Bacillus canaveralius sp. nov., an alkali-tolerant bacterium isolated from a spacecraft assembly facility

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Two Gram-positive, rod-shaped, alkali-tolerant (pH 10.5), endospore-forming bacteria (strains KSC SF8b^T and KSC SF10a) were isolated from surfaces within the Payload Hazardous Servicing Facility, where robotic spacecraft are assembled and tested before launch, at the Kennedy Space Center at Cape Canaveral. Based on 16S rRNA gene sequence similarities, these strains were shown to belong to the family *Bacillaceae* and the genus *Bacillus*. The highest 16S rRNA gene sequence similarity was ~97.5%, observed between the novel strains and *Bacillus selenatarsenatis* SF-1^T. Several phenotypic characteristics, such as growth with 10% NaCl and assimilation of melibiose and lactose, were useful in the discrimination of this novel species from the closely related alkali-tolerant species *Bacillus firmus* and *B. selenatarsenatis*. DNA–DNA hybridization studies revealed reassociation values of less than 45% between strain KSC SF8b^T and its closest genotypic neighbours. The combination of unique phenotypic and genotypic characteristics allowed the differentiation of these alkali- and halotolerant spore-forming strains from related *Bacillus* species, and a novel species, *Bacillus canaveralius* sp. nov., is proposed. The type strain is KSC SF8b^T (=ATCC BAA-1493^T=MTCC 8908^T).

The *Treaty on Principles Governing the Activities of States in the Exploration and Use of Outer Space, Including the Moon and Other Celestial Bodies*, also known as the UN Space Treaty of 1967, requires all space-faring nations to ‘pursue studies of outer space ...’ and ‘conduct exploration ... so as to avoid their harmful contamination ...’ (UN Treaty, 1967). After the United States of America ratified the treaty in 1967, the National Aeronautics and Space Administration (NASA) dictated strict control of terrestrial organisms contaminating robotic spacecraft intended to land, orbit, fly by or be in the vicinity of extraterrestrial bodies (Rummel, 2001). As a result, robotic spacecraft are assembled and encapsulated in clean rooms that follow stringent quality-control procedures. Extensive microbial

diversity studies of the clean rooms have revealed that microbes not only live but thrive under these relatively extreme conditions (Kempf *et al.*, 2005; La Duc *et al.*, 2007; Newcombe *et al.*, 2005, 2008; Venkateswaran *et al.*, 2003). There is growing concern that their hardy nature could allow these microbes to accompany robotic spacecraft into space and potentially contaminate extraterrestrial bodies (Horneck, 1993; Melosh, 1988; Newcombe *et al.*, 2005).

During a survey of assembly-room facilities across the United States of America in 2006, microbes were systematically isolated and enriched under various environmental conditions and identified using 16S rRNA gene sequence analysis (La Duc *et al.*, 2007). Two bacterial strains that were isolated from the Payload Hazardous Servicing Facility (PHSF) at the Kennedy Space Center at Cape Canaveral, KSC SF8b^T and KSC SF10a, survived heat shock protocols (NASA, 1980) and exhibited resistance to high concentrations of NaCl and MgCl₂ in aqueous solution (La Duc *et al.*, 2007). These organisms exhibited <97.5% 16S rRNA gene sequence similarity to all but two members of the genus *Bacillus* recorded in public databases. Based on phylogenetic analysis, these alkali- and halotolerant strains were considered to be potentially

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Abbreviations: NASA, National Aeronautics and Space Administration; PHSF, Payload Hazardous Servicing Facility.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains KSC SF8b^T and KSC SF10a are DQ870688 and DQ870686, respectively.

novel and they were characterized further using biochemical, physiological and molecular techniques.

