

# *Bacillus indicus* sp. nov., an arsenic-resistant bacterium isolated from an aquifer in West Bengal, India

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Strain Sd/3<sup>T</sup> (=MTCC 4374<sup>T</sup> = DSM 15820<sup>T</sup>), an arsenic-resistant bacterium, was isolated from a sand sample obtained from an arsenic-contaminated aquifer in Chakdah district in West Bengal, India (23° 3' N 88° 35' E). The bacterium was Gram-positive, rod-shaped, non-motile, endospore-forming and yellowish-orange pigmented. It possessed all the characteristics that conform to the genus *Bacillus*, such as it had A<sub>4</sub>β murein type (L-Orn-D-Asp) peptidoglycan variant, MK-7 as the major menaquinone and iso-C15:0 and anteiso-C15:0 as the major fatty acids. Based on its chemotaxonomic and phylogenetic characteristics, strain Sd/3<sup>T</sup> was identified as a species of the genus *Bacillus*. It exhibited maximum similarity (95%) at the 16S rRNA gene level with *Bacillus cohnii*; however, DNA–DNA similarity with *B. cohnii* was 60.7%. Strain Sd/3<sup>T</sup> also exhibited a number of phenotypic differences from *B. cohnii* (DSM 6307<sup>T</sup>). These data suggest that Sd/3<sup>T</sup> represents a novel species of the genus *Bacillus*. The name *Bacillus indicus* sp. nov. is proposed.

The ground-water and soil of the Bengal basin in India contains high levels of arsenic (Karim, 2000; Das *et al.*, 1996). The presence of arsenic in the environment may thus lead to the enrichment of arsenic-resistant bacteria. Bacteria belonging to the genera *Acidithiobacillus*, *Bacillus*, *Deinococcus*, *Desulfotobacterium* and *Pseudomonas* have already been reported to be resistant to arsenic (De Vicente *et al.*, 1990; Sato & Kobayashi, 1998; Dopson *et al.*, 2001; Niggemyer *et al.*, 2001; Prithivirajsingh *et al.*, 2001; Suresh *et al.*, 2004). In the current study, we report on the isolation and characterization of an arsenic-resistant bacterium from an arsenic contaminated aquifer located in the Bengal basin in India.

## Source of the organisms, media and growth conditions

Strain Sd/3<sup>T</sup> was isolated from a sand sample obtained from an arsenic-contaminated aquifer in Chakdah district in West Bengal, India (23° 3' N 88° 35' E) that yielded about  $3 \times 10^5$  c.f.u. (g soil)<sup>-1</sup>. The medium used for isolation was nutrient agar (0.5% peptone, 0.5% NaCl, 0.2% yeast extract, 0.2% beef extract and 1.5% agar, pH 7.0, all w/v) containing 5% (w/v) sodium arsenate. Nutrient agar

without sodium arsenate was used for growth and maintenance.

## Morphology, motility and biochemical and chemotaxonomic characteristics

Sd/3<sup>T</sup> was grown in nutrient broth and examined by phase-contrast microscopy ( $\times 1000$ ) to ascertain cell shape and the presence of motility. Biochemical tests were performed on cultures grown at 30 °C in Luria–Bertani broth (1.0% tryptone, 0.5% yeast extract, 0.9% NaCl, pH 7.2, all w/v). Hydrolysis of aesculin, nitrate reduction, indole test, Voges–Proskauer test, methyl red test and H<sub>2</sub>S production were performed as described by Lanyi (1987). Utilization of various carbon compounds, hydrolysis of casein, gelatin, starch and Tween 20 were performed as described by Smibert & Krieg (1994). Oxidase activity was qualitatively detected using the method described by Stanier *et al.* (1966). Further, the ability of the cultures to utilize carbon compounds as the sole carbon source was checked by supplementing minimal medium [1.05% K<sub>2</sub>HPO<sub>4</sub>, 0.45% KH<sub>2</sub>PO<sub>4</sub>, 0.1% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and 1.5% agar, all w/v] with 0.5% (w/v) of the filter-sterilized carbon compound. Nutrient agar was used to check the sensitivity of Sd/3<sup>T</sup> to different antibiotics, using commercial antibiotic discs (HiMedia, Mumbai, India).

Sd/3<sup>T</sup> was grown in nutrient broth at 30 °C for 48 h and the fatty acid methyl esters were extracted (Sato & Murata,

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The GenBank accession number for the 16S rRNA gene sequence of strain Sd/3<sup>T</sup> is AJ583158.



