The present invention relates to discovery and isolation of a biologically pure culture of a Bacillus odysseyi isolate with high adherence and sterilization resistant properties. B. odysseyi is a round spore forming Bacillus species that produces an exosporium. This novel species has been characterized on the basis of phenotypic traits, 16S rDNA sequence analysis and DNA-DNA hybridization. According to the results of these analyses, this strain belongs to the genus Bacillus and the type strain is 34hs-lT (=ATCC PTA-4993=NRRL B-30641=NBRC 100172). The GenBank accession number for the 16S rDNA sequence of strain 34hs-lT is AF526913.

1 Claim, 5 Drawing Sheets
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**FIGURE 7**

**FIGURE 8**
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<td>2. <em>B. fusiformis</em> ATCC 7055\textsuperscript{T}</td>
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<td>3. <em>B. silvestris</em> NRRL B-23336\textsuperscript{T}</td>
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<td>4. <em>B. pycnus</em> NRRL NRS-1691\textsuperscript{T}</td>
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<td>5. 'B. aminovorans' NRRL NRS-341</td>
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<td>6. <em>B. neidei</em> NRRL BD-101</td>
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<td>7. <em>B. sphaericus</em> NRRL BD-113</td>
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<td>8. <em>Sporosarcina aquimarina</em> SAFN-008</td>
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**FIGURE 9**
**BACILLUS ODYSSEYI ISOLATE**

**PRIORITY CLAIM**

This application is a non-provisional application, claiming the benefit of priority to provisional application No. 60/440,790, filed in the United States on Jan. 17, 2003, entitled “Bacterial spore-forming species that is extremely resistant to various sterilization methods.”

**GOVERNMENT RIGHTS**

This invention was made with Government support under Contract NAS7-1407 awarded by NASA. The Government has certain rights in the invention.

**FIELD OF INVENTION**

The present invention relates to an isolated biologically pure culture of a novel spore forming Bacillus species, and more particularly, to a Bacillus odysseyi isolate with high adherence and sterilization resistant properties.

**BACKGROUND OF INVENTION**

Several physiologically and phylogenetically distinct microorganisms have been encountered while examining microbial contamination of spacecraft surfaces. Some of these micro-organisms form round, exosporium-bearing spores, whose exosporia might be responsible for adaptation to the extreme clean conditions of, and direct adhesion to, spacecraft surfaces.

Such biofouling is a concern in not only space travel, but in a number of industries. Isolation, identification and understanding of the highly resistant and adhesive micro-organisms could be of significant use in industry, where biofouling is a major cause of reduction in productivity (resulting in a loss of over $6.5 billion in marine industries alone), and in medicine, where bacterial adhesion is often a primary step in human disease. In addition, purified exosporium components (proteins, lipids, etc.) could possibly be used in other ways, such as in sunscreens or to prolong the lives of convertible tops, tents, etc. as a UV-ray retardant spray.

Additionally, isolation of the microorganism would allow for formation of strategies for inactivating those resistance characteristics that interfere with sterilization of spacecraft materials; in particular, resistance to Hydrogen Peroxide (H₂O₂), Ultra Violet (UV), and γ-radiation and adhesion. An understanding of these mechanisms will guide the development of sterilization procedures that are targeted to the specific molecules responsible for resistance, and could eliminate the need for unduly harsh methods that jeopardize equipment. A need exists in the art for an improved sterilization procedure that would enable spacecraft to meet planetary protection requirements without a terminal heat sterilization step. This would support implementation of planetary protection policies for life detection missions.

**SUMMARY OF INVENTION**

The present invention relates to an isolated biologically pure culture of a novel spore forming Bacillus species, and more particularly, to a Bacillus odysseyi isolate with high adherence and sterilization resistant properties.

Additionally, because of its UV resistant properties, purified exosporium components (proteins, lipids, etc.) of B. odysseyi could be used in sunscreens or to prolong the lives of convertible tops, tents, etc. as a UV-ray retardant spray.

