Deinococcus indicus sp. nov., an arsenic-resistant bacterium from an aquifer in West Bengal, India

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An arsenic- and radiation-resistant bacterium, strain Wt/1a^T, was isolated from water from an arsenic-contaminated aquifer located in the Chakdah district of West Bengal, India. The bacterium stains Gram-negative and is rod-shaped, non-motile, non-sporulating and red-pigmented. Cell-wall peptidoglycan contains ornithine as the diamino acid, MK-8 is the major menaquinone, C_{15:1} and C_{16:1} are the major fatty acids and the DNA G + C content of the organism is 65.8 mol%. Based on these phenotypic and chemotaxonomic characteristics, strain Wt/1a^T was identified as a member of the genus *Deinococcus*. Strain Wt/1a^T exhibited maximum 16S rRNA gene sequence similarity (95 %) with *Deinococcus grandis*; however, strain Wt/1a^T exhibited only 14 % similarity to *D. grandis* IAM 13005^T at the DNA–DNA level. Furthermore, strain Wt/1a^T (compared to *D. grandis* IAM 13005^T) is more resistant to arsenate and arsenite, is positive for arginine dihydrolase, utilizes a number of carbon sources and exhibits quantitative differences in fatty acid composition and qualitative differences in lipid composition. Strain Wt/1a^T is identified as a novel species of the genus *Deinococcus*, for which the name *Deinococcus indicus* sp. nov. is proposed. The type strain of *Deinococcus indicus* is Wt/1a^T (=MTCC 4913^T = DSM 15307^T).

Arsenic contamination of water has been reported in various parts of the world, including India, where arsenic has contaminated the groundwater in the entire Bengal delta, which covers the Indian state of West Bengal and parts of Bangladesh. The source of arsenic in this site has been attributed to both the oxidation of pyrite/arsenopyrite in the aquifer (Bhattacharya *et al.*, 1997) and the reduction of ferrous-coated sand grains (Nickson *et al.*, 1998). Diverse microbial flora that is resistant to arsenic, including *Pseudomonas fluorescens* (de Vicente *et al.*, 1990; Prithivirajasingh *et al.*, 2001), *Bacillus subtilis* (Sato & Kobayashi, 1998), *Thermus aquaticus* and *Thermus thermophilus* (Gihring *et al.*, 2001), *Yersinia enterocolitica* and *Yersinia intermedia* (Bansal *et al.*, 2000), *Streptomyces noursei* (Friedrich *et al.*, 1984) and *Desulfotobacterium* sp. (Niggemeyer *et al.*, 2001), has been reported from various habitats.

Arsenic-contaminated water that was collected in sterile tubes from various aquifers in July 2001, when plated directly onto a nutrient agar plate and incubated at 30 °C for 2 days, yielded 2.21×10^3 c.f.u. ml⁻¹. These colonies represented 13 different morphotypes. Based on protein profiles, these 13 morphotypes were categorized into four groups (data not shown), as follows: group I (Wt/1a^T, Wt/1b and Wt/1c); group II (Wt/2a, Wt/2b, Wt/2c and Wt/2d); group III (Wt/3a, Wt/3b, Wt/3c, Wt/3d and Wt/3e); and group IV (Wt/4a and Wt/4b). All strains within each group had identical protein profiles, indicating that they were clonal in origin. In the present study, isolate Wt/1a^T, which was chosen as a representative strain of the three isolates that belong to group I, is subjected to polyphasic taxonomic studies. This red-pigmented bacterium was identified as a novel species of the genus *Deinococcus* and was assigned the name *Deinococcus indicus* sp. nov.

Strain Wt/1a^T was isolated from a shallow aquifer in the Bengal basin, Chakdah district, West Bengal, India (88° 35' S 23° 3' E). The medium used for isolation of the bacterium was nutrient agar [0.5 % (w/v) peptone, 0.3 % (w/v) beef extract, 0.5 % NaCl and 1.5 % (w/v) agar, pH 6.5]. Nutrient agar was used for growth, maintenance and biochemical tests. Optimum pH and temperature for growth were 6.5 and 30 °C, respectively. Cultures were grown in nutrient broth that contained either sodium

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Abbreviations: DPG, diphosphatidylglycerol; PC, phosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Wt/1a^T is AJ549111.

A table showing survival of *Deinococcus* species under UV light and a figure showing one-dimensional TLC of total lipid extracts from *Deinococcus* species are available as supplementary material in IJSEM Online.

arsenate (Na_2HAsO_4) or arsenic trioxide (As_2O_3) to determine the tolerance of the culture to arsenate and arsenite.