Table 1. cont.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Utilization of carbon source																			
D-Cellobiose	+	-	+	+	ND	ND	+	ND	ND	ND	ND	ND	+	ND	-	ND	+	ND	+
<i>meso</i> -Erythritol	+	-	-	-	ND	ND	ND	ND	ND	ND	-	ND	-	-	ND	ND	-	ND	-
Fumarate	-	+	-	-	+	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Glutarate	-	+	-	-	-	-	ND	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Inositol	+	-	+	+	ND	ND	-	-	ND	ND	+	ND	-	-	-	+	ND	ND	ND
Lactose	+	-	+	+	ND	ND	+	-	ND	+	+	ND	-	-	-	+	ND	-	-
D-Maltose	+	-	+	+	ND	ND	ND	ND	ND	ND	ND	ND	+	ND	+	ND	+	+	+
D-Mannose	+	-	-	-	ND	ND	ND	-	ND	ND	+	ND	+	+	-	-	+	ND	+
D-Melibiose	+	-	+	-	ND	ND	ND	-	ND	ND	+	ND	-	-	-	+	+	-	-
Pyruvate	-	+	-	-	-	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	ND	ND	-
Raffinose	+	-	+	-	ND	ND	-	-	ND	ND	+	ND	-	-	-	-	+	-	ND
L-Rhamnose	+	-	+	-	ND	ND	-	-	ND	ND	+	ND	-	-	-	+	ND	ND	ND
D-Ribose	+	-	-	+	ND	ND	+	-	ND	ND	+	ND	-	+	+	ND	+	-	ND
Sucrose	+	-	+	+	ND	ND	+	+	ND	+	+	ND	+	+	+	ND	+	+	+
L-Arginine	+	-	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
L-Proline	-	+	-	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
L-Serine	-	+	-	+	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
L-Histidine	+	-	+	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Acid production																			
D-Glucose	-	-	+	+	-	+	+	+	+	+	+	ND	+	+	+	ND	+	+	+
Lactose	-	-	-	-	ND	ND	+	+	-	+	ND	+	-	-	-	+	+	ND	ND
D-Mannitol	-	-	-	-	ND	ND	ND	+	+	+	-	-	-	+	+	+	+	ND	+
D-Mannose	+	-	+	-	ND	ND	ND	ND	-	ND	+	ND	-	-	-	ND	+	ND	+
D-Xylose	-	-	+	-	ND	ND	+	+	-	+	-	ND	+	ND	+	ND	-	ND	-
Sensitivity to antibiotics																			
Amoxicillin (10 µg disk)	R	S	R	R	ND	ND	ND	ND	ND	ND	ND	ND	S	ND	ND	ND	ND	ND	ND
Ampicillin (10 µg disk)	S	S	R	S	ND	ND	ND	R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	S

\*TSI, Triple-sugar iron agar.

1988) and analysed as described previously (Reddy *et al.*, 2002). Isoprenoid quinones were extracted according to the method described by Collins *et al.* (1977) and separated by HPLC using a SB-C<sub>18</sub> Zorbax reverse-phase column fixed to a Hewlett Packard Series 1100 HPLC as described previously (Tamaoka *et al.*, 1983; Suresh *et al.*, 2004). Polar lipids were analysed as described by Suresh *et al.* (2004). Peptidoglycan type was analysed according to Komagata & Suzuki (1987). Mol% G+C content of the DNA was determined as described earlier (Shivaji *et al.*, 1989; Reddy *et al.*, 2000).

Phenotypic and chemotaxonomic characteristics of Sd/3<sup>T</sup> are described in the species description. It is apparent that Sd/3<sup>T</sup>, which is Gram-positive, rod-shaped, non-motile, endospore-forming and which possesses A<sub>4</sub>β murein type (L-Orn-D-Asp) peptidoglycan variant (Schleifer & Kandler, 1972), MK-7 as the main respiratory quinone, iso-C15:0, anteiso-C15:0, iso-C14:0, iso-C16:0 and iso-C17:0 as the predominant fatty acids, phosphatidylglycerol, phosphatidylethanolamine and phosphatidyl diglycerol as the polar lipids and a DNA G+C content of 41.2 mol%, conforms to the characteristics of the genus *Bacillus* (Tables 1 and 2) (Kämpfer, 1994). To date, only a few *Bacillus* species with oval spores and A<sub>4</sub>β murein-type peptidoglycan have been reported (Spanka & Fritze, 1993). Phenotypic and chemotaxonomic characteristics that differentiate Sd/3<sup>T</sup> from related *Bacillus* species are listed in Tables 1 and 2.

