Bacillus nealsonii sp. nov., isolated from a spacecraft-assembly facility, whose spores are \(\gamma\)-radiation resistant

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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 cleanrooms. 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 \(\sim\)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, \(\gamma\)-radiation, \(\mathrm{H}_2\mathrm{O}_2\) and desiccation.

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 phenol-ethyl-bufluoride (pE2) rinse solution (Anonymous, 1980). The plate and rinse solution were sonicated for 2 min (25 kHz, 0°C) to disrupt any microbial film. 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 and transferred to a nutrient broth sporulation 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.

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 Ca60 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 (10⁶ 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 H₂O₂ protocol, developed by Riesenman & Nicholson (2000), was modified and used to examine H₂O₂ resistance in spores. Suitable aliquots of spore suspensions prepared in PBS were treated with H₂O₂ (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 H₂O₂-resistant spores.

For desiccation resistance, the spore suspension (20 μl) was dispensed onto pre-sterilized metals and glass-fibre discs (10⁵ 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 plateing 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 (Claud & Berkeley, 1986; Priest, 1993). The ability to grow at a NaCl concentration of 1–10% was determined in T₅₀, 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 dyeoxygen 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

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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 \(\mu\)m were collected on witness plates (Anonymous, 1989). The stainless steel witness plates accumulated mid-range size (26–100 \(\mu\)m) 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 \pm 1 c.f.u. cm\(^{-2}\)) microbes. When the isolated colonies were exposed to harsh conditions, such as UV, \(\gamma\)-radiation, \(\text{H}_2\text{O}_2\) 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 \(\mu\)m in length, 1 \(\mu\)m in diameter and are motile. On TSA medium incubated at 32 \(^\circ\)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 0.5 \(\mu\)m; 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*...
subtilis ATCC 6633T, Bacillus pumilus ATCC 7061T and Bacillus megaterium IAM 13418T did not show an extraneous layer when purified from MN agar. The extra layer (exosporium) was retained in Bacillus cereus JCM 1252T 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-92T 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-92T 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-92T exhibited resistance to 0.5 Mrad (5 kGy) γ-radiation (Co60), 200 J m⁻² UV (254 nm), 5% liquid H₂O₂ 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-92T spores exhibited classic UV inactivation kinetics, with a characteristic ‘shoulder’ extending to ~100 J m⁻², followed by strict exponential inactivation thereafter. FO-92T spores exhibited an LD₉₀ 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-92T were resistant to 5% liquid H₂O₂ (data not shown). Purified spores that were exposed to 5% liquid H₂O₂ for 30–60 min showed resistance, but prolonged incubation to 90 min eliminated the viability (data not shown).

**Optimum growth conditions**

Strain FO-92T 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 6633T, where spores were formed in 3–4 days on TSA (data not shown), strain FO-92T produced spores in 1 day.

**Phenotypic characterization**

The biochemical characterization of strain FO-92T is presented in Table 1. In addition to the characters shown, strain FO-92T produced catalase but hydrogen sulfide was not produced from thiosulfite. The carbon substrate profile of FO-92T, 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 4513T.

Bacillus species that produce acid from a variety of sugars, including glucose, are classified under rRNA group 1 (Prist, 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 (Prist, 1993). Strain FO-92T, isolated from JPL-SAF, exhibited the characteristics necessary to place it into the rRNA group 1.

**Cellular fatty acid composition**

Strain FO-92T 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-92T. This FAME profile identified strain FO-92T as Bacillus circulans DSM 11T. FAME analysis of other Bacillus species showed distinct profiles. For example, Bacillus licheniformis ATCC 14580T contained ~90% terminally branched saturated fatty acids, whereas Bacillus mycoides ATCC 6462T showed more monosaturated fatty acids. Although both B. subtilis IAM 1026T and strain FO-92T exhibited high levels of straight-chain saturated fatty acids, B. subtilis IAM 1026T contained high levels of pentadecanoic

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**Fig. 2.** Resistance of FO-92T 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.
uncertainties, the identification of strain FO-92T could not be identified correctly (Table 2). Because of these uncertainties, the identification of strain FO-92T and related species could not uncommon. 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-92T 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 rDNA 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-92T 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-92T. All phylogenetic analyses, based on 16S rDNA sequence, unambiguously demonstrated that FO-92T 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-92T. 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-92T shares a close phylogenetic 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-92T was most closely associated with members of the genus *Bacillus*.

The similarities in the 16S rDNA nucleotide sequences between FO-92T 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-92T and *B. circulans* ATCC 4513T and 2% between FO-92T and *Bacillus benzocevorans* DSM 5391T as well as *Bacillus firmus* IAM 12464. A very high sequence variation (5%) was noticed between FO-92T and both *B. subtilis* ATCC 6633T 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-92T. These 12 other species exhibited five subclusters in which three major clades each contained at least three species. The first clade comprised FO-92T, *B. circulans* ATCC 4513T and *B. benzocevorans* DSM 5391T. The second clade contained ‘*Bacillus macroides*’ strain dhr2, *Bacillus fumarioli* LMG 17492 and *Bacillus niacini* IFO 15566T, and the third clade included *Bacillus*
Table 2. Fatty acid methyl ester composition (%) of *B. nealsonii* FO-92\(^T\) and related species

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<th>3(^*)</th>
<th>4(^*)</th>
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\(^*\)Data from Kämpfer (1994).

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**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.

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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. benzoaevorans* 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-92T 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. Nealon, 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 alginate are not. It neither produces H2S 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-92T (≡ATCC BAA-519T=DSM 15077T), was isolated from dust particles collected at the Jet Propulsion Laboratory Spacecraft Assembly Facility.

**ACKNOWLEDGEMENTS**

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