Bacillus nealsonii sp. nov., isolated from a spacecraft-assembly facility, whose spores are γ-radiation resistant

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One of the spore-formers isolated from a spacecraft-assembly facility, belonging to the genus *Bacillus*, is described on the basis of phenotypic characterization, 16S rDNA sequence analysis and DNA–DNA hybridization studies. It is a Gram-positive, facultatively anaerobic, rod-shaped eubacterium that produces endospores. The spores of this novel bacterial species exhibited resistance to UV, γ-radiation, H_2O_2 and desiccation. The 16S rDNA sequence analysis revealed a clear affiliation between this strain and members of the low G+C *Firmicutes*. High 16S rDNA sequence similarity values were found with members of the genus *Bacillus* and this was supported by fatty acid profiles. The 16S rDNA sequence similarity between strain FO-92^T and *Bacillus benzoevorans* DSM 5391^T was very high. However, molecular characterizations employing small-subunit 16S rDNA sequences were at the limits of resolution for the differentiation of species in this genus, but DNA–DNA hybridization data support the proposal of FO-92^T as *Bacillus nealsonii* sp. nov. (type strain is FO-92^T =ATCC BAA-519^T =DSM 15077^T).

INTRODUCTION

The main focus of the National Aeronautics and Space Administration's planetary-protection efforts is the development of cleaning and sterilization technologies for spacecraft preparation prior to launch. Knowledge of the microbial diversity of spacecraft-assembly facilities, as well as any extreme characteristics these microbes might possess, is essential to the development of these technologies. The spacecraft-assembly facilities can be considered extreme environments created by the controlled air circulation, low humidity and low-nutrient conditions found in these clean-rooms. A wide variety of micro-organisms can survive under such conditions (Puleo *et al.*, 1973, 1975, 1977; Venkateswaran *et al.*, 2001).

In on-going investigations to determine and document possible microbial contamination on representative spacecraft components and accessories, several physiologically and phylogenetically novel micro-organisms were encountered (Venkateswaran *et al.*, 2001). Witness plates made of spacecraft-quality stainless steel were exposed for ~9 months at a Jet Propulsion Laboratory Spacecraft Assembly Facility (JPL-SAF) and the particulate materials collected revealed the presence of novel *Bacillus* species. Micro-organisms that exhibit resistance to an assortment of free radicals and conditions employed in emergent technologies for sterilization of spacecraft components are significant. Here, we describe *Bacillus nealsonii*, whose spores are resistant to UV, γ-radiation, H_2O_2 and desiccation.

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Abbreviations: FAME, fatty acid methyl ester; JPL-SAF, Jet Propulsion Laboratory Spacecraft Assembly Facility.

The GenBank/EMBL/DDBJ accession number for the 16S rDNA sequence of strain FO-92^T is AF234863.

Images of the Jet Propulsion Laboratory Spacecraft Assembly Facility are available as supplementary data in IJSEM Online (http://ijs.sgmjournals.org).

METHODS

Sample preparation and isolation of microbes from a spacecraft-assembly facility. The dimensions of the JPL-SAF are 25 m wide, 36 m long and 15 m high. Relative humidity was controlled at 40 ± 5 % with a cap at 45 % and the mean temperature was maintained at 20 ± 5 °C. This JPL-SAF was maintained by qualified

contamination control personnel with periodic checks to ensure a class 100 000 (the maximum number of particles of the size >0·5 μm per cubic foot of air) clean-room level. Stainless steel witness plates (type 304, no. 4 finish, 0·05–0·08 cm thick; size, 2·5 × 5 cm; Mechanical Workshop, JPL) were ultrasonically cleaned in acetone (5–10 min) followed by 2-propanol (5–10 min). After air drying, the plates were sterilized by heating at 175 °C for 2 h. The pre-sterilized witness plates were exposed in JPL-SAF on stands about 2 m high. This minimized contamination from human exhalation and sweat and ensured collection of dust particles that were naturally falling onto the witness plates. After a 9-month exposure, all 20 witness plates were individually placed into 50 ml polypropylene disposable sterile centrifuge tubes.

Microbial examination. Each retrieved witness plate was placed into 30 ml of sterile phosphate-buffered (pH 7·2) rinse solution (Anonymous, 1980). The plate and rinse solution were sonicated for 2 min (25 kHz, 0·35 W cm^{-2}). The rinse solution was aseptically divided into two 15 ml aliquots. One aliquot of the rinse solution, along with the witness plate, was subjected to heat-shock (80 °C for 15 min), while the other aliquot was not heated. Total aerobic counts in appropriate aliquots of samples were determined by the pour plate technique using tryptic soy agar (TSA; Difco) as the growth medium (32 °C for 3–7 days). Type strains of different *Bacillus* species were procured from established culture collections and used as controls when necessary to validate the procedures.