*Bacillus cohnii* DSM 6307<sup>T</sup> and *Bacillus flexus* DSM 1320<sup>T</sup>, obtained from DSMZ, Germany, and *Bacillus megaterium* MTCC 1684, obtained from Institute for Microbial Technology, Chandigarh, India, were used as reference strains in studies related to morphology, motility, biochemical tests,

identification of fatty acids and DNA–DNA hybridization. DNA–DNA hybridization was performed by the membrane filter method (Tourova & Antonov, 1987) as described by Shivaji *et al.* (1992) and Reddy *et al.* (2000).

### Phylogenetic analysis

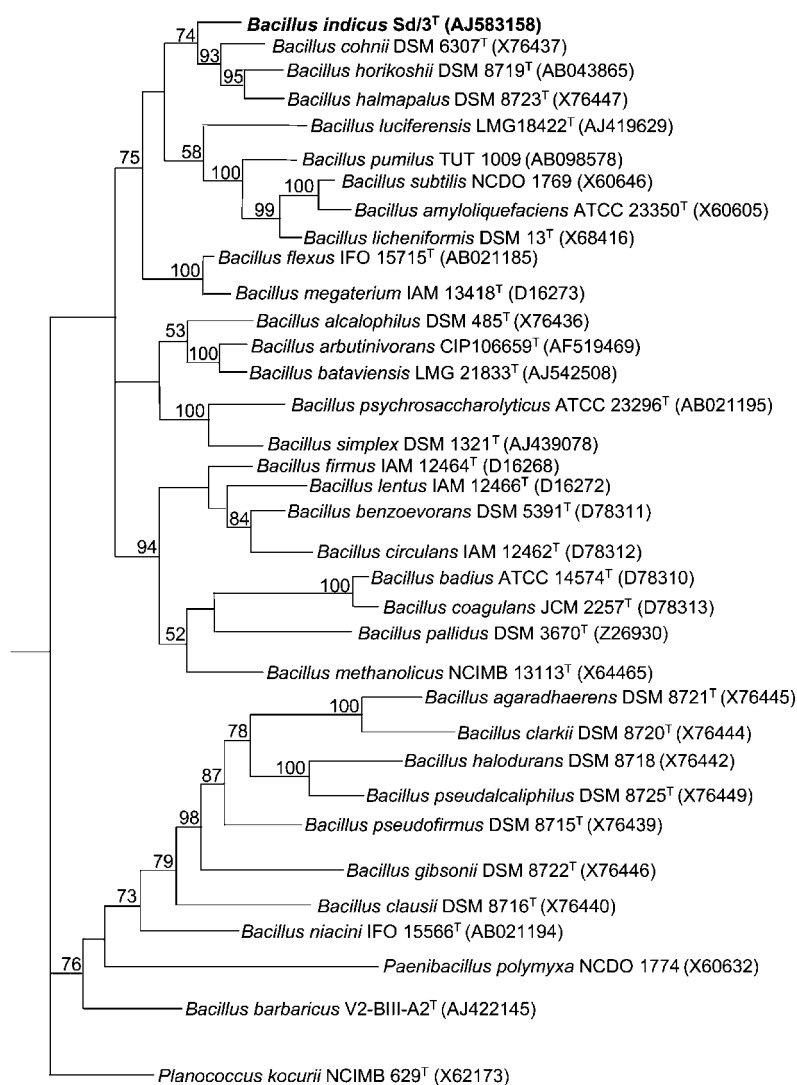
The 16S rDNA was amplified as described previously (Shivaji *et al.*, 2000) and the PCR product was purified using the QIA quick PCR purification kit (Qiagen). Sequencing of purified 16S rDNA was performed using the ABI PRISM Big Dye Terminator cycle sequencing kit (Applied Biosystems). Purified amplicons were electrophoresed on an automated DNA sequencer (ABI PRISM model, 3700). The partial (1473 bp) 16S rDNA was aligned with relevant sequences contained in EMBL database using CLUSTAL W (Thompson *et al.*, 1994). Pairwise evolutionary distances were calculated using the DNADIST program with the Kimura two-parameter model (Kimura, 1980). Phylogenetic trees were constructed by four different tree-making algorithms (UPGMA, KITSCH, FITCH and DNAPARS) in the PHYLIP software package (Felsenstein, 1993). The stability among the clades of a phylogenetic tree was assessed by taking 1000 replicates of the dataset and was analysed using the programs SEQBOOT, DNADIST, UPGMA and CONSENSE contained in the PHYLIP software package.

