**United States Patent**

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**Abstract:**

The present invention relates to discovery and isolation of a biologically pure culture of a *Bacillus pumilus* SAFR-032 isolate with UV sterilization resistant properties. This novel strain 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*. The GenBank accession number for the 16S rDNA sequence of the *Bacillus pumilus* SAFR-032 isolate is AY167879.

1 Claim, 4 Drawing Sheets
OTHER PUBLICATIONS


Ryzhov, V., et al., 2000, “Rapid characterization of spores of *Bacillus anthracis* sp. nov., a round-spore-forming *Bacillus* isolated from an ultraclean spacecraft assembly facility. Microbial Ecology 47:159-163.


Setlow, B., and P. Setlow. 1995. Binding to DNA protects alpha/beta-type, small, acid-soluble spore proteins of *Bacillus* and Clostridium species against digestion by their specific proteases as well as by other proteases. J Bacteriol 177:4149-51.


Figure 1. Dose response curve for 3 *Bacillus* species.
Figure 2. Resistance of 3 strains of *Bacillus* to UV radiation at the Mars solar constant.
Figure 3. Protection of *B. subtilis* by SAFR-32 from full spectrum irradiation at the Mars solar constant.
Scanning electron micrograph of *B. pumilus* SAFR-032 spores

Light microscope photograph of *B. pumilus* SAFR-032 cells

Transmission electron micrograph of a *B. pumilus* SAFR-032 spore
The present invention relates to an isolated biologically pure culture of a novel spore forming Bacillus species, and more particularly, to a Bacillus pumilus SAFR-032 isolate with high 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 for highly resistant bacterial isolates to study further to create 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.

1. **BACILLUS PUMILUS SAFR-032 ISOLATE**

PRIORITY CLAIM

This application is a non-provisional application, claiming the benefit of priority to provisional application No. 60/568,740, filed in the United States on May 6, 2004, entitled “UV Resistant Bacillus pumilus SAFR-032.”

GOVERNMENT RIGHTS

The invention described herein was made in the performance of work under a NASA contract, and is subject to the provisions of Public Law 96-517 (35 U.S.C. 202) in which the Contractor has elected to retain title.

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 pumilus SAFR-032 isolate with high 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 an Ultra Violet-ray retardant spray.

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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 pumilus SAFR-032 isolate with high sterilization resistant properties, having a GenBank accession number of AY167879.

2. 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 chart illustrating UV does response curves for three Bacillus species;

FIG. 2 is chart illustrating relative resistance of three strains of Bacillus to UV radiation at the Mars solar constant;

FIG. 3 is a chart illustrating the protection of B. subtilis by SAFR-032 from full spectrum irradiation at the Mars solar constant;

FIG. 4 is scanning electron microscopy image of Bacillus pumilus SAFR-032 spores;

FIG. 5 is a light microscopy image of Bacillus pumilus SAFR-032 spores;

FIG. 6 is a transmission electron microscopy image of Bacillus pumilus SAFR-032 spores;

Appendix A is an article co-authored by an inventor of the present invention, discussing Bacillus pumilus SAFR-032, entitled, “survival of spacecraft-associated microorganisms under simulated Martian UV irradiation;”

Appendix B is an article co-authored by an inventor of the present invention, discussing the identification and classification of Bacillus pumilus spores, entitled, “MALDI-TOFMS compared with other polyphasic taxonomy approaches for the identification and classification of Bacillus pumilus spores;” and

Appendix C is an article co-authored by an inventor of the present invention, discussing the UV resistance of Bacillus pumilus isolates, entitled, “extreme spore UV resistance of Bacillus pumilus isolates obtained from an ultraclean spacecraft assembly facility.”

DETAILED DESCRIPTION

The present invention relates to an isolated biologically pure culture of a novel spore forming Bacillus species, and more particularly, to a Bacillus pumilus SAFR-032 isolate with high UV and sterilization resistant properties.

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.
that can be used to infer natural relationships between

The most widely used informational macromolecule for bacterial

information. Each GenBank entry includes a concise description

that access to the cultures will be available during the vehicles. The question is whether forward contamination

pendency of this patent application to one determined by the 60 will be significantly increased by the current approach to

various modifications and variations occur to any given Protection protocols for the non-life detection Mars landing

organisms, and that the description described herein may be

altered to account for any modifications or variations. require that the rovers be heat-sterilized prior to launch.

The strain disclosed in this description will be deposited

instead of the Budapest Treaty for the Deposit of Microorganisms Le.,

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).

Several surveys on the microbial diversity of spacecraft

assembly facilities over a period of 3 years have lead to the

repeated isolation of Bacillus pumilus strains. Of these

strains tested, B. pumilus SAFR-032 spores were the most

resistant to UV irradiation (254 nm; FIG. 1) and the total flux

at the Mars simulated solar constant. Spores of B. pumilus

SAFR-032 showed highest resistance to all three UV band-

widths. LD90 of B. pumilus SAFR-032 under Mars solar UV

simulated solar constant was >360 sec, about 10 times greater than B. subtilis 168 (FIG. 2). B. pumilus SAFR-032 spores

are 2 to 3 times and ten times more resistant than a

previously patented "hardy" B. odyseyi spores to UV 254

nm and Mars UV simulated solar constant, respectively. B. pumilus is more resistant than any bacterium reported in the

literature to date.

