A bacterial spore-forming species that is extremely resistant to various sterilization methods

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A report presents a phenotypic and genotypic characterization of a bacterial species that has been found to be of the genus *Bacillus* and has been tentatively named *B. odysseensis* because it was isolated from surfaces of the Mars Odyssey spacecraft as part of continuing research on techniques for sterilizing spacecraft to prevent contamination of remote planets by terrestrial species. *B. odysseensis* is a Gram-positive, facultatively anaerobic, rod-shaped bacterium that forms round spores. The exosporium has been conjectured to play a role in the elevated resistance to sterilization. Research on the exosporium is proposed as a path toward improved means of sterilization, medical treatment, and prevention of biofouling.

*This work was done by Myron La Duc and Kasthuri Venkateswaran of Caltech for NASA's Jet Propulsion Laboratory.*

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A round spore-forming Bacillus species that possesses an exosporium was isolated from surfaces of the Mars Odyssey spacecraft. This novel species was characterized on the basis of phenotypic characterization, 16S rDNA sequence analysis, and DNA-DNA hybridization. Our assertion that this strain represents a distinct bacterial species within the genus Bacillus is supported by DNA-DNA hybridization studies and we are proposing the name B. odysseensis, in honor of the spacecraft from which the organism was isolated. B. odysseensis is a Gram-positive, rod-shaped eubacterium that produces endospores. Of 45 strains tested, B. odysseensis spores were the most consistently resistant and survived all of the challenges posed, those being exposure to conditions of desiccation (100% survival), H2O2 (26% survival), UV254 (10% survival at 660 J/m2), and g-radiation (0.4% survival). Ultra thin sections of B. odysseensis spores showed the presence of a very tightly associated exosporium, spore coat, cortex, and core.

The presence of an exosporium in spores of B. odysseensis is presumed to play a role in its elevated resistance properties.
compared to the model organism, B. subtilis strain 168, spores of strain B. odysseensis appeared to be quite resistant, with 3 times, 4 times, 6 times, and 10 times greater survival to UV (Nicholson et al., 2000), g-radiation (Venkateswaran et al., 2003), H2O2 (Kempf et al., 2002), and desiccation (Venkateswaran et al., 2003), respectively.

B. Solution

A key element of spore resistance is the coat, a multilayered protein shell that encases the spore. The coat of the best-studied spore-forming microbe, B. subtilis, is comprised of at least 45 proteins, most of which are poorly characterized. Several protective roles for the coat are well characterized, including resistance to large toxic molecules, ortho-phthalaldehyde, and UV radiation. In addition, this bacterium might be able to enzymatically decontaminate toxic molecules. Spores formed by many species, several of which we have isolated/identified, including B. anthracis, B. cereus, B. nealsonii, B. sphaericus, B. thuringiensis and B. odysseensis, are encased by an additional poorly studied structure known as the exosporium. A role for the exosporium has yet to be described, but it is plausible that it participates in resistance and/or attachment/adhesion. We found that one novel spore-former, B. nealsonii, isolated from JPL-SAF, exhibited resistance to 0.5 Mrad (5K Gy) g-radiation (cobalt60), 200 J/m2 UV (254 nm), 5% liquid H2O2, and severe desiccation. We also generated preliminary evidence that the exosporium of this species contributes resistance to these stresses. We anticipate that the exosporium and spore coat of this highly resistant bacilli that colonized the Mars Odyssey spacecraft might be responsible for the elevated resistance properties observed.

C. Detailed Description and Explanation

The cells of B. odysseensis are rod-shaped, 4 – 5 mm in length, 1 mm in diameter, and motile. They are Gram-positive, facultatively anaerobic, and endospore forming. Spores show an additional exosporium layer. Colonies on trypticase soy agar are regular, smooth, umbonate with undulate or lobate edges, and beige in color. Sodium ions are not essential and exhibited growth at 0 to 6% NaCl. Cells grow at a pH range of 6 to 9 with an optimum at 7. Cells are able to grow at 25 to 42 °C with optimum growth at 30 to 35 °C. They neither produce H2S from thiosulfite nor take part in denitrification. Based on 16S rDNA nucleotide sequences, this bacterium belongs to the class Firmicutes and is a member of the genus Bacillus. This bacterial strain, B. odysseensis, was isolated from the surface of the Mars Odyssey Spacecraft.
Morphology of *Bacillus odysseensis* spores