The bacterial culture was observed in the lag, exponential and stationary phases of growth under a phase-contrast microscope ($\times 1000$) to ascertain the shape and motility of the bacterium. Biochemical tests were performed as described by Lanyi (1987), Reddy *et al.* (2002a, b) and Smibert & Krieg (1994). Single carbon source assimilation tests were performed in a minimal basal salts medium [which contained 10.5 g K_2HPO_4 , 4.5 g KH_2PO_4 , 1 g $(\text{NH}_4)_2\text{SO}_4$ and 15 g agar (l medium prepared in distilled water) $^{-1}$]. Organic substrates were filter-sterilized (Millipore; catalogue no. PHWP02500) and added to the medium at a final concentration of 5 g l^{-1} before plates were poured. The sensitivity of the culture to different antibiotics was checked by using antibiotic discs that were supplied by HiMedia. SDS-PAGE was performed according to the method of Laemmli (1970). Isolation of DNA and determination of its G + C content were performed as described previously (Shivaji *et al.*, 1989; Reddy *et al.*, 2000). DNA–DNA hybridization was performed by the membrane filter method (Tourova & Antonov, 1987) as described previously (Shivaji *et al.*, 1992; Reddy *et al.*, 2000). To determine tolerance of the culture to UV radiation, the culture was grown to late-exponential phase and harvested at 5000 g for 5 min at 4 °C; the cell pellet was suspended in 10 ml phosphate buffer (pH 7.2), diluted serially and 0.1 ml was spread on nutrient agar plates. Plates (with their lids open) were then exposed to UV light (UV-B, 15 W $\times$ 4; Sanyo Denki) at a distance of 30 cm from the UV source for the required dose and were incubated subsequently at 30 °C for 5 days. The UV dose was monitored with a model RX003 UV detector (UVI Tech). *Deinococcus radiodurans* DSM 20539^T, *D. grandis* IAM 13005^T and *Escherichia coli* DH5 α were used as controls to evaluate the effect of UV radiation on the growth of the micro-organisms.

Cells were grown in nutrient broth at 30 °C and the fatty acid methyl esters (Sato & Murata, 1988) were analysed as described previously (Reddy *et al.*, 2002a). Isoprenoid quinones were extracted, separated by HPLC and identified as described previously (Reddy *et al.*, 2003). Peptidoglycan was prepared and analysed according to the method described by Komagata & Suzuki (1987). Polar lipids were extracted and analysed according to the method described by Counsell & Murray (1986). In this method, the bacterial cell pellet is extracted with chloroform:methanol (2:1) and separated by one-dimensional TLC, using a pre-coated silica gel TLC plate (Merck; catalogue no. 5721) and chloroform:acetone:methanol:acetic acid:water (10:4:2:2:1, v/v) as the solvent system. Total lipids were detected by spraying with 25 % H_2SO_4 in ethanol, followed by charring at 150 °C for 5 min and compared with phosphatidylinositol (PI), phosphatidylcholine (PC), phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG), which were run under similar conditions. Phosphoglycolipids were detected by staining with α -naphthol [a mixture

of 10.5 ml 15 % (w/v) α -naphthol in ethanol, 6.5 ml concentrated H_2SO_4 and 4.0 ml water], whereas ninhydrin (0.25 % in acetone) was used to detect aminoglycolipids.

The 16S rRNA gene was amplified (Shivaji *et al.*, 2000), purified with a QIAquick PCR purification kit (Qiagen) and sequenced by using an ABI PRISM BigDye Terminator cycle sequencing kit and an automatic DNA sequencer (ABI PRISM model 3700) (both from Applied Biosystems). The partial 16S rDNA sequence (1456 bp) was aligned with those of species of the genera *Deinococcus*, *Kocuria* and *Arthrobacter* by using CLUSTAL W (Thompson *et al.*, 1994). Pairwise evolutionary distances were computed by using the DNADIST program with the Kimura two-parameter model, as developed by Kimura (1980). Phylogenetic trees were constructed by using two tree-making algorithms: UPGMA and parsimony (DNAPARS; Felsenstein, 1993). 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 (UPGMA algorithm) and CONSENSE of the PHYLIP package.

