International Journal of Systematic and Evolutionary Microbiology (2004), **54**, 195–201 **DOI** 10.1099/ijs.0.02747-0

Bacillus odysseyi sp. nov., a round-spore-forming bacillus isolated from the Mars Odyssey spacecraft

Myron T. La Duc,[1] Masataka Satomi[2] and Kasthuri Venkateswaran[1]

[1]Biotechnology and Planetary Protection Group, Jet Propulsion Laboratory, California Institute of Technology, Pasadena, CA 91109, USA

[2]National Research Institute of Fisheries Science, Food Processing Division, Kanazawa-ku, Yokohama-City, Kanagawa 236-8648, Japan

Correspondence
Myron T. La Duc
mtladuc@jpl.nasa.gov

A round-spore-forming *Bacillus* species that produces an exosporium was isolated from the surface of the Mars Odyssey spacecraft. 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 is a Gram-positive, aerobic, rod-shaped, endospore-forming eubacterium. Ultrathin sections of the spores showed the presence of an exosporium, spore coat, cortex and core. 16S rDNA sequence similarities between this strain, *Bacillus fusiformis* and *Bacillus silvestris* were ~96 % and DNA–DNA reassociation values with these two bacilli were 23 and 17 %, respectively. Spores of the novel species were resistant to desiccation, H_2O_2 and UV and gamma radiation. Of all strains tested, the spores of this strain were the most consistently resistant and survived all of the challenges posed, i.e. exposure to conditions of desiccation (100 % survival), H_2O_2 (26 % survival), UV radiation (10 % survival at 660 J m^{-2}) and gamma radiation (0·4 % survival). The name proposed for this novel bacterium is *Bacillus odysseyi* sp. nov.; the type strain is 34hs-1^T (=ATCC PTA-4993^T=NRRL B-30641^T=NBRC 100172^T).

INTRODUCTION

Several physiologically and phylogenetically distinct micro-organisms have been encountered while examining microbial contamination of spacecraft surfaces (Venkateswaran *et al.*, 2001). 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.

Round-spore-forming *Bacillus* species were first described by Chester (1898). The presently recognized round-spore-forming species of *Bacillus*, *Marinibacillus* and *Sporosarcina* are *Bacillus fusiformis*, *Bacillus insolitus*, *Bacillus neidei*, *Bacillus pycnus*, *Bacillus sphaericus*, *Bacillus silvestris* and *Bacillus thermosphaericus* (Priest *et al.*, 1988; Rheims *et al.*, 1999; Nakamura, 2000; Nakamura *et al.*, 2002), *Marinibacillus marinus* (Rüger *et al.*, 2000) and *Sporosarcina globispora*, *Sporosarcina psychrophila* and *Sporosarcina pasteurii* (Yoon *et al.*, 2001). All of these round-spore-forming species fall into 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. It has been confirmed that the spheroid appearance arises from exosporia in spores of *B. sphaericus* (Neide, 1904), *B. fusiformis* (Priest *et al.*, 1988; Priest, 1993), *B. silvestris* (Rheims *et al.*, 1999) and a novel strain, 34hs-1^T, isolated from the surface of the Mars Odyssey spacecraft. Although exosporia are present in spores of *Bacillus anthracis*, *Bacillus cereus*, *Bacillus mycoides*, *Bacillus pseudomycoides*, *Bacillus thuringiensis* and *Bacillus weihenstephanensis*, spores of these species are not round (data not shown). A loosely attached extraneous layer resembling an exosporium was recently described in the ovoid-spore-forming *Bacillus nealsonii*, isolated from airborne particulates in a spacecraft assembly facility (Venkateswaran *et al.*, 2003).

Here, we describe *Bacillus odysseyi* sp. nov., isolated from the surface of the Mars Odyssey spacecraft, whose round spores are resistant to UV and gamma radiation, H_2O_2 and desiccation. The *Bacillus* strain isolated in this study was characterized based on a polyphasic taxonomic approach that examined its phenotypic and genotypic affiliations.