The PHSF is a Class 100 000 clean room (maximum number of particles $>0.5 \mu\text{m ft}^{-3}$ air; http://www.engineeringtoolbox.com/clean-rooms-iso-d_933.html). During the characterization of organisms isolated from surfaces within the PHSF, strains KSC SF8b^T and KSC SF10a were recovered from surface-swab samples 20 m apart. The isolation protocol consisted of 2 min in a sonication bath followed by 15 min heat-shock treatment at 80 °C (NASA, 1980). A detailed explanation of the sampling protocols, processing, and isolation is reported elsewhere (La Duc *et al.*, 2007). Strains KSC SF8b^T and KSC SF10a appeared as beige colonies on trypticase soy agar (TSA; Becton Dickinson). All further cultivations were carried out on TSA at 32 °C. Isolates were capable of growth at 15–40 °C but not at 50 °C. Cell morphology was observed under an Olympus light microscope at $\times 1000$ with cells grown for 3 days at 32 °C on TSA or in tryptic soy broth (TSB; Becton Dickinson).

Initial identification and phylogenetic affiliation of these novel strains were determined using 16S rRNA gene sequence analysis. Bacterial small-subunit rRNA genes were amplified by PCR with eubacterially biased primers B27F and B1492R (Lane, 1991) following conditions described by Ruimy *et al.* (1994). The PCR-amplified 16S rRNA gene fragments were purified using QIAquick columns (Qiagen) and were fully sequenced in both directions. The resulting continuous 1350 bp stretches of the 16S rRNA gene sequences from strains KSC SF8b^T and KSC SF10a were used to search GenBank using the BLAST algorithm (Zhang *et al.*, 2000) for similar sequences. 16S rRNA gene sequence similarity can indicate whether species are related and a recent publication suggested that the cut-off value of 16S rRNA gene sequence similarity be

raised from 97 to 98.7–99.0% for mandatory uniqueness testing (Stackebrandt & Ebers, 2006). All but two closely related species fell below the 98–99% sequence similarity value. Of the type strains of species of the genus with validly published names, *Bacillus selenatarsenatis* SF-1^T exhibited the highest 16S rRNA gene sequence similarity (97.5%) with strains KSC SF8b^T and KSC SF10a. All of the type strains of the other *Bacillus* species tested showed sequence similarities under 97.5%: *Bacillus circulans* ATCC 4513^T (97.2%), *Bacillus benzoovorans* ATCC 49005^T (97.1%) and *Bacillus firmus* IAM 12464^T (96.7%).

The top 100 most related sequences recovered from BLAST along with the most related *Bacillus* type strains were used for further phylogenetic analysis. Pairwise and multiple alignments and distance matrices of the aligned sequences were generated using MEGA4 software (Tamura *et al.*, 2007). The tree resulting from parsimony analysis of the most closely related sequences is given in Fig. 1. Bootstrap analysis based on 1000 replications was used to avoid sampling artefacts. The sequences of strains KSC SF8b^T and KSC SF10a were 99.77% similar to each other and formed a well-defined monophyletic cluster that was accompanied by high bootstrap values. An uncultured 16S rRNA gene clone JSC-7b from our earlier study grouped on the same branch with strains KSC SF8b^T and KSC SF10a (La Duc *et al.*, 2007). From this analysis, it is shown that strains KSC SF8b^T and KSC SF10a fall into the group of aerobic, low DNA G + C content, spore-forming members of *Bacillaceae*.

Five type strains, *B. firmus* IAM 12464^T, *B. benzoovorans* ATCC 49005^T, *B. circulans* ATCC 4513^T, *Bacillus foraminis* CV53^T and *B. selenatarsenatis* SF-1^T, consistently grouped with strain KSC SF8b^T via parsimony analyses and these species were selected for DNA–DNA hybridization analysis with strains KSC SF8b^T and KSC SF10a. Hybridization

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences from strains KSC SF8b^T and KSC SF10a and type strains of related recognized *Bacillus* species, generated using parsimony algorithms within the MEGA4 software package. Bootstrap percentages based on 1000 resamplings are shown at branch nodes. GenBank numbers are given in parentheses. *Paenibacillus barcinonensis* DSM 15478^T was used as the outgroup. Bar, 20 substitutions per nucleotide position.

analyses were performed with freshly isolated genomic DNA and carried out in triplicate using the membrane filter method (Tourova & Antonov, 1987). When hybridized with each other, strains KSC SF8b^T and KSC SF10a exhibited DNA–DNA reassociation values of >99 %, whereas the reassociation values of the novel isolates and *B. firmus* ATCC 14575^T (15.9 %), *B. circulans* ATCC 4513^T (44.8 %), *B. foraminis* LMG 23174^T (38.3 %), *B. benzoovorans* ATCC 49005^T (6.7 %) and *B. selenatarsenatis* JCM 14380^T (14.1 %) were all significantly below the 70 %

minimum reassociation value recommended for the delineation of bacterial species (Wayne, 1988). The G + C content of the genomic DNA was determined spectrophotometrically (Lambda 35; Perkin Elmer) using a thermal denaturation method (Mandel & Marmur, 1968).