Sporulation. *Bacillus* endospores were purified using the following two procedures. Cells of an overnight TSA culture were harvested, washed in sterile water and heat-shocked at 80 °C for 15 min. The heat-shock procedure killed vegetative cells but not mature spores. The heat-shocked samples were grown overnight on Difco nutrient agar supplemented with 5 p.p.m. $MnSO_4$ (MN agar), which triggers sporulation of the test microbe. About 200 μl of the heat-shocked samples was spread onto multiple MN agar plates to harvest sufficient quantities of the test isolate. The cells grown on agar were washed in sterile water and the heat-shock procedure, followed by growth on MN agar, was repeated until 99 % spores were obtained. The percentage of spores was determined by viewing the spore preparations using phase-contrast microscopy. Spores appear as bright bodies when viewed with a phase-contrast microscope. Purification of spores using this MN agar method resulted in the retention of a loosely attached extraneous layer around the spore coat. In addition, a nutrient broth sporulation medium (NSM) was used to produce spores (Nicholson & Setlow, 1990; Schaeffer *et al.*, 1965). A single purified colony of the strain to be sporulated was inoculated into liquid NSM. After 2–3 days of growth at 32 °C, the cultures were examined in wet mounts to determine the level of sporulation. Once the number of free spores in the culture was greater than the number of vegetative cells, the culture was harvested and the spores were purified. Spore purification was performed by treating the spores with lysozyme and washing with salt and detergent (Nicholson & Setlow, 1990). The chemical treatments used in this method removed the extraneous layer surrounding the spore coat. The purified spores were resuspended in sterile deionized water, heat-shocked (80 °C for 15 min) and stored at 4 °C in glass tubes.

Microscopy. The refractile nature of the spores was examined by phase-contrast microscopy using an Olympus microscope (BX-60). A Field-Emission Environmental Scanning Electron Microscope (ESEM; Philips XL30) was also used. Very high resolution/magnification and an excellent signal to noise ratio in regular high vacuum was achieved due to the field-emission electron source. Non-destructive examination of spores and vegetative cells was possible using this microscope. Specimen preparation procedures, which usually lead to sample artifacts, are not necessary when using the ESEM. In addition, standard scanning and transmission electron microscopy were used to examine the surface details and cross-sections, respectively, as per established methods (Cole & Popkin, 1981).

Characterization of spores for various physical and chemical conditions. Radiation dosimetry at the Co^{60} source was performed using an ion chamber with accuracy to the US Bureau of Standards (Coss, 1999) standard. All irradiations were carried out in glass vials using spore samples in water. The spores (10^8 spores ml^{-1}) were exposed to both 1 Mrad (50 rad s^{-1} for 330 min) and 0·5 Mrad (25 rad s^{-1} for 330 min.) and survival was quantitatively verified by growing the γ-radiation treated samples in TSA at 32 °C.

Purified spores were diluted in PBS (pH 7·2), placed into an uncovered Petri dish and exposed to UV radiation (254 nm; UV Products). At appropriate intervals, samples of spores were removed, diluted serially 10-fold in PBS and plated onto NSM agar. Plates were incubated at 37 °C for up to 5 days and colonies were counted.

A liquid H_2O_2 protocol, developed by Riesenman & Nicholson (2000), was modified and used to examine H_2O_2 resistance in spores. Suitable aliquots of spore suspensions prepared in PBS were treated with H_2O_2 (5 % final concentration) and incubated at room temperature (~25 °C) with gentle mixing. After 60 min incubation, a 100 μl sample was removed and diluted in a solution of bovine catalase (100 μg ml^{-1} in PBS). Serial 1 : 10 dilutions of the catalase-treated suspension were prepared in tryptic soy broth (TSB; Difco) to check viability and spread onto TSA for quantitative measurement of the H_2O_2-resistant spores.

For desiccation resistance, the spore suspension (20 μl) was dispensed onto pre-sterilized metals and glass-fibre discs (10^3 spores per disc; Millipore). After removing most of the water content by drying at room temperature (~40–50 % humidity in Pasadena, CA, USA) for 1 or 2 days, the colonies were counted on TSA medium. Briefly, the desiccated sample was placed in sterile PBS, mixed thoroughly and sonicated for 2 min before plating onto TSA medium. Plates were incubated at 32 °C for 2 days and the number of spores that survived was counted.

Identification

Phenotypic characterization and fatty acid analysis. Routine biochemical tests were carried out according to established procedures (Claus & Berkeley, 1986; Priest, 1993). The ability to grow at a NaCl concentration of 1–10 % was determined in T_1N_1 liquid medium (1 % Bacto tryptone and appropriate amount of NaCl) and the ability to grow without NaCl was determined in 1 % sterile tryptone water. The API CHB 50 kit and API 20E (bioMérieux) were used (75 biochemical tests). Identification of the test isolate was carried out by computing and comparing biochemical test results from the bioMérieux database. In addition, the commercially available Biolog identification system was also used, according to manufacturer's specifications. Fatty acid methyl ester (FAME) profiles were examined from overnight cultures grown at 32 °C in TSB, as described previously (Ringelberg *et al.*, 1994).

