Bacillus okhensis sp. nov., a halotolerant and alkalitolerant bacterium from an Indian saltpan

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A strictly aerobic, rod-shaped bacterium (0.6–0.8 × 2–3 µm), designated strain Kh10-101^T, was isolated from a saltpan (22° 15' N, 69° 1' E) in the vicinity of Port Okha, India. The creamish pigmented colonies of strain Kh10-101^T were round, flat and translucent with irregular margins and a smooth surface. The strain possessed up to three subpolar flagella, and was motile by a corkscrew motion. The strain grew optimally at 37 °C (temperature growth range 25–40 °C) in a complex glucose-containing medium with 5% NaCl (NaCl growth range 0–10%) at pH 9 (pH growth range pH 7–10), indicating that it was a mesophilic halotolerant alkaliphile. The strain was sensitive to lincomycin, meticillin, cefuroxime and cephalixin, but resistant to gentamicin, tetracycline and cotrimazine. Spores were not detected and cells were heat sensitive. The isolate metabolized a range of carbohydrates and hydrolysed casein, gelatin and starch. Growth was not observed on aromatic compounds, Tween 40 or Tween 80. Nitrate was not reduced and catalase was produced. Electron microscopic examination of thin sections revealed a single thick Gram-positive cell wall. The DNA G + C content was 41 ± 1 mol%. Phylogenetic analyses of the 16S rRNA gene sequence revealed that strain Kh10-101^T was a member of the sixth rRNA group of the genus *Bacillus*, which includes alkalitolerant, alkaliphilic and halotolerant species. The halotolerant obligate alkaliphile *Bacillus krulwichiae* is the closest relative of strain Kh10-101^T (96% similarity) but a number of phenotypic differences suggest that strain Kh10-101^T (=JCM 13040^T = ATCC BAA-1137^T) should be designated the type strain of a new species, for which the name *Bacillus okhensis* sp. nov. is proposed.

Haloalkaliphilic and alkalitolerant microbes occur naturally in alkaline and saline environments, and during the past decade, studies on the ecology, physiology and taxonomy of these organisms have revealed an impressive diversity. Haloalkaliphiles possess special adaptation mechanisms for survival under these two extremities. The members of the alkaliphilic and alkalitolerant *Bacillus* species form the sixth rRNA group (Nielsen *et al.*, 1994) and have attracted much attention as they secrete extracellular enzymes that are both active and stable at high salt concentrations and high pH. These properties make them an interesting tool for fundamental research and for biotechnological exploration (Margesin & Schinner, 2001; Demirjian *et al.*, 2001). The vast majority of haloalkaliphiles and alkalitolerant species have been isolated from athalassohaline environments (Duckworth *et al.*, 1996; Jones *et al.*, 1998; Pikuta *et al.*, 2003; Rees *et al.*, 2004) while thalassohaline environments have remained relatively less explored (Nielsen *et al.*, 1995; Suzuki *et al.*, 1997; Munoz *et al.*, 2001; Amoozgar *et al.*,

2003). We describe in this report the characterization of a new halotolerant and alkalitolerant *Bacillus* species that was isolated from a thalassohaline environment.

A soil sample was collected from a natural saltpan near the shore of Port Okha (22° 15' N, 69° 1' E) in sterile glass containers by scraping the soil surface. For initiating enrichment cultures, 1 g soil was diluted in 5 ml sterile distilled H₂O, and large suspended solids were allowed to settle by gravitation for 10 min, after which 1 ml of the relatively clear suspension was added to a flask containing 100 ml complex medium (CMB) consisting of (g l⁻¹): glucose, 10; peptone, 5; yeast extract, 5; KH₂PO₄, 5; NaCl, 10–25%. The medium was adjusted to pH 10.0 using sterile autoclaved Na₂CO₃ (20%, w/v). CMB medium became turbid after 48 h incubation at 37 °C and pure cultures were isolated by streaking CMB agar plates (CMB medium containing 2.5% agar). Single colonies that developed after 4 days incubation at 37 °C on CMB plates were restreaked onto fresh CMB agar plates. The isolation procedure was repeated three times to obtain the pure culture. One of the pure cultures, Kh10-101^T, was designated a type strain, and characterized further. The pure culture was maintained on CMB medium (pH 9;

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Kh10-101^T is DQ026060.

10%, w/v, NaCl) described above and stored at 4 °C. The strain was subcultured at monthly intervals. Colonies of Kh10-101^T were round, flat and translucent with irregular margins, a smooth surface and a creamish pigment.

Cellular characterization and sporulation tests performed as described previously (Kanso & Patel, 2003) showed that the cells of strain Kh10-101^T were rod-shaped (0.6–0.8 × 2–3 µm) and actively motile. Electron microscopy of negatively stained cells showed the presence of up to three subpolar flagella. The cells stained Gram-positive and thin sections revealed the presence of a typical thick Gram-positive cell wall. Microscopic examination did not reveal the presence of inclusion bodies or spores. The cells of strain Kh10-101^T were not viable after 10 min incubation at 70 °C, indicating the absence of heat-resistant bodies such as spores.