Electron micrographs of *Bacillus odysseensis* spores before and after γ-radiation and H₂O₂ exposure. ESEM (A, B, C) and TEM (D, E, F) micrographs showing spore surface and cross-section characteristics, respectively. (A, D) Purified spores showing intact, round structure. (B, E) Spores (1.1 x 10⁷) were exposed to γ-radiation (0.5 Mrad) and (C, F) 5% liquid H₂O₂ for 60 min. The intact spore with exosporium is designated as IS. The exosporium (EX), spore coat (SC), cortex, and spore core are shown in the longitudinal section of a spore (D, E, F). The exosporium removed from spore due to γ-radiation is shown in B. The spore removed exosporium due to H₂O₂ treatment is shown in C. Bar indicates 1 µm.
A bacterial spore-forming species that is extremely resistant to various sterilization methods

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A round spore-forming *Bacillus* species that produces an exosporium was isolated from surfaces of the Mars Odyssey spacecraft. This novel species has been characterized on the basis of phenotypic characterization, 16S rDNA sequence analysis, and DNA-DNA hybridization. Strain 34hs-1, according to 16S rDNA sequence analysis, belongs to the genus *Bacillus* and is a Gram-positive, facultatively anaerobic, rod-shaped, endospore-forming eubacterium. The 16S rDNA sequence similarity between strain 34hs-1 and *B. fusiformis* as well as *B. silvestris* was 96%. Our assertion that strain 34hs-1, whose name we propose to be *B. odysseensis* 34hs-1\(^{\text{T}}\), represents a distinct bacterial species within the genus *Bacillus* is supported by DNA-DNA hybridization studies. The spores of this novel bacterial species exhibited resistance to desiccation, H\(_2\)O\(_2\), UV, and \(\gamma\)-radiation. Of 45 strains tested, *B. odysseensis* spores were the most consistently resistant and survived all of the challenges posed, those being exposure to conditions of desiccation (100% survival), H\(_2\)O\(_2\) (26% survival), UV (10% survival at 660 J/m\(^2\)), and \(\gamma\)-radiation (0.4% survival). Ultra thin sections of *B. odysseensis* spores showed the presence of an exosporium, spore coat, cortex, and core. Microscopic analyses revealed the destruction of *B. odysseensis* spores by \(\gamma\)-radiation while the remnants of exosporia were left behind, whereas spores oxidized by H\(_2\)O\(_2\) formed “doughnut-like” structures. Further analysis showed highly-electron dense structures in the exosporia when compared to the H\(_2\)O\(_2\)-untreated control. The presence of exosporia in spores of *B. odysseensis* is presumed to play a role in its elevated resistance properties; however, other exosporium-bearing *Bacillus* species isolated in this study such as *B. nealsonii*, *B. cereus* and *B. thuringiensis*, along with their representative type strains, were susceptible to \(\gamma\)-radiation and H\(_2\)O\(_2\).
INTRODUCTION

Several physiologically and phylogenetically novel microorganisms were encountered while determining and documenting possible microbial contamination of spacecraft surfaces (Venkateswaran et al., 2001, 2003). Some of these novel species possessed round, exosporium-bearing spores, whose exosporia might be responsible for adhesion and adaptation to the extreme clean conditions of spacecraft surfaces.

Round-spore-forming Bacillus species were first described by Chester in 1898. The presently recognized round-spore species of Bacillus are, B. fusiformis, B. globisporus, B. psychrophilus, B. insolitus, B. marinus, B. neidei, B. pasteurii, B. pycnus, B. sphaericus, ‘B. aminovorans’, B. silvestris, and B. thermosphaericus (Nakamura, 2000; Rheims et al., 1999; Priest et al., 1988; Nakamura et al., 2002). All of these round-spore-forming species fall under the rRNA group 2 of the genus Bacillus (Ash et al., 1991). Though all of these species form round-spores, it is not clear whether the spore itself or the “exosporium” layer is responsible for the spheroid appearance. This spheroid manifestation was confirmed to be arising from an exosporium in spores of B. sphaericus (Neidei, 1904), B. fusiformis (Priest et al., 1988), B. silvestris (Rheims et al., 1999), and this new 34hs-1T strain. Although exosporia were present in B. anthracis, B. cereus, B. mycoides, B. pseudomycoides, B. thuringiensis, and B. weihenstephanensis, the spores of these species were not round (data not shown). A loosely attached extraneous layer resembling exosporia was recently described in the ovoid spore-forming B. nealsonii, isolated from the air-borne particulates within a spacecraft assembly facility (Venkateswaran et al., 2003).