The phylogenetic analyses, based on 16S rRNA gene sequence data, indicated that Sd/3<sup>T</sup> is most closely related to species of the genus *Bacillus* (Fig. 1). Sd/3<sup>T</sup> exhibited a similarity of 94% to *B. megaterium* MTCC 1684, *B. flexus* DSM 1320<sup>T</sup> and *Bacillus psychrosaccharolyticus* ATCC 23296<sup>T</sup> and 95% to *B. cohnii* DSM 6307<sup>T</sup>, *Bacillus pumilus* TUT1009, *Bacillus horikoshii* DSM 8719<sup>T</sup>, *Bacillus niacini*

**Table 2.** Fatty acid composition (%) of *B. indicus* sp. nov. (Sd/3<sup>T</sup>) and related *Bacillus* species

Strains: 1, *B. indicus* Sd/3<sup>T</sup> (present study); 2, *B. cohnii* DSM 6307<sup>T</sup> (present study); 3, *B. megaterium* MTCC 1684 (present study); 4, *B. flexus* DSM 1320<sup>T</sup> (present study); 5, *B. bataviensis* LMG 21833<sup>T</sup> (Heyrman *et al.*, 2004); 6, *B. pumilus* ATCC 7061<sup>T</sup> (Kämpfer, 1994); 7, *B. firmus* ATCC 14575<sup>T</sup> (Kämpfer, 1994); 8, *B. krulwichiae* IAM 15000<sup>T</sup> (Yumoto *et al.*, 2003); 9, *B. okuhidensis* JCM 10945<sup>T</sup> (Li *et al.*, 2002); 10, *B. luciferensis* LMG 18422<sup>T</sup> (Logon *et al.*, 2002); 11, *B. alcalophilus* JCM 5262<sup>T</sup> (Yumoto *et al.*, 2000). Fatty acid data for *B. arseniciselenatis*, *B. niacini*, *B. psychrosaccharolyticus*, *B. clarkii*, *B. halmपालus*, *B. horikoshii*, *B. veddri* and *B. selenitireducens* were not reported in the relevant references. *B. indicus* Sd/3<sup>T</sup> (1), *B. cohnii* (2), *B. megaterium* (3) and *B. flexus* (4) were grown in nutrient broth at 30 °C for the determination of fatty acid composition in the present study.

Fatty acid methyl esters	1	2	3	4	5	6	7	8	9	10	11
iso-C14:0	10.9	0.8	4.3	1.6	6.9	0.7	2.4	2.2	1.6	3.2	0.6
iso-C15:0	33.5	22.9	27.8	32.2	42.9	56.9	45.8	17.1	43.2	24.3	31.8
anteiso-C15:0	19.3	20.3	52.5	35.4	20.5	23.4	19.8	49.0	25.3	51.5	42.9
C15:0	1.3	0.2	—	—	—	—	0.3	1.1	—	—	0.3
iso-C16:0	11.0	7.0	1.4	8.6	2.4	0.4	2.9	0.8	3.7	4.4	1.0
C16:0	5.9	1.0	8.5	1.2	7.7	1.1	1.5	1.6	3.7	2.6	2.1
C16:1 (Δ7c)	—	—	—	6.4	2.3	—	4.5	9.8	—	—	1.5
C16:1 (9t)	1.5	2.1	—	—	—	—	—	—	—	—	—
C16:1 (9c)	2.3	7.8	—	—	—	—	—	—	—	—	—
iso-C17:0	10.8	35.3	1.5	2.1	1.4	5.2	2.9	2.9	7.4	Trace	4.3
anteiso-C17:0	2.9	2.2	3.2	3.4	1.1	3.5	3.7	4.9	9.3	2.9	11.2



**Fig. 1.** Neighbour-joining tree based on 16S rDNA (1473 bases) sequences showing the phylogenetic relationship between *B. indicus* sp. nov. (Sd/3<sup>T</sup>) and other related species of the genus *Bacillus* and related reference micro-organisms. Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are given at the nodes.