Both strains KSC SF8b^T and KSC SF10a exhibited characteristics consistent with a Gram-positive cell wall after Gram staining. A modified Schaeffer's medium (MSM) was used to induce sporulation as described by

Table 1. Phenotypic and chemotaxonomic characteristics of *Bacillus canaveralius* sp. nov. strains and related species

Strains: 1, KSC SF8b^T and KSC SF10a (*Bacillus canaveralius* sp. nov.; data from this study); 2, *B. firmus* IAM 12464^T (Heyrman *et al.*, 2004); 3, *B. circulans* ATCC 4513^T (Claus & Berkeley, 1986); 4, *B. foraminis* LMG 23174^T (Tiago *et al.*, 2006); 5, *B. selenatarsenatis* SF-1^T (Yamamura *et al.*, 2007); 6, *B. benzoovorans* NCIMB 12555^T (Pichinoty *et al.*, 1984); 7, *B. bataviensis* LMG 21833^T (Heyrman *et al.*, 2004); 8, *B. drentensis* LMG 21831^T (Heyrman *et al.*, 2004). All strains are rod-shaped. None of the strains grow at 50 °C, produce indole or arginine dihydrolase or utilize turanose. +, Positive; –, negative; v, variable (depending on strain and medium); w, weak; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8
Spore morphology	Oval	Oval	Oval	No spores	ND	Oval	Oval	Oval
Anaerobic growth	–	–*	+	–	+	–	+	+
Growth in:								
5 % NaCl	+	+	–	–	+	–	ND	ND
10 % NaCl	+	–†	–†	–	–	–†	ND	ND
H ₂ S production	–	–	–	–	+	ND	–	–
ONPG reaction	–	–	+	+	+	ND	+	+
Nitrate reduction	+	+	–	+	+	+	+	v
Tryptophan deaminase	+	–	+	ND	+	ND	–	–
Hydrolysis of:								
Aesculin	+	+	+	+	+	ND	ND	ND
Gelatin	–	+	+	+	+	–	+	–
Acid production from:								
Amygdalin	–	–	+	+	+	ND	v	v
L-Arabinose	+	–	+	+	–	–	–	–
Cellobiose	+	–	+	+	+	ND	+	–
L-Fructose	–	–	+	–	+	ND	v	–
Galactose	–	–§	+	+	–	ND	ND	ND
Glucose	–	+	+	+	+	–	+	+
Methyl α-D-glucoside	–	–	+	+	–	ND	v	v
Glycerol	–	+	v	–	–	ND	w	–
Glycogen	–	–	+	+	+	ND	–	–
Lactose	–	–	+	–	–	ND	+	+
D-Mannitol	–	+	+	+	–	–	+	–
Melezitose	–	–	+	+	–	ND	+	v
Melibiose	+	–	+	+	–	ND	v	+
Raffinose	–	–	+	+	–	ND	+	v
Rhamnose	–	–	v	+	–	ND	ND	ND
Ribose	–	–	+	+	–	ND	w	v
Salicin	–	–	+	+	+	ND	w	+
Sucrose	+	+	+	+	+	ND	v	v
D-Turanose	–	–	+	–	–	ND	+	v
Xylose	–	–	+	+	+	–	ND	ND
DNA G + C content (mol%)	40.5	36.1–47.4*	35.0	43.0	42.8	40.2–42.3‡	39.6–40.1	39.3–39.4

*Data from Claus & Berkeley (1986).

†Data from Priest & Alexander (1988).

‡Data from Klieve *et al.* (1999).