It follows that standard UV treatments which are effective

against B. subtilis spores may not be sufficient to inactivate

all spores such as SAFR-032. Hence the spores of B. subtilis
can not be reliably used as a biodosimetry model for the UV

inactivation of spores. SAFR-032 spores also exhibited resistance to H202. Upon exposure to 5% liquid H202

SAFR-32 spores experienced a 2 log decrease in population

compared to 4 log reduction exhibited by B. subtilis 168 spores. In addition, B. pumilus SAFR-032 spores were resistant to 0.5 Mrad gamma-radiation (25 rad/sec).

The goal of planetary protection as stated in NASA policy

is the prevention of forward and backward contamination. This policy applies directly to the control of terrestrial organisms contaminating spacecraft intended to land, orbit, flyby or be in the vicinity of extraterrestrial bodies. Planetary Protocols for the non-life detection Mars landing

missions such as, Mars Exploration Rovers (MER), did not require that the rovers be heat-sterilized prior to launch. Instead, NASA relied on a series of sequential sterilization steps using alcohol to maintain the cleanliness of the MER vehicles. The question is whether forward contamination will be significantly increased by the current approach to spacecraft sanitation such as used for the MARS landers.

Sporoform Bacillus subtilis have been shown to survive up to

6 years under interstellar space conditions. However, only

shielding from UV radiation enabled B. subtilis endospores to

survive the conditions long term. The solar flux at the Martian surface is considerably less than interstellar space and there is the potential that atmospheric conditions could
Microbes to the Martian conditions. Previous studies have suggested that the organisms associated with the facilities where assembly and encapsulation activities take place will indicate likely contamination of the spacecraft. Studies that follow existing planetary protection microbial isolation procedures that involve a heat-shock step have shown spore-formers to be the most common type of microbes isolated from surfaces of various spacecraft. Since most of the published information was based on the laboratory strains, predicting the actual survival and possible adaptation of terrestrial life on Mars is limited due to the lack of robust empirical data on the survival of indigenous spacecraft microbes to the Martian UV conditions. Previous studies have used model dosimetric strains to represent the potential survival of organisms under ~200 J/m² UV (14) and Mars solar UV irradiation conditions. The present invention produces data that indicates spores of *B. pumilus* SAFR-032 are far more resistant to Mars solar UV irradiation conditions than these model dosimetric strains. It may be necessary to consider these resistant organisms when investigating the survival of microorganisms under outer space or the Martian conditions.

The search for life on other planets will involve ultra-sensitive technologies that detect cells and biomarkers. Contamination of extraterrestrial bodies with cells or biomarkers originating from Earth (forward contamination) would seriously compromise the interpretation that life signatures. Recent data indicate the routine meteorite exchange between Earth and Mars and living microbes, particularly bacterial spores, may survive interplanetary transfer. Consequently, current planetary protection protocols require that spacecraft be constructed and assembled under conditions as nearly as possible approaching sterility. To achieve these conditions, robotic spacecraft are assembled in clean rooms where air circulation is controlled and strict hygienic practices are implemented to minimize microbial contamination. In addition a number of sterilants including vaporized hydrogen peroxide (H₂O₂) and ultraviolet radiation (UV) are under consideration. As part of the NASA planetary protection program, recent monitoring of microbial diversity in the relatively extreme environment (low nutrient, controlled humidity, periodic disinfection) NASA JPL-SAF resulted in the isolation of a number of microbial species inhabiting various parts of the facility. The predominant strains of spore-forming bacteria identified by biochemical testing and 16S rDNA analysis as being most closely related to *Bacillus pumilus*. Not only were *B. pumilus* spores found to survive in the JPL-SAF, but were also recently recovered from hardware surfaces and air particles aboard the International Space Station (ISS). It follows that spores of *B. pumilus* are capable of escaping current spacecraft disinfection regimens and may be inadvertently transported into space.

A key element of spore resistance is a multilayered protein shell that encases the spore called the spore coat. 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. It has only recently been shown that SAFR-032 can be mixed with UV susceptible species such as *B. subtilis* 168, allowing the susceptible species to survive longer (FIG. 3). These results make a strong argument that the physical makeup of SAFR-032 rather than biochemical reactions is responsible for its heightened resistance.

A goal associated with the present invention is to produce new strategies for inactivating resistant organisms like SAFR-032. Identifying the particular component of the spore that allows this heightened resistance can guide the development of sterilization procedures that are targeted to the specific molecules responsible for resistance, and avoid using unduly harsh methods that jeopardize equipment. An important specific long-term goal is an improved sterilization procedure that will enable NASA to meet planetary protection requirements without a terminal heat sterilization step. This would support implementation of planetary protection policies for life detection missions.

Typically hospitals and government agencies use biological indicators to ensure the quality control of sterilization processes (http://www.ravenlabs.com/bis.html). The spores of SAFR-032 that are more resistant to several sterilization procedures would serve as a better biological indicator than those in use currently.

As such, the present invention comprises an isolated biologically pure culture of *Bacillus pumilus* SAFR-032, under GenBank accession number AY167879. For further illustration, FIGS. 4, 5, and 6 illustrate micrograph images of the *Bacillus pumilus* SAFR-032 spores.

For a further description of *B. pumilus* SAFR-032, including both a phenotypic and genotypic description, see Appendices A, B, and C. Appendices A, B, and C are incorporated herein as though set forth fully herein. As can be appreciated by one in the art, the Appendices provide an enabling description of the isolation and classification of *B. pumilus* SAFR-032.

What is claimed is:

1. An isolated biologically pure culture of *Bacillus pumilus* SAFR-032 deposited under ATCC accession number PTA-7603.

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