16S rDNA sequencing. Purified genomic DNA (Johnson, 1981) from liquid cultures was quantified and ~10 ng of DNA was used as the template for PCR amplification. Universal primers (Bact 11 and 1,492) were used to amplify the 1·5 kb PCR fragment by protocols established by Ruimy *et al.* (1994). Amplicons were sequenced directly following purification on Qiagen columns. The identity of a given PCR product was verified by sequencing using the dideoxy chain termination method with the Sequenase DNA sequencing kit (United States Biochemical) and an ABI 373A automated sequencer (Perkin-Elmer). The phylogenetic relationships of organisms covered

in this study were determined by comparison of individual 16S rDNA sequences to other existing sequences in GenBank. Evolutionary trees were constructed using PAUP (Swofford, 1990).

DNA–DNA hybridization. Cells were suspended in 0·1 M EDTA (pH 8·0) and digestion of the cell wall was carried out by treating the cells with lysozyme (final concentration, 2 mg ml^{-1}). DNA was isolated by standard procedures (Johnson, 1981). DNA–DNA homology was studied by microplate hybridization methods (Ezaki *et al.*, 1989) with photobiotin labelling and colorimetric detection, using 1,2-phenylenediamine (Sigma) as the substrate and streptavidine-peroxidase conjugate (Boehringer Mannheim) as the colorimetric enzyme (Satomi *et al.*, 1997).

RESULTS AND DISCUSSION

Microbial and particle contamination of JPL-SAF

Particles of the size 11–150 μm were collected on witness plates (Anonymous, 1989). The stainless steel witness plates accumulated mid-range size (26–100 μm) particles and the abundance of particles decreased when the particle size decreased (data not shown). Microbial contamination transferred through particulate materials was not high, in terms of microbial load, in this well-controlled facility. The particles trapped on stainless steel witness plates harboured an equivalent number of both vegetative (5 c.f.u. cm^{-2}) and spore-forming (6 ± 1 c.f.u. cm^{-2}) microbes. When the isolated colonies were exposed to harsh conditions, such as UV, γ-radiation, H_2O_2 and desiccation, some spore-formers showed resistance. Among these spore-formers, a strain, designated as FO-92^T, exhibited distinct spore morphology and was further characterized for its phylogenetic affiliation.

Morphological and physiological characteristics

Strain FO-92^T is a Gram-positive, facultatively anaerobic, rod-shaped, spore-forming bacterium. Cells are 4–5 μm in length, 1 μm in diameter and are motile. On TSA medium incubated at 32 °C, young colonies are beige, irregular, with a diameter of 3–4 mm, rough, umbonate with undulate or lobate edges. Endospores of strain FO-92^T are oval ($1 \times 0{\cdot}5$ μm; Fig. 1a), with one spore per cell. Spores purified using the MN agar procedure contain a distinctive extraneous layer (Fig. 1b). Cross-sections of the MN agar-purified spores clearly show a loosely arranged layer outside the spore coat (Fig. 1c, d). This structure resembles the exosporium of the *Bacillus cereus* group (data not shown). This extraneous layer can be removed from the FO-92^T spores by washing with detergents and salts using the Nicholson & Setlow (1990) protocol. Spores of *Bacillus*