IFO 15566<sup>T</sup> and *Bacillus bataviensis* LMG 21833<sup>T</sup> following BLAST analysis. In the present study comparisons were made between Sd/3<sup>T</sup> and only *B. cohnii* and *B. megaterium* because the former had a similar A<sub>4</sub>β murein-type (L-Orn-D-Asp) peptidoglycan, and because the latter is a representative species of the genus. All the other species, which exhibited 94–95% similarity at the 16S rRNA gene level, differ from Sd/3<sup>T</sup> in peptidoglycan type and other phenotypic characteristics (Table 2). The 16S rRNA gene sequence of Sd/3<sup>T</sup> shows a deletion of 14 bases between bases 55–68 compared to *B. cohnii* DSM 6307<sup>T</sup>. Furthermore, at the DNA–DNA level, Sd/3<sup>T</sup> shares 60.7% (mean of three replicate values of 58, 60 and 64) and only 34.3% (mean of three replicate values of 32, 34 and 37) relatedness with *B. cohnii* DSM 6307<sup>T</sup> and *B. megaterium* MTCC 1684, respectively. Thus, based on the less than 95% similarity between Sd/3<sup>T</sup> and the reported species of *Bacillus* at the 16S rRNA gene level, it is proposed to assign novel species status to Sd/3<sup>T</sup>. In addition, Sd/3<sup>T</sup> differs from both *B. cohnii* DSM 6307<sup>T</sup> and *B. megaterium* MTCC 1684 in that the colonies are yellowish-orange in colour and are unable to grow in the presence of

5% (w/v) NaCl but can grow in the presence of 3 mM arsenite (As III) and 20 mM arsenate (As V). Additionally, Sd/3<sup>T</sup> is positive for arginine dihydrolase, does not hydrolyse Tween 20 and utilizes *meso*-erythritol, D-mannose, D-ribose and L-arginine (Table 2). Phenotypic characteristics that differentiate Sd/3<sup>T</sup> from related *Bacillus* species are listed in Table 2. Based on the phenotypic differences and the phylogenetic data it is proposed to classify Sd/3<sup>T</sup> as a novel species of the genus *Bacillus*, for which the name *Bacillus indicus* sp. nov. is proposed.

#### Description of *Bacillus indicus* sp. nov.

*Bacillus indicus* (in' di.cus L. masc. adj. *indicus* pertaining to India, Indian).

Cells are aerobic, Gram-positive, non-motile rods measuring approximately 0.9–1.2 μm wide and 3.3–5.3 μm long. Strain Sd/3<sup>T</sup> has sub-terminal endospores in a slightly swollen sporangium. Colonies on nutrient agar are yellowish-orange pigmented, circular, raised, smooth, convex and 3.0–4.0 mm in diameter. The pigment in acetone exhibits

three absorption maxima at 404, 428 and 451 nm, characteristic of carotenoids. Grows in the range of 15–37 °C (optimum 30 °C) but not at 40 °C. Grows between pH 6 and 7 and tolerates up to 2.0% (w/v) NaCl. Positive for catalase, gelatinase, amylase, arginine dihydrolase and aesculin. Does not hydrolyse Tween 20 or urea. Does not reduce nitrate to nitrite and is negative for indole production, Voges–Proskauer test and citrate utilization. Utilizes D-cellobiose, meso-erythritol, inositol, lactose, D-melibiose, D-maltose, D-mannose, sucrose, L-rhamnose, D-ribose, raffinose, L-arginine, L-tryptophan and L-tyrosine but not arabinose, fumarate, glutarate, pyruvate, L-proline and L-serine as sole carbon sources. Sensitive to the antibiotics ampicillin (10 µg), chloramphenicol (30 µg), kanamycin (30 µg), nalidixic acid (30 µg), neomycin (30 µg), rifampicin (30 µg), streptomycin (10 µg) and tetracycline (30 µg). Resistant to amoxycillin (10 µg). The major fatty acids are iso-C14:0 (10.9%), iso-C15:0 (33.5%), anteiso-C15:0 (19.3%), iso-C16:0 (11.0%), C16:0 (5.9%) and iso-C17:0 (10.8%). The main proportion of the polar lipids consists of phosphatidylglycerol, diphosphatidylglycerol and phosphatidylethanolamine. The major respiratory quinone is MK-7. The cell wall is an A4β-murein with ornithine as the diamino acid and aspartic acid as the interpeptide bridge. The G+C content of DNA is 41.2 mol%. The type strain is Sd/3<sup>T</sup> (=MTCC 4374<sup>T</sup>=DSM 15820<sup>T</sup>), and was isolated from sand of an arsenic-contaminated aquifer in West Bengal, India.

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