Bacillus Odysseyi Isolate

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Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

Ampl. No.: 10759,327
Filed: Jan. 17, 2004

Prior Publication Data
US 2004/0158042 A1 Aug. 12, 2004

Related U.S. Application Data
Provisional application No. 60/440,790, filed on Jan. 17, 2003.

References Cited
La Duc, M. et al (Characterization of microbes intensively associated with the Mars Odyssey Orbiter and it’s assembly facility, Abstracts of the general meeting of the American Society for Microbiology, 2002; 102: 389).
Venkateswaran, K. et al (Bacillus nealsonii sp. nov., isolated from a spacecraft-assembly facility, whose spores are gamma-radiation resistant, International Journal of Systematic and Evolutionary Microbiology, Jul. 2002; 52: 165-172).*


* cited by examiner

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ABSTRACT

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-1T (=ATCC PTA-49937=NRRRL B-30641=f=NBRC 100172). The GenBank accession number for the 16S rDNA sequence of strain 34hs-1T is AF526913.

1 Claim, 5 Drawing Sheets
### Table

<table>
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<th>Characteristic</th>
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<td>16S rDNA sequence similarity (%)</td>
<td>NA</td>
<td>96·4</td>
<td>95·7</td>
<td>93·1</td>
<td>92·4</td>
<td>91·5</td>
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</table>

**FIGURE 7**

10 changes

- *Bacillus pycnus* NRRL NRRL-1691T (AF168531)
- *Kurthia zeplii* NCIMB 9878T (X70321)
- *Kurthia gibsonii* NCIMB 9758T (X70320)
- *Bacillus neidii* NRRL BD-101 (AF169508)
- *Sporosarcina psychrophila* ATCC 23304T (X60634)
- *Sporosarcina globispora* NCIMB 11434T (X60644)
- *Filibacter limicola* DSM 13886T (AJ292316)
- *Sporosarcina aquimaris* SW28T (AF202056)
- *Sporosarcina pasteurii* NCIMB 8841T (X50631)
- *Planococcus psychrotolerans* JCM 11080 (AF324659)
- *Bacillus insolitus* DSM 5T (X60642)
- *Caryophanon tenuex* NCDO 2324T (X70315)
- *Caryophanon fatum* NCIMB 9533T (X70314)
- *Bacillus silvestris* HR3-23T (AJ006086)
- *Bacillus sphæricus* DSM 28T (AJ310084)
- *Bacillus fusiformis* DSM 2988T (AJ310083)
- *Bacillus odysseyi* 34hs-1T (AF526913)
- *Bacillus subtilis* ATCC 6833 (AB018486)

**FIGURE 8**
<table>
<thead>
<tr>
<th>Strain</th>
<th>Similarity (%) ( t )</th>
<th>1</th>
<th>2</th>
<th>3</th>
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<td>5. 'B. aminovorans' NRRL NRS-341</td>
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<td>16</td>
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<td>6. <em>B. neidei</em> NRRL BD-101</td>
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<td>ND</td>
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<td>15</td>
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<td>7. <em>B. sphaericus</em> NRRL BD-113</td>
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<td>ND</td>
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<td>100</td>
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<td>8. <em>Sporosarcina aquimarina</em> SAFN-008</td>
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<td>ND</td>
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<td>8</td>
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</table>

**FIGURE 9**
1

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 descriptions 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-hs1 from related species;
FIG. 8 is a chart illustrating a phylogenetic tree of round-spore forming Bacillus and other species closely related to strain 34-hs1 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-hs1 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. 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.
A. Method

The methods for collection, isolation and characterization described herein are for illustrative purposes only. It must be understood that the species \textit{B. odysseyi} may be collected and isolated from any surface where the bacteria is present and that there may be other techniques for characterizing the species. The following techniques are non-limiting examples for completing the described process or technique.

(i) Sample Preparation and Isolation of Microbes from the Mars Odyssey Spacecraft.

Components of the Mars Odyssey spacecraft were manufactured in various geographic locations. The spacecraft underwent several months of assembly in the Spacecraft Assembly Facility at the Jet Propulsion Laboratory, Pasadena, Calif., USA, before it was transported to the Kennedy Space Center, Fla., USA. Samples were taken from 25 different surface areas (25 cm$^2$) of the spacecraft using sterile, water-moistened polyester swabs during final assembly and encapsulation in the Spacecraft Assembly and Encapsulation Facility II, Kennedy Space Center (February 2001). Upon collection of a surface sample, each swab was placed individually into sterile water, creating a final volume of 10 ml (i.e. swab plus sterile water). The 25 samples were pooled into one sterile container and processed immediately. All samples were analyzed for both spore-formers and total cultivable heterotrophs.

(ii) Microbial Examination.

As a non-limiting example of microbial examination, samples were sonicated for 2 minutes (min.) and heat-shocked at 80$^\circ$ C. for 15 min., at which time appropriate aliquots were plated into Petri dishes and total aerobic spores were enumerated by pour-plate techniques using Tryptic Soy Agar (TSA) as the growth medium (32$^\circ$ 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$^\circ$ C. for up to 7 days. Isolates were selected, purified, and stored in glycerol at $80^\circ$ C. Identification of purified strains was accomplished by ribosomal Deoxyribonucleic Acid (rDNA) sequencing (see below). Type strains of various \textit{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$^\circ$ 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$^\circ$ C. for 15 min.) to ensure inactivation of the vegetative population and stored at 4$^\circ$ 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 unco-
treated samples on TSA at 32° C. streptavidin-peroxidase conjugate as the colorimetric
to examine H2O2 resistance in spores. Known concentra-
tions were included and worked up alongside test
samples of spores were removed, diluted serially tenfold in PBS and plated onto NSM agar
plates. 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. Known concentra-
tions 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 soni-
cating 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
100%.