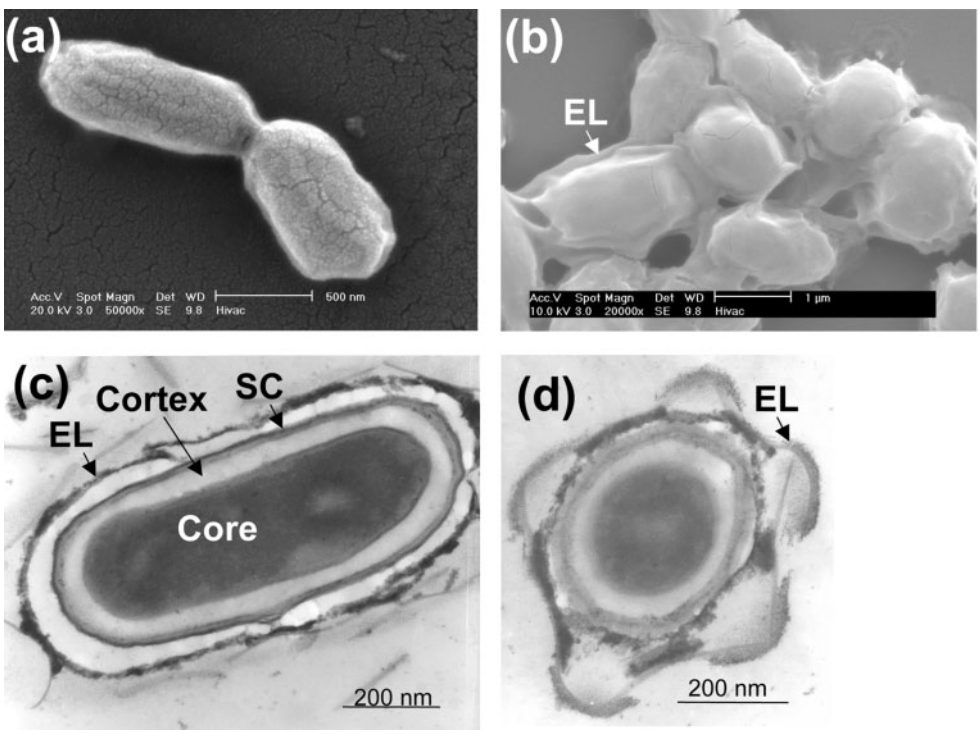

Fig. 1. Environmental scanning electron micrograph (a, b) and transmission electron micrograph (c, d) of *B. nealsonii* FO-92^T spores. Spores (b–d) were purified on MN agar (see Methods). (a) Purified spores as per the protocol of Nicholson & Setlow (1990); (b) MN agar-purified spores retaining the extraneous layer (EL); (c) longitudinal section of a spore where the extraneous layer (EL), spore coat (SC), cortex and spore core are shown; (d) cross-section of a spore where the loosely attached extraneous layer is observed. The extraneous layer was removed by salt and detergent washes (a). Cross-sections of spores purified according to the method of Nicholson & Setlow (1990) did not possess the extraneous layer (data not shown).

subtilis ATCC 6633^T, *Bacillus pumilus* ATCC 7061^T and *Bacillus megaterium* IAM 13418^T did not show an extraneous layer when purified from MN agar. The extra layer (exosporium) was retained in *Bacillus cereus* JCM 1252^T and *B. sphaericus* 34hs1 even after the chemical treatments (Nicholson & Setlow, 1990) used to purify the spores (data not shown). The characterization and the physiological role of this extraneous layer of strain FO-92^T spores is not discussed in this paper. However, the resistance of the spores with and without extraneous layers against various treatments was measured.

Resistance of FO-92^T spores to various physical and chemical conditions

The resistance of *Bacillus* spores to a variety of conditions is common as seen in our control experiments (data not shown) and in other studies (for a review, see Nicholson *et al.*, 2000). The spores of FO-92^T exhibited resistance to 0·5 Mrad (5 kGy) γ-radiation (Co^{60}), 200 J m^{-2} UV (254 nm), 5 % liquid H_2O_2 and desiccation conditions. However, 1 Mrad γ-radiation was lethal and no spore germination was observed. Spores with the extraneous layer showed a 4-log reduction whereas spores without the extraneous layer showed a 5-log reduction at 0·5 Mrad γ-radiation. Although preliminary experiments suggest a protective role of the extraneous layer against γ-radiation, more detailed studies are warranted. The FO-92^T spores exhibited classic UV inactivation kinetics, with a characteristic 'shoulder' extending to ~100 J m^{-2}, followed by strict exponential inactivation thereafter. FO-92^T spores exhibited an LD_{90} value (the 90 % lethal dose) of ~200 J m^{-2} (Fig. 2), in good agreement with UV resistance values obtained for spores of the model organism, *B. subtilis* strain 168 (Nicholson *et al.*, 2000). The vegetative cells of strain FO-92^T were resistant to 5 % liquid H_2O_2 (data not shown). Purified spores that were exposed to 5 % liquid H_2O_2 for 30–60 min showed resistance, but prolonged incubation to 90 min eliminated the viability (data not shown).

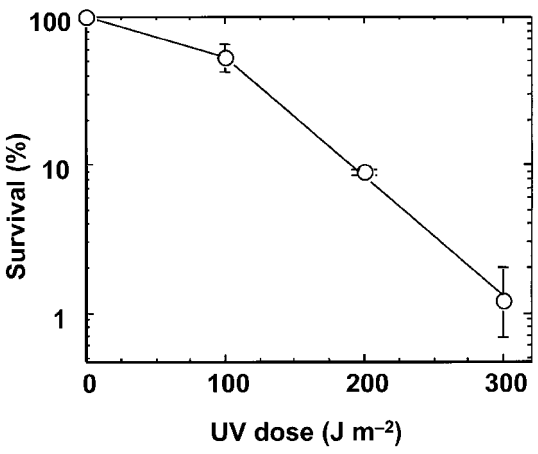

Fig. 2. Resistance of FO-92^T spores to 254 nm UV radiation. Results shown are the means and standard deviations of three experiments. Spores purified by the Nicholson & Setlow (1990) protocol were used in this experiment.

Optimum growth conditions

Strain FO-92^T grew at 25–60 °C, with optimum growth at 30–35 °C and over the pH range of 6–10 (optimum 6–7). This strain did not require Na^+ for growth and was as desiccation resistant as other spore-formers. However, it is interesting to note that the centre of an overnight colony on TSA (at 32 °C) predominantly consisted of spores when compared to the periphery of the colony (data not shown). Such an immediate response in triggering sporulation during nutrient-depleted conditions is common in *Bacillus* species. But, when compared to *B. subtilis* ATCC 6633^T, where spores were formed in 3–4 days on TSA (data not shown), strain FO-92^T produced spores in 1 day.

Phenotypic characterization

The biochemical characterization of strain FO-92^T is presented in Table 1. In addition to the characters shown, strain FO-92^T produced catalase but hydrogen sulfide was not produced from thiosulfite. The carbon substrate profile of FO-92^T, as measured by the BioLog system, showed an identification match for *Bacillus*. Phenotypically, as measured by the API system, this strain resembles *B. circulans* ATCC 4513^T.