METHODS

Sample preparation and isolation of microbes from the Mars Odyssey spacecraft. Components of the Mars Odyssey spacecraft were manufactured in various geographical locations. The spacecraft underwent several months of assembly in the Spacecraft Assembly Facility at the Jet Propulsion Laboratory, Pasadena, CA,

The GenBank accession number for the 16S rDNA sequence of strain 34hs-1^T is AF526913.

USA, before it was transported to the Kennedy Space Center, FL, USA. Samples were taken from 25 different surface areas (25 cm^2) of the spacecraft using sterile, water-moistened polyester swabs (Texwipe) 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 (final volume 10 ml). The 25 samples were pooled into one sterile container and processed immediately. All samples were analysed for both spore-formers and total cultivable heterotrophs.

Microbial examination. Samples were sonicated for 2 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; Difco) as the growth medium (32 °C for 2 days) (Anonymous, 1980). Samples that were not heat-shocked were enumerated for total aerobic cultivable heterotrophs on TSA. 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 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.

Sporulation. A nutrient sporulation medium (NSM) was used to produce spores (Schaeffer *et al.*, 1965; Nicholson & Setlow, 1990). 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 (Nicholson & Setlow, 1990). These chemical treatments did not remove the exosporium surrounding the spore coat. Purified spores were resuspended in sterile deionized water, heat-shocked (80 °C for 15 min) to ensure inactivation of the vegetative population 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). Non-destructive examination of spores and vegetative cells was also exploited using a field-emission environmental SEM (Phillips XL30). In addition, standard SEM and TEM were utilized to examine surface details and cross-sections, respectively, according to established methods (Cole & Popkin, 1981).

Characterization of spores for various physical and chemical conditions. Radiation dosimetry at the Co^{60} source was performed using an ion chamber with accuracy to the USA Bureau of Standards (Coss, 1999). All irradiations were carried out in glass vials using spore samples in water. Spores (10^8 spores ml^{-1}) were exposed to both 1 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 (10^6 spores ml^{-1}) were diluted in PBS (pH 7·2), placed in an uncovered Petri dish and exposed to UV radiation (254 nm; UV Products). 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 H_2O_2 protocol, developed by Riesenman & Nicholson (2000), was modified and used to examine H_2O_2 resistance in spores. Known concentrations of spore suspensions prepared in PBS (10^8 spores ml^{-1}) were treated with H_2O_2 (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; Difco) to check viability and spread onto TSA for quantitative measurement of H_2O_2-resistant spores.

To test desiccation resistance, spore suspensions (20 µl) were dispensed onto pre-sterilized metal and glass-fibre discs (10^3 spores per disc; Millipore). 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 water bath (Branson Ultrasonics) 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.

Systematic characterization

(i) Phenotypic characterization. The ability to grow in NaCl concentrations of 1–10 % was determined in T_1N_1 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 (Colwell & Grimes, 2000). The commercially available Biolog identification system was used, according to the manufacturer's specifications, to characterize utilization of various carbon substrates. In addition, API NE test strips (bioMérieux) were used to characterize the strain further.

(ii) 16S rDNA sequencing. Approximately 10 ng purified DNA (Johnson, 1981) from liquid cultures was used as a template for PCR amplification. Universal primers (Bact 11 and 1492) were used to amplify the 1·5 kb PCR fragment according to protocols established by Ruimy *et al.* (1994). Purified amplicons were sequenced (MWG Biotech) and the identity of a given PCR product was verified by bi-directional sequencing analysis. The phylogenetic relationships of organisms covered in this study were determined by comparison of individual 16S rDNA sequences to existing sequences in public databases (http://www.ncbi.nlm.nih.gov/). Evolutionary trees based on parsimony and maximum-likelihood analyses were constructed with PAUP software (Swofford, 1990).