Here, we describe B. odysseensis, isolated from the Mars Odyssey Spacecraft surface, whose round spores are resistant to conditions of UV, γ-radiation, H₂O₂, and desiccation. The Bacillus strain isolated in this study was characterized based on a polyphasic taxonomical approach that examined its phenotypic and genotypic affiliations.

METHODS

Sample preparation and isolation of microbes from the Mars Odyssey spacecraft. Samples were collected from 25 surface areas of the spacecraft. Samples collected from the spacecraft were taken from selected surface areas (25 cm²) at various locations using sterile water-moistened, sterile polyester swabs (Catalog #TX761; Texwipe, Upper Saddle River, NJ). Each swab sample was then placed individually into sterile water (final volume 10 mL). After return to the lab, these 25 samples were pooled into one sterile container and processed.

Microbial examination. Total microbial populations were examined using direct count epifluorescent microscopy following DAPI and Baclight staining (manufacturer, city state). All samples were analyzed for both spore-formers and total cultivable heterotrophs. Samples were sonicated for 2 min. and heat-shocked at 80°C for 15 min. Appropriate aliquots of samples were placed into petri dishes and total aerobic spore
counts were enumerated by the pour plate techniques using tryptic soy agar (TSA; Difco Laboratories, Detroit, MI) as the growth medium (32°C for 2 days)(1, 2). In addition, samples that were not heat-shocked were enumerated for total aerobic cultivable heterotrophs in TSA. Colony-forming units (CFUs) were counted after incubation at 32°C for up to 7 days. Isolates were selected, purified, and stored at −80°C for further processing and analysis. Identification of purified strains was determined based on 16S rDNA sequencing (see below). Type strains of various Bacillus species were procured from appropriate culture collections and used as controls.

Sporulation. 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 NSM liquid medium. After several (2 to 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 salts and detergent (Nicholson & Setlow, 1990). The chemical treatments used in this method did not remove the exosporia surrounding the spore coat. The purified spores were resuspended in sterile deionized water, heat-shocked (80°C for 15 minutes), 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; Olympus, Nepa, CA). The Field-Emission Environmental Scanning Electron Microscope (ESEM; Phillips XL30; Potomac, MD) 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 SEM and transmission electron microscopy (TEM) 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 cobalt 60 source was performed using an ion chamber with accuracy to the US Bureau of Standards (Coss, 1999). All irradiations were carried out in glass vials using spore samples in water. The spores (10^8 spores/ml) were exposed to both 1 Mrad (50 rad/sec for 330 min.) and 0.5 Mrad (25 rad/sec 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 phosphate-buffered saline (PBS; pH 7.2), placed into an uncovered petri dish, and exposed to UV radiation (254 nm; UV Products, model #UVG-11, San Gabriel, CA). At appropriate intervals, samples of spores were removed, diluted serially tenfold in PBS, and plated onto NSM agar medium. Plates were incubated at 37°C for up to 5 days and colonies were counted.
A liquid \( \text{H}_2\text{O}_2 \) protocol, developed by Riesenman and Nicholson (2000), was modified and used to examine \( \text{H}_2\text{O}_2 \) resistance in spores. Suitable aliquots of spore suspensions prepared in PBS were treated with \( \text{H}_2\text{O}_2 \) (5% final concentration) and incubated at room temperature (~25 °C) with gentle mixing. After 60 minutes incubation, a 100 µl sample was removed and diluted in a solution of bovine catalase (100 µg/ml 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 \( \text{H}_2\text{O}_2 \) resistant spores.

For desiccation resistance, the spore suspension (20 µL) was dispensed onto pre-sterilized metals and glass-fiber discs (10³ spores per disc) (Cat # AP 4001000; Millipore Corp., Bedford, MA). After removing most of the water content by drying at room temperature (~40 to 50% humidity in Pasadena, CA) 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.

(i) Phenotypic characterization. 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 to 10% was determined in T\( _{11} \) liquid medium (1% Bacto tryptone and appropriate amount of NaCl), and the ability to grow without NaCl was determined in 1% sterile tryptone water.