Bacillus species that produce acid from a variety of sugars, including glucose, are classified under rRNA group 1 (Priest, 1993). Most of these species were able to grow at least weakly in the absence of oxygen. Spores of these species were ellipsoidal and did not swell the mother cell. These species are considered the 'subtilis group' because of their similar physiological properties (Priest, 1993). Strain FO-92^T, isolated from JPL-SAF, exhibited the characteristics necessary to place it into the rRNA group 1.

Cellular fatty acid composition

Strain FO-92^T contained straight-chain and terminally branched saturated and mono-unsaturated fatty acids with a composition of 18, 73 and 9 %, respectively (Table 2). Among the fatty acids measured, tetradecanoic acid (14 : 0), 13-methyl pentadecanoic acid (15 : 0 iso) and 12-methyl tetradecanoic acid (15 : 0 anteiso) were the major fatty acids in FO-92^T. This FAME profile identified strain FO-92^T as *Bacillus circulans* DSM 11^T. FAME analysis of other *Bacillus* species showed distinct profiles. For example, *Bacillus licheniformis* ATCC 14580^T contained ~90 % terminally branched saturated fatty acids, whereas *Bacillus mycoides* ATCC 6462^T showed more monosaturated fatty acids. Although both *B. subtilis* IAM 1026^T and strain FO-92^T exhibited high levels of straight-chain saturated fatty acids, *B. subtilis* IAM 1026^T contained high levels of pentadecanoic

Table 1. Biochemical characteristics of *B. nealsonii* FO-92^T and related species

Strain: 1, *B. licheniformis* ATCC 14580^T; 2, *B. subtilis* IAM 1026^T; 3, *B. pumilus* ATCC 7061^T; 4, *B. mycoides* ATCC 6462^T; 5, *B. circulans* ATCC 4513^T; 6, *B. firmus* ATCC 14575^T; 7, *B. nealsonii* FO-92^T. All strains are Gram-positive rods, facultatively anaerobic and do not denitrify. None produces lysine or ornithine decarboxylases, urease, tryptophan deaminase, hydrogen sulfide or indole. None utilizes glucose, inositol, sucrose, citrate, sorbitol, rhamnose, melibiose or arabinose as sole carbon source. All ferment L-arabinose, D-glucose, D-fructose, D-mannose, mannitol, methyl α-D-glucoside, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, sucrose and trehalose. None ferments methyl α-D-mannoside, inulin, xylitol, L-fucose, erythritol, L-xylose, methyl β-xyloside, L-sorbose, dulcitol, D-fucose, L-arabitol or 5-ketogluconate.

Test	1	2	3	4	5	6	7
Enzyme production:							
β-Galactosidase	+	−	−	+	+	+	+
Arginine dihydrolase	+	−	−	−	−	−	−
Cytochrome oxidase	+	+	+	−	−	−	−
Acetoin production	−	−	+	−	−	−	−
Gelatin liquefication	−	+	−	−	−	−	−
Utilization of:							
Mannitol	−	−	−	−	+	+	−
Amygdalin	−	−	+	−	+	−	−
Fermentation of:							
Glycerol	+	+	+	−	+	+	+
Ribose	+	+	+	−	−	−	−
D-Xylose	−	−	+	+	+	−	+
Adonitol	−	+	−	−	−	−	−
Galactose	−	−	+	−	+	−	+
Rhamnose	−	−	−	−	−	−	+
Inositol	+	+	+	−	+	−	+
Sorbitol	+	+	−	−	+	−	+
N-Acetylglucosamine	+	−	+	+	+	+	+
Lactose	−	−	+	−	−	−	+
Melibiose	−	+	+	−	+	−	+
Melezitose	−	−	−	−	+	−	+
Raffinose	−	+	−	−	−	−	+
Starch	−	+	−	−	+	−	+
Glycogen	−	+	−	−	+	−	−
Gentiobiose	−	+	+	−	+	−	+
D-Turanose	−	+	+	−	+	+	+
D-Lyxose	−	−	−	−	+	−	+
D-Tagatose	+	−	+	+	−	−	+
D-Arabitol	−	−	−	−	−	−	+
Gluconate	+	−	−	−	+	−	+
2-Ketogluconate	−	−	−	−	−	−	+

acid (15 : 0). Unfortunately, different culture conditions can result in high variability within FAME profiles (Venkateswaran *et al.*, 1999). FAME analysis is ambiguous because type strains of some of the *Bacillus* species could not be identified correctly (Table 2). Because of these uncertainties, the identification of strain FO-92^T could not be conclusively determined by fatty acid profiles.

16S rDNA sequence analysis

Molecular methods are less susceptible to artifactual misinterpretation than culture-based approaches. Studies have revealed that organisms with less than 97 % similarity over the 16S rRNA gene do not yield DNA reassociation values of more than 60 % (Stackebrandt & Goebel, 1994). While the gene sequence of the small subunit of the 16S rRNA molecule is acceptable for defining phylogenetic relationships between distinctly related organisms (Woese, 1987), this molecule at times lacks the specificity required for the differentiation of close relatives (Fox *et al.*, 1992; Venkateswaran *et al.*, 1998, 1999; Yamada *et al.*, 1999). Strain FO-92^T closely resembled *B. circulans* by conventional phenotypic characterization and FAME profiles. In order to confirm the species identity, molecular phylogeny was carried out on this strain.

