United States Patent
Venkateswaran et al.

BACILLUS ODYSSEYI ISOLATE

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U.S. Cl. .......................... 435/252.5; 424/93.46
Field of Classification Search ........................ None
See application file for complete search history.

References Cited

La Duc, M. et al (Characterization of microbes interactively associated with the Mars Odyssey Orbiter and its 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 g-radiation resistant, International Journal of Systematic and Evolutionary Microbiology, Jul. 2002: 53: 165-172).*

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-lT (=ATCC PTA-4993T=NRRL B-30641T=NBRC 100172T). The GenBank accession number for the 16S rDNA sequence of strain 34hs-lT is AF526913.

1 Claim, 5 Drawing Sheets
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<td>91.5</td>
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**FIGURE 7**

![Phylogenetic tree showing the relationship between various bacterial strains.](image)

**FIGURE 8**

![Close-up view of a bacterial culture.](image)
<table>
<thead>
<tr>
<th>Strain</th>
<th>Similarity (%) ( t ) labelled DNA from:</th>
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<tr>
<td>1. <em>B. odysseyi</em> 34hs-1\textsuperscript{T}</td>
<td>100 18 17 17</td>
</tr>
<tr>
<td>2. <em>B. fusiformis</em> ATCC 7055\textsuperscript{T}</td>
<td>23 100 17 15</td>
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<tr>
<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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<tr>
<td>8. <em>Sporosarcina aquimarina</em> SAFN-008</td>
<td>7 ND ND 8</td>
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</table>

**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 g-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-hsl 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-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 to 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 stain 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.

(2) Introduction

This specification describes Bacillus odysseyi sp. nov., isolated from the surface of the Mars Odyssey spacecraft, whose round spores are resistant to Ultra Violet (UV) and gamma radiation, Hydrogen Peroxide (H_2O_2) and desiccation. The Bacillus strain isolated and described herein was characterized based on a polyphasic taxonomic approach that examined its phenotypic and genotypic affiliations. It is readily apparent to those skilled in the art that within nature, various modifications and variations occur to any given organism and that the description described herein may be altered to account for any modifications or variations.

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 B-30641.

The subject culture has been deposited under conditions that assure that access to the cultures will be available during the pendency of this patent application to one determined by the Commissioner of Patents and Trademarks to be entitled thereto under 37 C.F.R. 1.14 and 35 U.S.C. 122. The deposits are available as required by foreign patent laws in countries wherein counterparts of the subject application, or its progeny, are filed. However, it should be understood that the availability of the deposits does not constitute a license to practice the subject invention in derogation of patent rights granted by governmental action.

Further, the subject culture deposits will be stored and made available to the public in accord with the provisions of the Budapest Treaty for the Deposit of Microorganisms i.e., they will be stored with all the care necessary to keep them viable and uncontaminated for a period of at least five years after the most recent request for the furnishing of a sample of a deposit, and in any case, for a period of at least thirty (30) years after the date of deposit or for the enforceable life of any patent which may issue disclosing the cultures. The depositor acknowledges the duty to replace the deposit(s) should the depository be unable to furnish a sample when requested due to the condition of the deposit(s). All restrictions on the availability to the public of the subject culture deposits will be irrevocably removed upon the granting of a patent disclosing them.
(TEM) were utilized to examine surface details and cross sections, respectively, according to established methods.

(v) Characterization of Spores for Various Physical and Chemical Conditions.

As a non-limiting example of spore characterization, radiation dosimetry at the Co60 source was performed using an ion chamber with accuracy to the USA Bureau of Standards. All irradiations were carried out in glass vials using spore samples in water. Spores (10⁸ spores ml⁻¹) were exposed to both 1 Mega rad (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 gamma radiation-treated samples on TSA at 32°C.

Purified spores (10⁹ spores ml⁻¹) were diluted in Phosphate Buffered Saline (PBS) (pH 7.2), placed in an uncovered Petri dish and exposed to UV radiation (254 nm). 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 H₂O₂ protocol, developed by Riesemman & 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 (10³ 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. A liquid H₂O₂ protocol, developed by Riesemman & 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.

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Bacillus badius.

pared 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 showed an identification match for cutoff value required to place strains within the Same spe-

rides, but preferred pyruvate, amino acids, purine Or PYri- DNA-DNA hybridization, but -97% 16s rDNA sequence

sphaericus prolonged incubation (>3 days), arabinose was assimilated; of at least two determinations between the two

denitrification or acetoin production. 34hs-1 did not ferment 4o formed between 34hs-1 and round-spore-forming

The branching order of the phylogenetic tree shown in FIG. 8, 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.

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-3507T; Strain 3 is B. sphaericus DSM 28T; Strain 4 is B. pycnus NRRL NRS-1691TT; 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, orminthine 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; however, this is not a discriminatory phenotypic trait. Hydrogen sulfide was not produced from thioglycollate. The carbon substrate utilization profile of 34hs-1, as measured by the Biolog system, showed an identification match for Bacillus badius. Furthermore, most of the Biolog-generated phenotypic characteristics were similar to those of both B. sphaericus and B. fusformis shown in FIG. 7. Strain 34hs-1 did not metabolize common hexoses, pentoses or disaccharides, but preferred pyruvate, amino acids, purine or pyrimidine bases and related compounds as carbon and energy sources. Most round spored Bacillus species, including strain 34hs-1, are not able to grow in the absence of oxygen.

(v) Phenotypic Characterization.

The 16S rDNA sequences of all known Firmicutes were compared with that of 34hs-1. All phylogenetic analyses, based on 16S rDNA sequences, unambiguously demonstrated that 34hs-1 belonged to the low-G+C-containing Gram-positive bacteria. The 16S rDNA sequences of all known members of the Gram-positive bacteria were compared with that of 34hs-1. Bootstrapping (500 replicates) analysis was performed to avoid sampling artifacts. The resulting analyses indicated that 34hs-1 shares a close phylogenetic relationship with Bacillus species belonging to rRNA group 2. Neighbor-joining, parsimony and maximum-likelihood analyses were undertaken on this subset of bac- teria, using several subdomains of the 16S rDNA. In all analyses, strain 34hs-1 was most closely related to members of the genus Bacillus.

Similarities in 16S rDNA sequence between 34hs-1 and closely related Bacillus species, recognized by GenBank BLAST searches, were 95–96%. GenBank is a nucleotide sequence database maintained by the National Center for Biotechnology Information, located at 8600 Rockville Pike, Bethesda, Md. 20894. Sequence variation of ~3.5% was found between 34hs-1 and B. fusformis ATCC 7055T and B. sphaericus DSM 28T. A very high sequence variation (8%) was observed between 34hs-1 and B. subtilis ATCC 6633. Such a high degree of dissimilarity within a well-described genus is not uncommon. Likewise, B. badius, the strain most phenotypically similar to 34hs-1, was only 91.5% similar in 16S rDNA sequence.

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. 8, 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-23336T 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.seyi. L. n. Odyssey the Odys- sey; 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 H₂S from thiosulfite and is not involved in denitrification. The type strain, strain 34hs-1 (ATCC PTA-4993<sup>T</sup>=NRRL B-30641=NBRC 100172<sup>T</sup>), 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.