**BRIEF DESCRIPTION OF THE DRAWINGS**

The objects, features and advantages of the present invention will be apparent from the following detailed description of the various aspects of the invention in conjunction with reference to the following drawings, where:

FIG. 1 is a light microscopy image of sporulating vegetative cells showing terminal swelling of mother cells;

FIG. 2 is a microscopy image of a purified spores showing an intact spore with exosporium;

FIG. 3 is a microscopy image of a longitudinal section of an untreated spore, showing the exosporium, spore coat, cortex and spore core;

FIG. 4 is a microscopy image of spores after being exposed to gamma radiation;

FIG. 5 is a microscopy image of spores after being exposed to H₂O₂;

FIG. 6 is a microscopy image of spores after being exposed to both gamma radiation and H₂O₂;

FIG. 7 is a table illustrating characteristics for differentiating B. odysseyi 34-hsl from related species;

FIG. 8 is a chart illustrating a phylogenetic tree of round-sporing forming Bacillus and other species closely related to strain 34-hsl based on maximum likelihood and parsimony analysis of 16S rDNA nucleotide sequences; and

FIG. 9 is a table illustrating DNA-DNA hybridization between B. odysseyi sp. Nov. 34-hsl and related species.

**DETAILED DESCRIPTION**

The present invention relates to an isolated biologically pure culture of a novel spore forming Bacillus species, and more particularly, to Bacillus odysseyi.

The following description, taken in conjunction with the referenced drawings and/or tables, is presented to enable one of ordinary skill in the art to make and use the invention. Various modifications will be readily apparent to those skilled in the art, and the general principles defined herein may be applied to a wide range of aspects. Thus, the present invention is not intended to be limited to the aspects presented, but is to be accorded the widest scope consistent with the principles and novel features disclosed herein. Furthermore, it should be noted that unless explicitly stated otherwise, the figures included herein are illustrated qualitatively and without any specific scale, and are intended to generally present the concept of the present invention.

In order to provide a working frame of reference, first a glossary of terms used in the description and claims is given as a central resource for the reader. Next, a discussion of various aspects of the present invention is provided to give an understanding of the specific details.

(1) **Glossary**

Before describing the specific details of the present invention, a centralized location is provided in which various terms used herein and in the claims are defined. The glossary provided is intended to provide the reader with a general understanding for the intended meaning of the terms, but is not intended to convey the entire scope of each term. Rather, the glossary is intended to supplement the rest of the specification in more clearly explaining the terms used.

The strain disclosed in this description has been deposited in the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 North University Street, Peoria, Ill. 61604, U.S.A., as NRRL
The deposit was received by NRRL on Feb. 4, 2003, and was given an accession number by the International Depository Authority of NRRL B-30641. The deposit has been made and received by the International Depository Authority under the provisions of the Budapest Treaty, and all restrictions upon public access to the deposit will be irrevocably removed upon the grant of a patent on this application.

API 20NE Test Strips—The term "API 20NE" refers to test strips that are used for 24–48 hour identification of gram-negative Enterobacteriaceae.

DNA-DNA hybridization—The term "DNA-DNA hybridization" refers to a technique that provides for genetic comparisons integrated over the entire genome of two species.

Gram-positive—The term "gram positive" refers to bacteria that are stained dark blue or violet by gram staining, in contrast to gram negative bacteria which are not stained dark blue or violet by gram staining. The strain is caused by a higher amount of peptidoglycan in the cell wall, which typically lacks the secondary membrane and lipopolysaccharide layer found in other bacteria.

(ii) Microbial Examination.

