et al.*, 1994). Pairwise evolutionary distances were computed by using the DNADIST program with the Kimura two-parameter model (Kimura, 1980). Phylogenetic trees were constructed by using NEIGHBOR, UPGMA, KITSCH, FITCH and DNAPARS of the PHYLIP package (Felsenstein, 1993). The stability among the clades of a phylogenetic tree was assessed by taking 1000 replicates of the dataset, which were analysed by using the programs SEQBOOT, DNADIST, NEIGHBOR and CONSENSE of the PHYLIP package.

Phylogenetic analysis based on the 16S rRNA gene sequence, indicated that strain Con a/3^T is closely related to *B. barbaricus* DSM 14730^T (97%); lower 16S rRNA gene sequence similarity was observed with other *Bacillus* species, including *Bacillus flexus* DSM 1320^T (94%), *Bacillus cohnii* DSM 6307^T (94%), *Bacillus megaterium* IAM 13418^T (94%), *Bacillus bataviensis* DSM 15601^T (94%), *Bacillus niacini* DSM 2923^T (94%), *Bacillus novalis* LMG 21837^T (94%), *Bacillus benzoovorans* DSM 5391^T (94%), *Bacillus lentus* NCIMB 8773^T (94%), *Bacillus gelatini* DSM 15865^T (93%), *Bacillus naganoensis* ATCC 53909^T (93%) and *Bacillus indicus* Sd/3^T (91%). The neighbour-joining phylogenetic tree further confirmed that strain Con a/3^T is phylogenetically related to species of *Bacillus* and forms a clade with *B. barbaricus* (DSM 14730^T), with a bootstrap value of 100% (Fig. 1; also see Supplementary Figure C in IJSEM Online). Phylogenetic trees constructed by using KITSCH, FITCH, UPGMA and DNAPARS also yielded similar stable groupings.

Fig. 1. Neighbour-joining tree based on 16S rRNA (1465 bases) gene sequences showing the phylogenetic relationships between *B. arsenicus* sp. nov. (Con a/3^T) and the nearest neighbours of the genus *Bacillus* and related reference micro-organism. Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are given at the nodes.

By comparison, strain Con a/3^T exhibited a similarity of only 60 % (mean of 57 and 63 %) with *B. barbaricus* (Täubel *et al.*, 2003) at the DNA–DNA level. DNA–DNA similarity was not done with those species showing more than 3 % difference at the 16S rRNA gene sequence level. Therefore, it would appear that, based on the more than 3 % difference at the 16S rRNA gene sequence level and less than 70 % similarity at the DNA–DNA level with the closest related *Bacillus* species, strain Con a/3^T can probably be classified as representing a novel species according to accepted criteria (Stackebrandt & Goebel, 1994). This probability is supported by the number of phenotypic differences observed between strain Con a/3^T and the type strain of the closely related species of *B. barbaricus* (DSM 14730^T) (Table 1), and the type strains of other arsenic-resistant species of *Bacillus*, namely *Bacillus arseniciselenatis* (DSM 15340^T), *Bacillus selenitireducens* (DSM 15326^T) and *B. indicus* (Sd/3^T). Significant differences in the levels of the fatty acids C_{13:0}, iso-C_{15:1}, iso-C_{16:0}, C_{16:1}(δ 7cis), iso-C_{15:0} and anteiso-C_{15:0} further differentiate strain Con a/3^T from *B. barbaricus* (DSM 14730^T), when grown under identical conditions (Table 2). Moreover, strain Con a/3^T was able to grow in the presence of 20 mM arsenate [As(V)] and 0.3 mM arsenite [As(III)], unlike the above species. Strain Con a/3^T also differs from *B. indicus* Sd/3^T, an arsenic-resistant bacterium (Suresh *et al.*, 2004a), in that *B. indicus* Sd/3^T is resistant to higher concentrations of arsenite (3 mM), and the similarity at the 16S rRNA gene level is only 91 %. Therefore, based on the phenotypic, chemotaxonomic and phylogenetic characteristics, strain Con a/3^T merits recognition as representing a novel species, for which the name *Bacillus arsenicus* sp. nov. is proposed.

Description of *Bacillus arsenicus* sp. nov.

Bacillus arsenicus (ar.sen.i'cus. N.L. masc. adj. *arsenicus* pertaining to arsenic).

Cells are strictly aerobic, Gram-positive, motile, sporulating and rod-shaped, 1.4–2.0 μ m long and 0.3–0.5 μ m wide. Endospores form subterminally in non-swelling sporangia. Colonies on nutrient agar are cream, circular, raised, smooth, convex and 1–2 mm in diameter. Optimum growth occurs at 30 °C. Growth occurs at 20–40 °C, but not at 45 °C. Growth occurs at pH 5.5–8.0. Tolerates up to 1.0 % NaCl. Positive for catalase, amylase and protease, but negative for arginine dihydrolase. Hydrolyses starch and gelatin but not urea, Tween 20 or aesculin. Does not reduce nitrate to nitrite and is negative for the methyl red test, indole production, Voges–Proskauer test, citrate utilization and H₂S production. Utilizes D-fructose, D-glucose, D-maltose and L-histidine, but not D-cellobiose, L-arabinose, fumarate, glutarate, L-arginine, aspartic acid, creatine or tyrosine as the sole carbon source. Acid is produced from D-glucose and D-maltose, but not from lactose or D-xylose. Strain Con a/3^T is sensitive to the antibiotics chloramphenicol (30 μ g), kanamycin (30 μ g), nalidixic acid (30 μ g), neomycin (30 μ g), rifampicin (30 μ g), streptomycin (10 μ g) and tetracycline (30 μ g), and is resistant to amoxicillin

(10 μ g) and ampicillin (10 μ g). Other characteristics are listed in Table 1. The major fatty acids are iso-C_{15:0}, anteiso-C_{15:0}, iso-C_{16:0} and C_{16:1}(δ 7cis) (Table 2), and the phospholipids present are phosphatidylglycerol, diphosphatidylglycerol and phosphatidylethanolamine. The major respiratory quinone is MK-7. The diamino acid found in the cell peptidoglycan is diaminopimelic acid. The G + C content of the DNA is 35.0 mol%.

The type strain is Con a/3^T (=MTCC 4380^T=DSM 15822^T=JCM 12167^T).

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