The 16S rDNA sequences of all known *Firmicutes* were compared with that of FO-92^T. All phylogenetic analyses, based on 16S rDNA sequence, unambiguously demonstrated that FO-92^T belonged to the low G+C Gram-positive bacteria. The 16S rDNA sequences of all known members of the Gram-positive bacteria were compared with that of FO-92^T. Their phylogenetic relationships were then analysed and the study was repeated with several different subdomains of the 16S rDNA sequence. Bootstrapping (500 replicates) analysis was performed to avoid sampling artifacts. The resulting analyses indicated that FO-92^T shares a close phylogenic relationship with *Bacillus* species. Neighbour-joining, parsimony and maximum-likelihood analyses were undertaken on this subset of bacteria, using several subdomains of the 16S rDNA. In all analyses, FO-92^T was most closely associated with members of the genus *Bacillus*.

The similarities in the 16S rDNA nucleotide sequences between FO-92^T and the top 17 closely related *Bacillus* species, recognized by GenBank BLAST searches, were between 95 and 98·7 %. A sequence variation of ~1 % was found between FO-92^T and *B. circulans* ATCC 4513^T and 2 % between FO-92^T and *Bacillus benzoevorans* DSM 5391^T as well as *Bacillus firmus* IAM 12464. A very high sequence variation (5 %) was noticed between FO-92^T and both *B. subtilis* ATCC 6633^T and *B. pumilus* OM-F6. Such a high degree of dissimilarity within a well-described genus is not uncommon.

A phylogenetic tree based on 16S rDNA sequences is shown in Fig. 3. The branching order of this tree showed two distinct clusters in which one clade consisted of the *B. subtilis* group and another stock formed with 12 other species, including strain FO-92^T. These 12 other species exhibited five subclusters in which three major clades each contained at least three species. The first clade comprised FO-92^T, *B. circulans* ATCC 4513^T and *B. benzoevorans* DSM 5391^T. The second clade contained '*Bacillus macroides*' strain dhr2, *Bacillus fumarioli* LMG 17492 and *Bacillus niacini* IFO 15566^T, and the third clade included *Bacillus*

Table 2. Fatty acid methyl ester composition (%) of *B. nealsonii* FO-92^T and related species

Strain: 1, *B. circulans* DSM 11^T; 2, *B. firmus* DSM 12^T; 3, *B. megaterium* DSM 32^T; 4, *B. simplex* DSM 1321; 5, *B. pumilus* DSM 27^T; 6, *B. subtilis* DSM 10^T; 7, *B. subtilis* IAM 1026^T; 8, *B. licheniformis* ATCC 14580^T; 9, *B. mycoides* ATCC 6462^T; 10, *B. nealsonii* FO-092^T.

Fatty acid	1*	2*	3*	4*	5*	6*	7	8	9	10
Straight-chain saturated										
14:0	2·9	1·1	1·6	1·5	0·5	0·2	1·1		3·0	12·3
15:0	1·0	0·3	0·1	0·6		0·2				
16:0	2·7	1·5	2·6	4·1	1·4	2·0	10·2	5·3	6·2	5·2
18:0		0·1			0·1		1·0			
Terminally branched saturated										
12:0 iso									1·4	
13:0 iso	0·2				0·1	0·1			7·6	2·9
14:0 iso	4·0	2·4	6·0	3·2	0·7	0·9	2·3	1·0	5·0	6·6
15:0 iso	13·9	45·8	33·6	9·3	56·9	33·1	13·2	19·7	20·9	26·4
16:0 iso	4·4	2·9	0·9		1·4	2·0	4·6	4·8	10·2	2·1
17:0 iso	1·3	2·9	1·3	1·0	5·2	10·5	7·8	6·3	6·9	
13:0 anteiso	0·8		0·7		0·5	0·6			1·9	
15:0 anteiso	58·4	19·8	43·4	68·7	23·4	35·7	40·0	41·2	4·5	32·2
17:0 anteiso	4·8	3·7	3·0	3·2	3·5	7·7	12·2	16·1	2·2	3·0
Mono-unsaturated										
16:1ω7*c* alcohol	0·2	4·5	0·9	1·8	0·4	0·1			4·6	1·8
16:1ω11*c*	2·6	3·2	2·9	2·3	1·0	1·2	4·8	2·1	4·0	7·5
iso 17:1ω10*c*	0·1	0·1					1·6	1·4	11·8	
Sum of 15:0 iso 2-OH/16:1ω7*c*	0·8	1·2	1·0	1·8	0·6	0·5			7·1	
Sum of 17:1 anteiso B/iso I	0·3	7·2	0·9	1·0	4·3	4·7	1·2	2·0	2·7	

*Data from Kämpfer (1994).