As a non-limiting example of microbial examination, samples were sonicated for 2 minutes (min.) and heat-shocked at 80°C for 15 min., at which time appropriate aliquots were placed into Petri dishes and total aerobic spores were enumerated by pour-plate techniques using Tryptic Soy Agar (TSA) as the growth medium (32°C for 2 days). Samples that were not heat-shocked were enumerated for total aerobic cultivable heterotrophs on TSA. Colony forming units (c.f.u.) were counted after incubation at 32°C for up to 7 days. Isolates were selected, purified, and stored in glycerol at −80°C. Identification of purified strains was accomplished by ribosomal DeoxyriboNucleic Acid (rDNA) sequencing (see below). Type strains of various Bacillus species for use as controls were either procured from the American Type Culture Collection or received as gifts from the USDA Research Centre (National Center for Agricultural Utilization Research) collection.

(iii) Sporulation.

As a non-limiting example of sporulation, a Nutrient Sporulation Medium (NSM) was used to produce spores. A single purified colony of the strain to be sporulated was inoculated into NSM liquid medium. After 2–3 days growth at 32°C, cultures were examined in wet mounts to ascertain the level of sporulation. Once the number of free spores was greater than the number of vegetative cells, the culture was harvested and spores were purified. Spores were purified by treating with lysozyme and washing with salts and detergents. These chemical treatments did not remove the exosporium surrounding the spore coat. Purified spores were re-suspended in sterile de-ionized water, heat-shocked (80°C for 15 min.) to ensure inactivation of the vegetative population and stored at 4°C in glass tubes.

(iv) Microscopy.

As a non-limiting example of microscopy, the refractile nature of the spores was examined by phase-contrast microscopy. Non-destructive examination of spores and vegetative cells was also exploited using a field-emission environmental Scanning Electron Microscope (SEM). In addition, a standard SEM and a Transmission Electron Microscope...
phate Buffered Saline (PBS) (pH 7.2), placed in an un-15 C. Results and Discussion were dispensed onto presterilized metal and glass-fibre affiliation.

By Riesenman for Polymerase Chain Reaction (PCR) amplification is well documented. Spores of 34hs-1 exhibited resistance to 60 W,,, (254 nm W radiation), gamma radiation, 5% liquid PBS (1 0' spores ml-l) were treated with H2O2 (5% final concentration) and quantitative measurement of H2O2-resistant spores. 30 one strain, designated 34hs-1, exhibited distinct spore morphology and was characterized further for its phylogenetic affiliated.

After 60 min incubation, 100 pl was removed and diluted in PBS and plated onto NSM agar medium. Plates were incubated at 32° 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. Known concentrations of spore suspensions prepared in PBS (106 spores ml-1) were treated with H2O2 (5% final concentration) and incubated at room temperature (~25° C.) with gentle mixing. After 60 min incubation, 100 µl was removed and diluted in bovine catalase (100 µg ml-1) in PBS. Serial 1:10 dilutions of the catalase treated suspension were prepared in Tryptic Soy Broth (TSB) to check viability and spread onto TSA for quantitative measurement of H2O2-resistant spores.

To test desiccation resistance, spore suspensions (20 µl) were dispensed onto pre-sterilized metal and glass-fibre discs (106 spores per disc). The spore inoculated discs were incubated in a glass desiccation chamber with a relative humidity of 15% for 1 or 2 days before c.f.u. were counted on TSA medium. Briefly, the desiccated sample was placed in sterile PBS, vortexed thoroughly and placed in a sonicating bath for 2 min at room temperature before plating onto TSA medium. Plates were incubated at 32° C. for 2 days and the number of spores that survived was counted. Untreated aliquots of purified spores at equivalent concentrations were included and worked up alongside test aliquots for all treatments as a means of determining relative percentage survivability.

B. Systematic Characterization

(i) Phenotypic Characterization. The ability to grow in NaCl concentrations of 1–10% was determined in Luria–Bertani liquid medium (1% Bacto tryptone containing the appropriate amount of NaCl) and the ability to grow without NaCl was determined in 1% sterile tryptone water. The commercially available Biolog identification system was used, according to the manufacturer’s specifications, to characterize utilization of various carbon substrates. In addition, API 20NE test strips were used to characterize the strain further.