The optimal conditions for growth of strain Kh10-101^T were tested in a medium containing (per 100 ml double-distilled H₂O): 1 g D-glucose, 0.5 g KH₂PO₄, 0.5 g peptone, 0.5 g yeast extract and 5 g NaCl. The strain grew optimally at pH 9 (pH growth range pH 7–10) with 5% NaCl (NaCl growth range 0–10%) at 37 °C (temperature growth range 25–40 °C). The generation time was determined to be 2.5 h under optimal growth conditions (CMB medium, pH 9, containing 5% NaCl and incubation temperature of 37 °C).

To determine antibiotic sensitivity, an active culture of strain Kh10-101^T was inoculated onto CMB agar plates (pH 9) containing 10% (w/v) NaCl following which Hi-media octadiscs impregnated with antibiotics were placed on their surface. The plates were incubated at 37 °C for up to 48 h

and the zones of inhibition around the antibiotic discs were recorded. Strain Kh10-101^T was found to be highly sensitive to lincomycin, meticillin, cefuroxime and cephalexin but resistant to gentamicin, tetracycline and cotrimazine.

Biolog GP2 plates (Hayward) were used to characterize the biochemical capacity of the strain using the manufacturer's recommended procedures but with the following modification. A culture of strain Kh10-101^T was centrifuged and the cell pellet was washed and resuspended in a tube of Inoculating Fluid (IF GN/GP) supplied with the Biolog plates, to which was added 1.25 g NaCl and 1.2 g Na₂CO₃. The resulting fluid was filter-sterilized prior to use. Cell suspension (150 µl) was then added to the wells of Biolog GP2 plates, and the plates were incubated at 37 °C and read after 24 h as recommended by the manufacturer. Strain Kh10-101^T could metabolize 3-methyl glucose, D-fructose, D-fructose 6-phosphate, D-galactose, D-glucose, D-galacturonic acid, D-mannose, D-psicose, D-ribose, D-tagatose, D-xylose, gentiobiose, lactulose, L-arabinose, L-fucose, L-rhamnose, palatinose and turanose. CMB agar plates lacking glucose but containing casein (2.5%), gelatin (0.004%) or starch (0.01%) were used to test for caseinase, gelatinase and amylase activities, respectively, using the methods described by Barrow & Feltham (1993). Strain Kh10-101^T was capable of hydrolysing casein, gelatin and starch. Amylase production occurred at pH 7–10 and 0–10% (w/v) NaCl at an optimum of 5% NaCl and pH 9.

Catalase activity was positive as determined by dropping H₂O₂ on to colonies that developed on CMB agar plates. Aromatic compounds (*p*-methylhydroxybenzoate, *p*-ethylhydroxybenzoate, *p*-propylbenzoate and sodium benzoate)

Fig. 1. Position of strain Kh10-101^T (=JCM 13040^T=ATCC BAA-1137^T) within the radiation of members of the family *Bacillaceae*, phylum *Firmicutes*. Its close relationship to members of the sixth rRNA group represented by alkalitolerant and alkaliphilic members is shown as a box. Bar indicates 1 nucleotide substitution per 100 nucleotides.

tested as carbon sources (0.03 %) in a medium (pH 9.0) containing (per 100 ml) 0.05 g peptone, 0.05 g yeast extract, 1 g KH_2PO_4 and 5 g NaCl were found not to be used. No growth was observed with Tween 40 or Tween 80.

Genomic DNA was prepared using a modified method (Marmur & Doty, 1961) in which achromopeptidase was added at a final concentration of 1 mg ml^{-1} for cell lysis and RNase was added at a final concentration of 20 $\mu\text{g ml}^{-1}$ to digest RNA (Kanso & Patel, 2003). The DNA was dissolved overnight at 4 °C in $0.1 \times \text{SSC}$ to a concentration of 20 $\mu\text{g ml}^{-1}$. *Escherichia coli* genomic DNA prepared in the same manner was used as reference DNA. The DNA G+C content of strain Kh10-101^T was 41 ± 1 mol%, determined by the thermal denaturation temperature (T_m) using a Cintra20 spectrophotometer (GBC Scientific Equipment).