(ii) 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 per protocols established by Ruimy et al. (1994). Amplicons were sequenced directly following purification on Qiagen columns (Qiagen, Valencia, CA). 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 Corp., Cleveland, OH) and an ABI 373A automated sequencer (Perkin-Elmer Corp., Foster City, CA). The phylogenetic relationships of organisms covered in this study were determined by comparison of individual 16S rDNA sequences to other existing sequences in the public database (GenBank; http://www.ncbi.nlm.nih.gov/). Evolutionary trees were constructed with PAUP software (Swofford, 1990). The GenBank nucleotide accession number for the 16S rDNA of strain 34hs-1\(^T\) is XXXXXX.

(iii) DNA-DNA hybridization. Cells were suspended in 0.1M EDTA (pH 8.0) and digestion of the cell wall was carried out by treating the cells with lysozyme (final concentration, 2 mg/ml). DNA was isolated by standard procedures (Johnson, 1981). DNA-DNA homology was studied by microplate hybridization methods (Ezaki et al., 1989) with photobiotin labeling and colorimetric detection, using 1,2-phenylenediamine
(Sigma, St. Louis, MO) as the substrate and streptavidine-peroxidase conjugate (Boehringer-Mannheim, Germany) as the colorimetric enzyme (Satomi et al., 1997).

RESULTS AND DISCUSSION

Microbial contamination of the Mars Odyssey Spacecraft. The microbial population of the large surface area of the spacecraft showed, on average (25 determinations), total heterotrophs and spore-formers at 28±8.6 and 2±1.5 CFU per 25 cm$^2$, respectively (data not shown). Only one of every two aliquots (4 mL per plate, 30 plates total) arising from this pooled sample yielded any spore-formers. A schematic of the spacecraft depicting sampling locations can be viewed at the JPL website (http://mars.jpl.nasa.gov/odyssey/newsroom/presskits/odysseyarrival1.pdf). When the isolated colonies were exposed to harsh conditions, such as UV, γ-radiation, H$_2$O$_2$, and desiccation, some spore-formers showed resistance. Among these spore-formers, a strain, designated as 34hs-1$^T$, exhibited distinct spore morphology and was further characterized for its phylogenetic affiliation.

Morphological and physiological characteristics. Strain 34hs-1$^T$ is a Gram-positive, facultatively anaerobic, rod-shaped, spore-forming bacterium. Cells are 4 to 5 µm in length, 1 µm in diameter, and are motile. On TSA medium incubated at 32°C, young colonies are beige, regular, with a diameter of 3 to 4 mm, fairly smooth, umbonate with undulate or lobate edges. Endospores of strain 34hs-1$^T$ (1 x 0.5 µm) are terminal, round (Fig. 1A), with one spore per cell. Ultra thin sections of 34hs-1$^T$ spores showed the presence of an exosporium, spore coat, cortex, and core (Fig. 1D). This structure resembles the exosporium of the B. cereus group (data not shown). Unlike the spores of B. nealsonii, the exosporia of 34hs-1$^T$ spores cannot be removed by washing with detergents and salts using the Nicholson and Setlow (1990) protocol. Microscopic analyses revealed the destruction of 34hs-1$^T$ spores by γ-radiation while the remnants of exosporia were left behind (Fig. 1B), whereas spores oxidized by H$_2$O$_2$ formed “doughnut-like” structures (Fig. 1C). Further analysis showed highly-electron dense structures in the exosporia of γ-irradiated and H$_2$O$_2$-treated (Fig. 1E, F) spores when compared to the untreated control (Fig. 1D). The characterization and the physiological role of this exosporium of strain 34hs-1$^T$ spores will not be discussed in this communication.

Resistances of 34hs-1$^T$ 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 (review, Nicholson et al., 2000). The spores of 34hs-1$^T$ exhibited resistance to UV (254 nm), γ-radiation, 5% liquid H$_2$O$_2$, and desiccation conditions. The 34hs-1$^T$ spores did not exhibit classic UV inactivation kinetics, the characteristic "shoulder" was missing and inactivation did not take effect until well after 400 J/m$^2$. The 34hs-1$^T$ spores exhibited an LD$_{90}$ value (the 90% lethal dose) of ~660 J/m$^2$. Spores of 34hs-1$^T$ also survived 0.5Mrad γ-radiation (0.4% survival).
Purified spores exposed to 5% liquid H$_2$O$_2$ showed resistance and about 26% of the initial inoculum (1.1x10$^7$ mL$^{-1}$) was viable after 60 min. exposure. Finally, desiccation had no effect on the viability of the 34hs-1$^T$ spores. When compared to the model organism, B. subtilis strain 168, spores of strain 34hs-1$^T$ appeared to be quite resistant, with 3 times, xx times, 6 times, and 10 times greater survival to UV (Nicholson et al., 2000), γ-radiation (Venkateswaran et al., 2003), H$_2$O$_2$ (Kempf et al., 2002), and desiccation (data not shown), respectively.