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 survival.

B. Systematic Characterization
(i) Phenotypic Characterization.
The ability to grow in NaCl concentrations of 1–10% was
determined in TN4 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 specifi-
cations, to characterize utilization of various carbon sub-
strates. In addition, API 20NE test strips were used to
counter the strain further.

(ii) 16S rDNA Sequencing.

Approximately 10 nanograms (ng) of purified Deoxyri-
boNucleic Acid (DNA) from liquid cultures was used as a
template for Polymerase Chain Reaction (PCR) amplifica-
tion. 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 data-
bases, such as the database of the National Center for
Biotechnology Information. Evolutionary trees based on
parsimony and maximum-likelihood analyses were con-
structed with Phylogenetic Analysis Using Parsimony
(PAUP) software.

(iii) DNA-DNA Hybridization.

Cells were suspended in 0.1 Mole (M) of Ethylenedi-
amine 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 detec-
tion, 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 Space-
craft.

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 cm2, respectively. Isolates were identified by
16S rDNA sequence analysis as species of Actinobacteria,
Bacillus, Curtobacterium, Delftia, Microbacterium and Ral-
stonia. Additionally, all fungal isolates were identified as
Aureobasidium pullulans by 18S rDNA sequence analysis.

(ii) Morphological and Physiological Characteristics.

Strain 34hs-1 is a Gram-positive, aerobic, rod-shaped,
sporo-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 Spores 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
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. by the Biolog system, showed an identification match for cutoff value required to place strains within the Same sphen 2. Neighbor-joining, parsimony and maximum-
P pH 6-10 (optimum at pH 7) and 2542° C. (optimum ridides, but preferred pyruvate, amino acids, purine Or PYri-
sphaericus phenotypic characteristics were similar to those of both
however, this is not a discriminatory phenotypic trait. selected species, None of the prolonged incubation (>3 days), arabinose was assimilated; of at least two determinations between the two
thine decarboxylase, or arginine dihydrolase. It did not show As shown in FIG, NRRL BD-87T; and Strain 6 is
the Surface Of Mars. This presents a large problem to those A maximum-likelihood phylogenetic tree based on 16s rDNA sequences of several round-spore-forming bacilli, as well as some asporogenous genera, is shown in FIG. 8. Strain number and GenBank accession numbers are shown following the species name respectively. Numbers above the lines are percentage bootstrap values of 500 replications of that branch of the tree. Bar, 10 changes among 1.5 kb, meaning that 10 base pairs changed from one organism to another and required 1,500 million years of evolution.
The branching order of the phylogenetic tree shown in FIG, showed three distinct clusters, in which one clade contained Kurthia species, another group was formed from species of Sporosarcina, Filibacter and Planococcus and a final grouping was composed of species of Bacillus and Caryophanon, including strain 34hs-1. The round-spore-forming Bacillus group was very tightly bound phylogenetically; all members of this clade shared sequence similarities of >95%. Strain 34hs-1 exhibited the characteristics necessary to place it in Bacillus RNA group 2. To differentiate these closely related species more accurately, DNA-DNA hybridization was performed. (vi) DNA-DNA Hybridization.
As shown in FIG. 9, DNA-DNA hybridization was performed between 34hs-1 and round-spore-forming Bacillus and Sporosarcina species. The values shown in FIG. 9 are means of at least two determinations between the two selected species. None of the Bacillus species that showed very high 16S rDNA sequence similarities (~96%) exhibited >70% DNA-DNA re-association values with 34hs-1, i.e. the cutoff value required to place strains within the same species. In particular, the hybridization value between 34hs-1 and B. silvestris NRRL B-23361T was only 17%, whereas their 16S rDNA sequences were 96.4% similar. Also, strain 34hs-1 and B. sphaericus NRRL BD-113 showed 17% DNA-DNA hybridization, but ~97% 16S rDNA sequence similarity. Based on DNA-DNA re-association values, strain 34hs-1 represents a novel Bacillus species, Bacillus odysseyi sp. nov. (3) Conclusion Bacillus odysseyi (o. dys.seo.yi. L. n. Odysse; N.L. gen. n. odysseyi pertaining to the Mars Odyssey spacecraft, from which the organism was isolated).
Cells are rod-shaped, 4–5 mm in length, approximately 1 mm in diameter and motile. Furthermore, the B. odysseyi cells are Gram-positive and aerobic, form terminal endospores, and the spores show an additional exosporium layer. Colonies on TSA are round, smooth, flat with entire edges and beige in color. Sodium ions are not essential for growth; growth occurs in 0–5% NaCl. B. odysseyi grows at pH 6–10 (optimum at pH 7) and 25–42° C. (optimum 30–35° C.). With the exception of arabinose, breakdown of
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 dihydrolase, lysine or ornithine decarboxylase, lipase, amylase or alginate. 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=NBR 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.

* * * *