The methods used for 16S rRNA gene amplification and sequencing have been reported previously (Andrews & Patel, 1996). Partial sequences generated in this investigation were assembled and the consensus sequence was corrected manually for errors using BioEdit version 5.0.1 (Hall, 1999). The most closely related sequences against GenBank and Ribosomal Database Project II were identified using BLAST (Altschul *et al.*, 1997) and the Sequence Match program (Maidak *et al.*, 2001), extracted, aligned and manually adjusted according to the 16S rRNA secondary structure using BioEdit. Sequence uncertainties were omitted and phylogenetic reconstruction was achieved using TreeCon (Van de Peer & De Wachter, 1994) in which pairwise evolutionary distances were computed from percentage similarities (Jukes & Cantor, 1969) and phylogenetic trees were constructed from the evolutionary distances using

Table 1. Differential table comparing *Bacillus okhensis* sp. nov. Kh10-101^T and *Bacillus krulwichiae* AM31D^T

Both strains grew on D-fructose, D-galactose, D-glucose, glycerol and maltose. Both produced catalase and hydrolysed starch. The temperature growth range for both strains was 25–40 °C. Neither strain could grow on cellobiose, melibiose, *myo*-inositol, raffinose or sorbitol.

Character	<i>Bacillus okhensis</i> Kh10-101 ^{T*}	<i>Bacillus krulwichiae</i> AM31D ^{T†}
Location isolated	Saltpan	Soil
Morphology	Rod (0.6–0.8 × 2–2.9 μm)	Rod (0.5–0.7 × 1.5–2.6 μm)
Motile	+	–
Flagella	Terminal tuft of flagella	Peritrichous flagella
Colony morphology	White round irregular edges	Colourless circular
Spore formation	Not observed	Ellipsoidal
pH growth range	pH 7–10	pH 8–10
NaCl growth range	0–10 %	0–14 %
G + C content (mol%)	41 ± 1	40.6–41.5
Nitrate reduction	–	+
Anaerobic growth	–	+
Hydrolysis of:		
Gelatin	+	Variable
Casein	+	Variable
Tween 40	–	+
Tween 80	–	+
Utilization of:		
D-Mannose	+	–
L-Rhamnose	+	–
Lactose	+	–
D-Xylose	–	+
Mannitol	–	+
Sucrose	–	+
Trehalose	–	+
Aromatic compounds‡	–*	+
Benzoic acid	–*	+

*This study.

†Yumoto *et al.* (2003).

‡The aromatic compounds used are indicated in the main text.

the neighbour-joining method (Saitou & Nei, 1987). Tree topology was re-examined by the bootstrap method of resampling (Felsenstein, 1985) using 1000 bootstraps. BLAST results indicated a 96% similarity to the sixth 16S rRNA group represented by the cluster of haloalkaliphilic and alkalitolerant members of the genus *Bacillus* and other related bacteria with the closest relative being *Bacillus krulwichiae* (96% similarity) (Fig. 1). In addition to the phylogenetic distance separating strain Kh10-101^T and *B. krulwichiae*, a great number of phenotypic differences also suggest that strain Kh10-101^T is a different species (Table 1). For example, strain Kh10-101^T did not utilize Tween 40, Tween 80, aromatic compounds or a range of carbohydrates (mannitol, trehalose and sucrose) but *B. krulwichiae* did. Also, strain Kh10-101^T used lactose, L-rhamnose and D-mannose but *B. krulwichiae* did not. Strain Kh10-101^T was a halotolerant alkaliphile and grew at neutral pH and with NaCl concentrations of up to 10% whereas *B. krulwichiae* was a halotolerant obligate alkali-philic which grew with NaCl concentrations of up to 14%.

The DNA G+C content of strain Kh10-101^T was 41 ± 1 mol% and is similar to that for *B. krulwichiae*. Based on the phylogeny and phenotypic differences, we propose to designate strain Kh10-101^T a new species of *Bacillus*, *Bacillus okhensis* sp. nov.

Description of *Bacillus okhensis* sp. nov.

Bacillus okhensis (ok.hen'sis. N.L. masc. adj. *okhensis* pertaining to Port Okha, a port of the Dwarka region in India, where the type strain was isolated).

Cells are Gram-positive rods (0.6–0.8 × 2–3 µm) with a subterminal flagella tuft allowing vigorous forward propulsion. Spores are not observed and cells are sensitive to heat. Colonies are round and creamish with irregular edges. Growth occurs at pH 7–10 and 0–10% (w/v) NaCl. Grows optimally with 5% NaCl (NaCl growth range 0–10%). Sensitive to lincomycin, meticcillin, cefuroxime and cephalixin but resistant to gentamicin, tetracycline and cotrimazine. Utilizes 3-methyl glucose, D-fructose, D-fructose 6-phosphate, D-galactose, D-glucose, D-galacturonic acid, D-mannose, D-psicose, D-ribose, D-tagatose, D-xylose, gentiobiose, lactulose, L-arabinose, L-fucose, L-rhamnose, palatinose and turanose but not Tween 40 or 80, glycerol, mannitol, sucrose, adenosine or dextrin. Hydrolyses casein, starch and gelatin, produces catalase and can not reduce nitrate. The DNA G+C content is 41.6 ± 1 mol%.

The type strain, Kh10-101^T (= JCM 13040^T = ATCC BAA-1137^T), was isolated from an Indian saltpan.

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