Cells of Wt/1a^T stain Gram-negative and are rod-shaped, non-motile, non-sporulating and red-pigmented. Cell-wall peptidoglycan contains ornithine as the diamino acid (A3 β variant), MK-8 is the organism's major respiratory quinone, C_{15:1} and C_{16:1} are the major fatty acids and the DNA G + C content is 65.8 mol%. Based on the above characteristics, strain Wt/1a^T was identified as a member of the genus *Deinococcus* (Murray & Brooks, 1986; Rainey *et al.*, 1997). A BLAST sequence similarity search and phylogenetic studies corroborated the above data and confirmed that strain Wt/1a^T belongs to the genus *Deinococcus*. Furthermore, the 16S rRNA gene sequence of strain Wt/1a^T possessed all the signature nucleotides, namely C, G, T, G, T, A, G, C and C at positions 657, 749, 757, 1050, 1208, 1421, 1429, 1471 and 1479, respectively, that are characteristic features of the genus *Deinococcus* (Rainey *et al.*, 1997).

Phylogenetic analysis based on the 16S rRNA gene sequence indicated that strain Wt/1a^T is related closely to *D. grandis* IAM 13005^T (Oyaizu *et al.*, 1987; Rainey *et al.*, 1997), with 95 % sequence similarity. The topology of the phylogenetic tree (Fig. 1) indicated that strain Wt/1a^T forms a robust clade with *D. grandis* IAM 13005^T, with a bootstrap resampling value of 100 %. Furthermore, the phylogenetic affiliation of strain Wt/1a^T with *D. grandis* IAM 13005^T is confirmed by its rod shape and Gram-staining (both isolates stain Gram-negative), whereas other species of the genus are Gram-positive and spherical in shape. However, when the 16S rRNA gene sequence of strain Wt/1a^T (1453 bp) is compared with that of *D. grandis* IAM 13005^T, 73 bases were observed to be different and strain Wt/1a^T shared only 14 % DNA–DNA reassociation with *D. grandis* IAM 13005^T. Furthermore, strain Wt/1a^T showed low 16S rRNA gene sequence similarity values of 91.94, 91.67, 93.51, 91.6, 89.39 and 91.5 % with *Deinococcus radiopugnans*, *Deinococcus murrayi*, *D. radiodurans*, *Deinococcus radiophilus*,

Fig. 1. UPGMA phenogram showing the phylogenetic relationship between *Deinococcus indicus* Wt/1a^T, other species of the genus *Deinococcus* and related reference micro-organisms, based on 16S rRNA gene sequence analysis. Bootstrap values are given at nodes. Branch-lengths in the phenogram are not to scale.

Deinococcus geothermalis and *Deinococcus proteolyticus*, respectively.

Strain Wt/1a^T also differed from *D. grandis* IAM 13005^T with respect to a number of physiological characteristics (Table 1). It had higher amounts of the fatty acids C_{16:0} and C_{16:1} and reduced amounts of C_{14:0} and C_{15:1} (Table 2). In addition, lipid analysis indicated that strain Wt/1a^T had seven lipids with *R_f* values of 0.21, 0.27, 0.42, 0.70, 0.76, 0.84 and 0.89 (identical to the lipid profile of *D. grandis* IAM 13005^T), but differed in that it did not contain lipids 1–3, with *R_f* values of 0.11, 0.12 and 0.17, respectively, as observed in *D. grandis* IAM 13005^T (see Supplementary Figure, available in IJSEM Online). Furthermore, none of the lipids matched with PI, PC, PG or DPG, although one of the phosphoglycolipids (lipid 4, with an *R_f* value of 0.21) showed similar (but not identical) chromatographic behaviour to PI. The fact that lipid 4 and PI are different is also evident from the fact that unlike lipid 4, which stains positively with α -naphthol, PI does not stain [see Supplementary Fig. (B), available in IJSEM Online]. In addition to the above differences, strain Wt/1a^T grew in the presence of 10 and 0.2 mM arsenate and arsenite, respectively, whereas *D. grandis* IAM 13005^T is highly sensitive to both arsenate and arsenite (Table 1). Cultures of *D. radiodurans* DSM 20539^T, *D. grandis* IAM 13005^T and strain Wt/1a^T were found to be more resistant to UV radiation than *E. coli* DH5 α . When the cultures were exposed to varying doses of UV radiation, it was observed that the survival of *E. coli* decreased by 99% when exposed to 0.32 J cm⁻², whereas at the same dose, survival in strain Wt/1a^T, *D. grandis* IAM 13005^T and *D. radiodurans* DSM 20539^T was 66, 81 and 85%, respectively. At higher UV doses, *E. coli* DH5 α did not survive, but 2–4% survival was observed in the remaining three cultures, even when they were exposed to 5.87 J cm⁻² (see

Table 1. Phenotypic differences between *D. indicus* Wt/1a^T and *D. grandis* IAM 13005^T

Taxa: 1, *D. indicus* Wt/1a^T; 2, *D. grandis* IAM 13005^T. Cells were cultured in appropriate media at 30 °C for all studies. Both species are resistant to 1 mM Na₂HAsO₄. +, Positive; –, negative; R, resistant; S, sensitive.