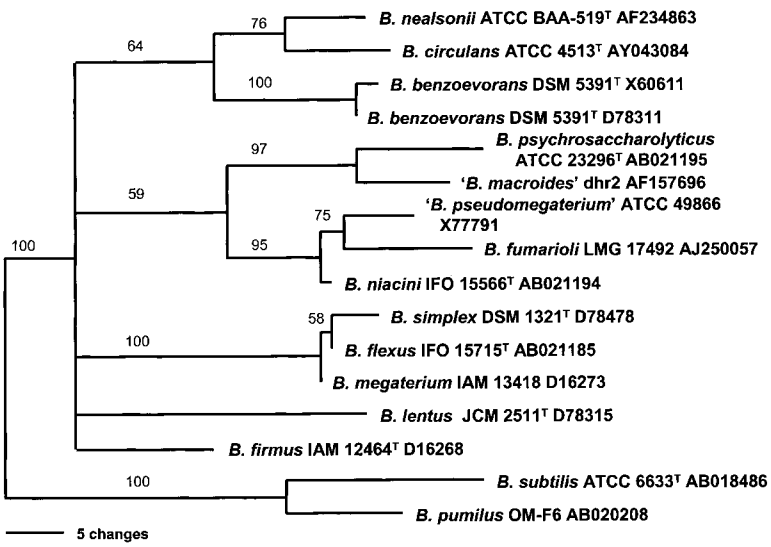

Fig. 3. Phylogenetic tree of various species of *Bacillus* based on 16S rDNA nucleotide sequences. The numbers after the name of the bacteria are the GenBank nucleotide accession numbers and the numbers above the lines are the percentage bootstrap values of that branch of the tree.

simplex DSM 1321^T, *Bacillus flexus* IFO 15715^T and *Bacillus megaterium* IAM 13418. Because of the inadequacy of 16S rDNA analysis for species differentiation, DNA–DNA hybridization was performed.

DNA–DNA hybridization

DNA–DNA hybridization was performed between FO-92^T and 18 strains, comprising 12 *Bacillus* species. None of the *Bacillus* species that showed very high similarities with the 16S rDNA sequences (~97 %) exhibited >70 % DNA–DNA reassociation values that would place the strain within the same species. Particularly, the similarity between FO-92^T and *B. circulans* ATCC 4513^T was only 16 %. This pair showed 98·7 % similarity in their 16S rDNA sequences. Similarly, FO-92^T and *B. benzoevorans* ATCC 49005^T showed only 15 % DNA–DNA hybridization values whereas

this pair exhibited ~98 % similarity in their 16S rDNA sequence. Based on the DNA–DNA reassociation values, FO-92^T is a novel *Bacillus* species.

Description of *Bacillus nealsonii* sp. nov.

Bacillus nealsonii (neal′son.i.i. N.L. gen. n. *nealsonii* referring to Kenneth H. Nealson, a well-known American microbiologist).

Cells are rod-shaped, 4–5 μm in length, 1 μm in diameter and motile. Gram-positive, facultatively anaerobic and forms endospores. Spores show an additional extraneous layer similar to an exosporium. Colonies on TSA are irregular, rough, umbonate with undulate or lobate edges and beige in colour. Sodium ions are not essential and it grows at 0–8 % NaCl. Grows at pH 6–10, optimum pH 7. Grows at 25–60 °C, optimum 30–35 °C. Catalase and β-galactosidase are produced, but gelatinase, arginine dihydrolase, lysine and ornithine decarboxylases, lipase, amylase and alginase are not. It neither produces H_2S from thiosulfite nor denitrifies. Based on 16S rDNA nucleotide sequences, this bacterium belongs to the class *Firmicutes* and is a member of the genus *Bacillus*. The type strain, FO-92^T (=ATCC BAA-519^T =DSM 15077^T), was isolated from dust particles collected at the Jet Propulsion Laboratory Spacecraft Assembly Facility.

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REFERENCES

Anonymous (1980). NASA Standard Procedures for the Microbiological Examination of Space Hardware. Jet Propulsion Laboratory communication NHB 5340.1B, 1980. Pasadena, CA: National Aeronautics and Space Administration.

Anonymous (1989). Contamination control procedure for the tape lift sampling of surfaces. Jet Propulsion Laboratory communication GSFC-TLS-PR-7324-01. Pasadena, CA: National Aeronautics and Space Administration.

Claus, D. & Berkeley, R. C. W. (1986). Genus *Bacillus* Cohn, 1872. In *Bergey's Manual of Sytematic Bacteriology*, vol. 2, pp. 1105–1139. Edited by P. H. A. Sneath, N. S. Mair, M. E. Sharpe & J. G. Holt. Baltimore: Williams & Wilkins.

Cole, R. M. & Popkin, T. J. (1981). Electron microscopy. In *Manual of Methods for General Bacteriology*, pp. 34–51. Edited by P. Gerhardt, R. G. E. Murray, R. N. Costilaw, E. W. Nester, W. A. Wood, N. R. Krieg & G. B. Phillips. Washington, DC: American Society for Microbiology.

Coss, J. R. (1999). *Test Procedure for Total Ionizing Dose Radiation Testing of Piece-Parts.* # D-15827. Pasadena, CA: National Aeronautics and Space Administration.

Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in micro-dilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.