Optimum growth conditions. Strain 34hs-1$^T$ grew between 25 to 42 °C, with optimum growth at 35 °C, and over the pH range of 4 to 9 (optimum 6 to 7). This strain did not require Na$^+$ for growth and was as desiccation resistant as other spore-formers.

The 16S rDNA sequences of all known Firmicutes were compared with that of 34hs-1$^T$. All phylogenetic analyses, based on 16S rDNA sequence, unambiguously demonstrated that 34hs-1$^T$ 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 34hs-1$^T$. Their phylogenetic relationships were then analyzed 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 34hs-1$^T$ shares a close phylogenetic relationship with Bacillus species. Neighbor-joining, parsimony, and maximum likelihood analyses were undertaken on this subset of bacteria, using several subdomains of the 16S rDNA. In all analyses, 34hs-1$^T$ was most closely associated with members of the genus Bacillus.

The similarities in the 16S rDNA nucleotide sequences between 34hs-1$^T$ and the top 17 closely related Bacillus species, recognized by GenBank “BLAST” searches, were between 95 and 96%. A sequence variation of ~4% was found between 34hs-1$^T$ and B. fusiformis ATCC 4513$^T$, and 2% between 34hs-1$^T$ and B. benzoevorans DSM 5391$^T$ as well as B. firmus IAM 12464. A very high sequence variation (5%) was noticed between 34hs-1$^T$ 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.

DNA-DNA hybridization. DNA-DNA hybridization was performed between 34hs-1$^T$ and 18 strains, comprising 12 Bacillus species. None of the Bacillus species that showed very high similarities in 16S rDNA sequence (~97%) exhibited >70% DNA-DNA reassociation values that would place the strain within the same species. Particularly, the similarity between 34hs-1$^T$ and B. circulans ATCC 4513$^T$ was only 16%. This pair showed 98.7% similarity in their 16S rDNA sequences. Similarly, 34hs-1$^T$ and B. benzoevorans ATCC 49005$^T$ showed only 15% DNA-DNA hybridization values whereas this pair exhibited ~98% similarities in their 16S rDNA sequence. Based on the DNA-DNA reassociation values, 34hs-1$^T$ is a novel Bacillus species.

Description of Bacillus odysseensis

Bacillus odysseensis (o.dys.se.en’ sis, pertaining to the Mars Odyssey (L. Odyssea) spacecraft, from which the organism was isolated)
The cells of the type strain are rod-shaped, 4 – 5 µm in length, 1 µm in diameter, and motile. They are Gram-positive, facultatively anaerobic, and endospore forming. Spores show an additional exosporium layer. Colonies on TSA are regular, smooth, umbonate with undulate or lobate edges, and beige in color. Sodium ions are not essential and exhibited growth at 0 to 6% NaCl. Cells grow at a pH range of 6 to 9 with an optimum at 7. Cells are able to grow at 25 to 42 ºC with optimum growth at 35 ºC. Based on 16S rDNA nucleotide sequences, this bacterium belongs to the class Firmicutes and is a member of the genus Bacillus. The type strain, 34hs-1^T (= ATCC XXXXXX ^T), was isolated from the surface of the Mars Odyssey Spacecraft.

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Fig. 1. Electron micrographs of Bacillus odysseensis spores before and after γ-radiation and H_2O_2 exposure. ESEM (A, B, C) and TEM (D, E, F) micrographs showing spore surface and cross-section characteristics, respectively. (A, D) Purified spores showing intact, round structure. (B, E) Spores (1.1 x 10^7) were exposed to γ-radiation (0.5 Mrad) and (C, F) 5% liquid H_2O_2 for 60 min. The intact spore with exosporium is designated as IS. The exosporium (EX), spore coat (SC), cortex, and spore core are shown in the longitudinal section of a spore (D,E,F). The exosporium removed from spore due to γ-radiation is shown in B. The spore removed exosporium due to H_2O_2 treatment is shown in C. Bar indicates 1 µm.
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