(ii) 16S rDNA Sequencing. Approximately 10 nanograms (ng) of purified Deoxyribonucleic Acid (DNA) from liquid cultures was used as a template for Polymerase Chain Reaction (PCR) amplification. Universal primers (Bact 11 and 1492) were used to amplify the 1.5 kilobase pair (kb) PCR fragment. Purified amplicons were sequenced and the identity of a given PCR product was verified by bi-directional sequencing analysis. The phylogenetic relationships of organisms covered in this description were determined by comparison of individual 16S rDNA sequences to existing sequences in public databases, such as the database of the National Center for Biotechnology Information. Evolutionary trees based on parsimony and maximum-likelihood analyses were constructed with Phylogenetic Analysis Using Parsimony (PAUP) software.

(iii) DNA–DNA Hybridization. Cells were suspended in 0.1 Mole (M) of Ethylenediamine Tetraacetic Acid (EDTA) (pH 8.0) and cell walls were digested by lysozyme treatment (final concentration, 2 mg ml-1). DNA was isolated by standard procedures. DNA–DNA relatedness was studied by microplate hybridization methods with photobiotin labeling and colorimetric detection, using 1,2-phenylenediamine as the substrate and streptavidin-peroxidase conjugate as the colorimetric enzyme.

C. Results and Discussion

(i) Microbial Examination 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 28.0±8.6 and 2.0±1.5 c.f.u. per 25 cm2, respectively. Isolates were identified by 16S rDNA sequence analysis as species of Acinetobacter, Bacillus, Carbohydractera, Delftia, Microbacterium and Ralstonia. Additionally, all fungal isolates were identified as Aureobasidium pullulans by 18S rDNA sequence analysis. When purified strains arising from isolated colonies were screened for resistance to harsh conditions, such as UV, gamma radiation, H2O2 and desiccation, several spore-forming isolates showed resistance. Of the 45 strains identified, one strain, designated 34hs-1, exhibited distinct spore morphology and was characterized further for its phylogenetic affiliation.

(ii) Morphological and Physiological Characteristics. Strain 34hs-1 is a Gram-positive, aerobic, rod-shaped, spore-forming bacterium. Cells are 4–5 mm long, 1 mm in diameter and motile. On TSA medium incubated at 32° C., young colonies are beige, round, ~3 mm in diameter, fairly smooth and flat with entire edges. As shown in FIG. 1, endospores of strain 34hs-1 (1 mm in diameter) are terminal, with one spore per cell, and swell the mother cell.

Additionally, as shown in FIG. 2, the endospores are round. An intact spore is shown, labeled as IS. As shown in FIG. 3, ultrathin sections of spores of strain 34hs-1 showed the presence of an exosporium (labeled in FIG. 3 as EX), a spore coat (labeled in FIG. 3 as SC), a cortex (labeled in FIG. 3 as Cortex) and a core (labeled in FIG. 3 as Core).

As shown in FIG. 4, microscopic analyses revealed the partial destruction of 34hs-1 spores by gamma radiation, although remnants of exosporia were left behind; some spores oxidized by H2O2 formed ‘doughnut-like’ structures (Shown in FIG. 5). Further analysis showed highly electron-dense structures in the exosporia (EX) of gamma-irradiated and H2O2-treated (Shown in FIG. 6) spores when compared with the untreated control shown in FIG. 3.