§Data from this study.

Kempf *et al.* (2005). A single colony of each strain was inoculated in 10 ml MSM medium and incubated for 24–48 h at 28 °C before being observed under phase-contrast light microscopy (Olympus microscope BX-90). Endospore-forming, terminally swollen cells, with phase-bright spore bodies, were observed. Biochemical characterizations were accomplished using the API 20E, API 20NE and API 50 CH (using API 50 CHB/E suspension medium) systems (bioMérieux), according to the manufacturer's instructions. The API 20E tests were used to determine reactions for ornithine decarboxylase, H₂S and tryptophan deaminase. API 20NE tests were used to determine nitrate reduction, aesculin hydrolysis, β -galactosidase production and glucose fermentation. Both API 20E and 20NE were used to test for ONPG hydrolysis, indole production, gelatinase and urease. API 50 CH was used to determine acid production from and assimilation of carbohydrates. TSA supplemented with NaCl was used to measure tolerance to 0.5, 5, 10 and 15 % (w/v) NaCl. To test the ability to grow under anaerobic conditions, cultures were spread on TSA and incubated in a GasPack jar (Becton Dickinson).

The biochemical characteristics that distinguish KSC SF8b^T and KSC SF10a from closely related alkali-tolerant species are listed in Table 1. Strains KSC SF8b^T and KSC SF10a were oxidase-negative, catalase-positive, facultatively anaerobic, spore-forming, rod-shaped bacteria that could grow in standard growth media augmented to 15 % NaCl (w/v). They could also grow in medium augmented to pH 10.5. Gelatin hydrolysis and H₂S production distinguished KSC SF8b^T and KSC SF10a from their closest phylogenetic relative *B. selenatarsenatis*. Likewise, the novel isolates exhibited growth in the presence of 0.5–15 % NaCl whereas *B. selenatarsenatis* did not grow in the presence of >5 % NaCl (Yamamura *et al.*, 2007). Acid production from several carbon substrates could distinguish the novel strains from all of the other strains tested.

Based on the evidence gathered during this study, strains KSC SF8b^T and KSC SF10a exhibit characteristics that distinguish them from phenotypically and phylogenetically related taxa (Fig. 1, Table 1). Hence, they represent a novel species of the genus *Bacillus*, for which the name *Bacillus canaveralius* sp. nov. is proposed.

Description of *Bacillus canaveralius* sp. nov.

Bacillus canaveralius [ca.na.ve.ra'li.us. N.L. masc. adj. *canaveralius* pertaining to (Cape) Canaveral, isolated from walls and floors of the Kennedy Space Center at Cape Canaveral].

Cells are Gram-positive, endospore-forming, motile rods (1 × 3–5 µm). Colonies are beige and circular with an entire margin and a raised elevation. Growth occurs at 15–45 °C (optimum, 32 °C) and pH 7.0–10.5 (optimum, pH 7.4). Growth occurs in the presence of 0.5–15 % NaCl. Aerobic chemoheterotroph. No growth occurs

under strict anaerobic conditions and fermentation of glucose, production of H₂S or liquefaction of gelatin do not occur. Reduces nitrate to nitrite. Hydrolyses aesculin. Produces catalase and α -galactosidase, but not oxidase, urease, arginine dihydrolase, lysine and ornithine decarboxylases or indole. Assimilates cellobiose, D-glucose, lactose, D-mannitol, D-mannose, melibiose, N-acetylglucosamine, potassium gluconate, adipic acid, malic acid and sucrose. Produces acid from D-fructose, methyl α -D-glucopyranoside, cellobiose, D-mannose, melibiose and sucrose, but not from amygdalin, L/D-arabinose, D-mannitol, melezitose, turanose, D-galactose, D-glucose, glycerol, glycogen, L-fructose, methyl α -D-glucoside, raffinose, L-rhamnose, ribose, salicin or D-xylose. The G + C content of the type strain is 40.6 mol%.

The type strain, KSC SF8b^T (=ATCC BAA-1493^T=MTCC 8908^T), was isolated from walls and floors of the Kennedy Space Center at Cape Canaveral, FL, USA.

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