Characteristic	1	2
Resistance to arsenic:		
As(V) (Na ₂ HAsO ₄):		
5 mM	+	–
10 mM	+	–
As(III) (As ₂ O ₃) (0.2 mM)	+	–
Biochemical characteristics:		
Arginine dihydrolase	+	–
Triple sugar/iron agar (change to yellow)	+	–
Utilization as carbon source:		
L-Arabinose	+	–
D-Cellobiose	+	–
Lactose	+	–
D-Mannose	+	–
D-Melibiose	+	–
L-Rhamnose	+	–
D-Raffinose	+	–
L-Tryptophan	+	–
L-Aspartic acid	+	–
L-Arginine	+	–
L-Histidine	+	–
Sensitivity to antibiotics:		
Amoxycillin	R	S
Ampicillin	R	S
Nalidixic acid	S	R
Chemotaxonomic characteristics:		
Lipid 1 (<i>R_f</i> value, 0.11)	–	+
Lipid 2 (<i>R_f</i> value, 0.12)	–	+
Lipid 3 (<i>R_f</i> value, 0.17)	–	+

Supplementary Table in IJSEM Online). Thus, based on physiological, chemotaxonomic and phylogenetic differences, it is proposed to assign novel species status to strain Wt/1a^T, for which the name *Deinococcus indicus* sp. nov. is proposed, with Wt/1a^T (= MTCC 4913^T = DSM 15307^T) as the type strain.

Description of *Deinococcus indicus* sp. nov.

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

Cells stain Gram-negative and are non-motile and rod-shaped. Colonies on nutrient agar medium are red-pigmented, smooth, convex, circular, uniform-edged and 1–2 mm in diameter. Optimum growth occurs at 30 °C; growth occurs at 20–37 °C and pH 6–7. Tolerates up to 1.0% NaCl and is resistant to UV radiation (5.87 J cm⁻²). Strictly aerobic and positive for catalase, gelatinase, amylase, arginine dihydrolase, aesculin and casein hydrolysis and

Table 2. Fatty acid composition (%) of *D. indicus* Wt/1a^T and *D. grandis* IAM 13005^T

Taxa: 1, *D. indicus* Wt/1a^T; 2, *D. grandis* IAM 13005^T. Cultures were grown in nutrient broth at 30 °C and used for analysis of fatty acid methyl esters. –, Absent.

Fatty acid	1	2
Straight-chain:		
C _{14:0}	1.93	3.85
C _{16:0}	12.15	7.21
C _{18:0}	0.08	–
Branched-chain:		
iso-C _{15:0}	0.06	0.18
anteiso-C _{15:0}	4.78	4.82
anteiso-C _{17:0}	9.77	10.52
Unsaturated:		
C _{15:1}	12.45	22.44
C _{16:1}	33.65	19.56
C _{17:1}	10.7	11.29
C _{18:1}	0.26	0.24

reduction of nitrate to nitrite, but negative for oxidase, lipase, urease, indole production, Voges–Proskauer test and citrate utilization. A number of compounds are utilized as sole carbon sources (Table 1), including D-maltose, sucrose, D-ribose and L-tyrosine, but not acetate, cellulose, D-fructose, D-galactose, D-glucose, *meso*-inositol, pyruvate, D-sorbose, L-glycine, creatinine, L-alanine or L-cysteine. Sensitive to the antibiotics bacitracin, chloramphenicol, kanamycin, nalidixic acid, neomycin, penicillin, rifampicin, rifamycin, streptomycin and tetracycline, but resistant to ampicillin and amoxycillin. Fatty acid composition of the type strain is given in Table 2; there are seven unknown polar lipids. Major respiratory quinone is MK-8 and cell-wall peptidoglycan contains ornithine as the diamino acid. DNA G+C content is 65.8 mol%.

The type strain is Wt/1a^T (= MTCC 4913^T = DSMZ 1537^T).

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