(iii) Resistance of Strains of Strain 34hs-1 to Various Physical and Chemical Conditions. The resistance of Bacillus spores to a variety of conditions is well documented. Spores of 34hs-1 exhibited resistance to classical UV254 (254 nm UV radiation), gamma radiation, 5% liquid H2O2 and desiccation conditions. Spores of 34hs-1 did not exhibit classic UV254 inactivation kinetics: the characteristic ‘shoulder’ was missing and inactivation did not take effect until well after 400 J m⁻². Spores of strain 34hs-1 exhibited an LD₅₀ (90% lethal dose) of ~660 J m⁻². Spores of 34hs-1 also survived 0.5 Mrad gamma radiation (0.4% survival). Purified spores exposed to 5% liquid H2O2 showed resis-
Bacillus badius. Compared with that of 34hs-1. Bootstrapping (500 replicates) endospores, and the spores show an additional exosporium no effect on viability of the 34hs-1 spores. When compared of the genus ml-l) viable after 60 min exposure. Finally, desiccation had analyses, strain 34hs-1 was most closely related to members of the Surface Of Mars. This presents a large problem to those with Earth-derived biomatter. A maximum-likelihood phylogenetic tree based on 16s rDNA sequences of each of the shown strains with that of Bacillus. Strain 34hs-1 grew between 25 and 42°C, with optimum growth at 30–35°C, and over the pH range 6–10 (optimum 6–7). It did not require Na+ for growth. Biochemical characterization of strain 34hs-1 is presented in FIG. 7; where Strain 1 is B. odysseyi 34hs-1; Strain 2 is B. fusformis NRRL NRS-350T; Strain 3 is B. sphaericus DSM 28T; Strain 4 is B. pycnus NRRL NRS-1691T; Strain 5 is B. neidei NRRL BD-87T; and Strain 6 is B. badius ATCC 14574T. The row in the table labeled “16S rDNA sequence similarity (%),” refers to the percent similarity of the 16S rDNA sequences of each of the shown strains with that of B. odysseyi 34hs-1.

This strain produced catalase, but not cytochrome oxidase, gelatinase, urease, tryptophan deaminase, lysine, ornithine decarboxylase, or arginine dihydrolase. It did not show denitrification or acetoin production. 34hs-1 did not ferment glucose or utilize glucose as a sole carbon source. After prolonged incubation (>3 days), arabinose was assimilated; of at least two determinations between the two sources. 34hs-1 represents a novel strain 34hs-1, are not able to grow in the absence of oxygen.

(v) Phenotypic Characterization.

Strain 34hs-1 grew between 25 and 42°C, with optimum growth at 30–35°C, and over the pH range 6–10 (optimum 6–7). It did not require Na+ for growth. Biochemical characterization of strain 34hs-1 is presented in FIG. 7; where Strain 1 is B. odysseyi 34hs-1; Strain 2 is B. fusformis NRRL NRS-350T; Strain 3 is B. sphaericus DSM 28T; Strain 4 is B. pycnus NRRL NRS-1691T; Strain 5 is B. neidei NRRL BD-87T; and Strain 6 is B. badius ATCC 14574T. The row in the table labeled “16S rDNA sequence similarity (%),” refers to the percent similarity of the 16S rDNA sequences of each of the shown strains with that of B. odysseyi 34hs-1.

This strain produced catalase, but not cytochrome oxidase, gelatinase, urease, tryptophan deaminase, lysine, ornithine decarboxylase, or arginine dihydrolase. It did not show denitrification or acetoin production. 34hs-1 did not ferment glucose or utilize glucose as a sole carbon source. After prolonged incubation (>3 days), arabinose was assimilated; of at least two determinations between the two sources. 34hs-1 represents a novel strain 34hs-1, are not able to grow in the absence of oxygen.
sugars to acids does not occur following prolonged incubation. *B. odysseyi* prefers pyruvate, amino acids, purine or pyrimidine bases and related compounds as carbon and energy sources. *B. odysseyi* is catalase-positive, but does not produce gelatinase, arginine diliydrolase, lysine or ornithine decarboxylase, lipase, amylase or alginase. The organism does not produce H$_2$S from thiosulfite and is not involved in denitrification. The type strain, strain 34hs-1 (ATCC PTA-4993$^{T}$=NRRL B-30641=NBRC 100172$^{T}$), was isolated from the surface of the Mars Odyssey spacecraft.

What is claimed is:

1. An isolated biologically pure culture of *Bacillus odysseyi* strain 34hs-1 deposited under accession number NRRL B-30641.

* * * * *