Fox, G. E., Wisotzkey, J. D. & Jurtshuk, P., Jr (1992). How close is close. 16S rRNA sequence identity may not be sufficient to guarantee species identity. *Int J Syst Bacteriol* **42**, 166–170.

Johnson, J. L. (1981). Genetic characterization. In *Manual of Methods for General Bacteriology*. pp. 450–472. Edited by P. Gerhardt, R. G. E. Murray, R. N. Costilaw, E. W. Nester, W. A. Wood, N. R. Krieg & G. B. Phillips. Washington, DC: American Society for Microbiology.

Kämpfer, P. (1994). Limits and possibilities of total fatty acid analysis for classification and identification of *Bacillus* species. *Syst Appl Microbiol* **17**, 86–98

Nicholson, W. L. & Setlow, P. (1990). Sporulation, germination and outgrowth. In *Molecular Biological Methods for Bacillus*, pp. 391–450. Edited by C. R. Harwood & S. M. Cutting. Chichester: Wiley.

Nicholson, W. L., Munakata, N., Horneck, G., Melosh, H. J. & Setlow, P. (2000). Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. *Microbiol Mol Biol Rev* **64**, 548–572.

Priest, F. G. (1993). Systematics and ecology of *Bacillus*. In *Bacillus subtilis and other Gram-positive Bacteria*, pp. 3–33. Edited by A. L. Sonenshein, J. A. Hoch & R. Losick. Washington, DC: American Society for Microbiology.

Puleo, J. R., Oxborrow, G. S., Fields, N. D., Herring, C. M. & Smith, L. S. (1973). Microbiological profiles of four Apollo spacecraft. *Appl Microbiol* **26**, 838–845.

Puleo, J. R., Favero, M. S., Oxborrow, G. S. & Herring, C. M. (1975). Method for collecting naturally occurring airborne bacterial spores for determining their thermal resistance. *Appl Microbiol* **30**, 786–790.

Puleo, J. R., Fields, N. D., Bergstrom, S. L., Oxborrow, G. S., Stabekis, P. D. & Koukol, R. (1977). Microbiological profiles of the Viking spacecraft. *Appl Environ Microbiol* **33**, 379–384.

Riesenman, P. J. & Nicholson, W. L. (2000). Role of the spore coat layers in *Bacillus subtilis* spore resistance to hydrogen peroxide, artificial UV-C, UV-B and solar UV radiation. *Appl Environ Microbiol* **66**, 620–626.

Ringelberg, D. B., Townsend, G. T., DeWeerd, K. A., Suflita, J. M. & White, D. C. (1994). Detection of the anaerobic dechlorinating microorganism *Desulfomonile tiedjei* in environmental matrices by its signature lipopolysaccharide branched-long-chain hydroxy fatty acids. *FEMS Microbiol Ecol* **14**, 9–18.

Ruimy, R., Breittmayer, V., ElBaze, P., Lafay, B., Boussemart, O., Gauthier, M. & Christen, R. (1994). Phylogenic analysis and assessment of the genera *Vibrio*, *Photobacterium*, *Aeromonas*, and *Plesiomonas* deduced from small-subunit rRNA sequences. *Int J Syst Bacteriol* **44**, 416–426.

Satomi, M., Kimura, B., Mizoi, M., Sato, T. & Fujii, T. (1997). *Tetragenococcus muriaticus* sp. nov., a new moderately halophilic lactic acid bacterium isolated from fermented squid liver sauce. *Int J Syst Bacteriol* **47**, 832–836.

Schaeffer, P., Millet, J. & Aubert, J.-P. (1965). Catabolic repression of bacterial sporulation. *Proc Natl Acad Sci U S A* **54**, 704–711.

Stackebrandt, E. & Goebel, B. M. (1994). A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.

Swofford, D. (1990). PAUP: phylogenetic analysis using parsimony, version 3.0. Computer program distributed by the Illinois Natural History Survey, Champaign, IL, USA.

Venkateswaran, K., Dohmoto, N. & Harayama, S. (1998). Cloning and nucleotide sequence of the *gyrB* gene of *Vibrio parahaemolyticus* and its application in detection of the pathogen in shrimp. *Appl Environ Microbiol* **64**, 681–687.

Venkateswaran, K., Moser, D. P., Dollhopf, M. E. & 10 other authors (1999). Polyphasic taxonomy of the genus *Shewanella* and description of *Shewanella oneidensis* sp. nov. *Int J Syst Bacteriol* **49**, 705–724.

Venkateswaran, K., Satomi, M., Chung, S., Kern, R., Koukol, R., Basic, C. & White, D. (2001). Molecular microbial diversity of a spacecraft assembly facility. *Syst Appl Microbiol* **24**, 311–320.

Woese, C. R. (1987). Bacterial evolution. *Microbiol Rev* **51**, 221–271.

Yamada, S., Ohashi, E., Agata, N. & Venkateswaran, K. (1999). Cloning and nucleotide sequence analysis of *gyrB* of *Bacillus cereus, B. thuringiensis, B. mycoides* and *B. anthracis* and their application to the detection of *B. cereus* in rice. *Appl Environ Microbiol* **65**, 1483–1490.