(iii) DNA–DNA hybridization. Cells were suspended in 0·1 M EDTA (pH 8·0) and cell walls were digested by lysozyme treatment (final concentration, 2 mg ml^{-1}). DNA was isolated by standard procedures (Johnson, 1981). DNA–DNA relatedness was studied by microplate hybridization methods (Ezaki *et al.*, 1989) with photobiotin labelling and colorimetric detection, using 1,2-phenylenediamine (Sigma) as the substrate and streptavidin–peroxidase conjugate (Boehringer Mannheim) as the colorimetric enzyme (Satomi *et al.*, 1997).

RESULTS AND DISCUSSION

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 cm^2, respectively. Isolates were identified

by 16S rDNA sequence analysis as species of *Acinetobacter*, *Bacillus*, *Curtobacterium*, *Delftia*, *Microbacterium* and *Ralstonia* (La Duc *et al.*, 2003). 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, H_2O_2 and desiccation, several spore-forming isolates showed resistance (La Duc *et al.*, 2003). Of the 45 strains identified, one strain, designated 34hs-1^T, exhibited distinct spore morphology and was characterized further for its phylogenetic affiliation.

Morphological and physiological characteristics

Strain 34hs-1^T is a Gram-positive, aerobic, rod-shaped, spore-forming bacterium. Cells are 4–5 μm long, 1 μm in diameter and motile. On TSA medium incubated at 32 °C, young colonies are beige, round, $\sim$3 mm in diameter, fairly smooth and flat with entire edges. Endospores of strain 34hs-1^T (1 μm in diameter) are terminal (Fig. 1a), round (Fig. 1b), with one spore per cell and swell the mother cell (Fig. 1a). Ultrathin sections of spores of strain 34hs-1^T showed the presence of an exosporium, spore coat, cortex and core (Fig. 1c). Microscopic analyses revealed the partial destruction of 34hs-1^T spores by gamma radiation, although remnants of exosporia were left behind (Fig. 1d); some spores oxidized by H_2O_2 formed 'doughnut-like' structures (Fig. 1e). Further analysis showed highly electron-dense structures in the exosporia of gamma-irradiated and H_2O_2-treated (Fig. 1f) spores when compared with the untreated control (Fig. 1c). The characterization and physiological role of the exosporium of strain 34hs-1^T spores is not discussed here.

Resistance of spores of strain 34hs-1^T to various physical and chemical conditions

The resistance of *Bacillus* spores to a variety of conditions is well documented, as seen in our control experiments (data not shown) and in other studies (for a review see Nicholson *et al.*, 2000). Spores of 34hs-1^T exhibited resistance to UV (254 nm), gamma radiation, 5 % liquid H_2O_2 and desiccation conditions. Spores of 34hs-1^T did not exhibit classic UV_{254} 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^T exhibited an LD_{90} (90 % lethal dose) of $\sim$660 J m^{-2}. Spores of 34hs-1^T also survived 0·5 Mrad gamma radiation (0·4 % survival). Purified spores exposed to 5 % liquid H_2O_2 showed resistance, with nearly 26 % of the initial inoculum ($1{\cdot}1 \times 10^7$ ml^{-1}) viable after 60 min exposure. Finally, desiccation had no effect on viability of the 34hs-1^T spores. When compared with the model organism, *Bacillus subtilis* strain 168, spores of strain 34hs-1^T appeared to be quite resistant, respectively exhibiting 3, 10, 6 and 10 times greater survival of UV (Nicholson *et al.*, 2000), gamma radiation (Venkateswaran *et al.*, 2003), H_2O_2 (unpublished data) and desiccation (data not shown). It is apparent that micro-organisms, now shown to withstand appreciable doses of several sterilants, are present on the surface of a spacecraft being sent to Mars, a pristine extraterrestrial system. Perhaps even more intriguing than their ability to withstand decontamination strategies imposed by mankind is the possibility of such resistances allowing them to survive the highly oxidative UV and gamma radiation-rich environments that they will encounter en route to and on the surface of Mars. This presents a large problem to those concerned with planetary protection, i.e. ensuring appropriate levels of spacecraft cleanliness in order to avoid (i) compromising the integrity of *in situ* and/or sample-return missions and (ii) contamination of pristine extraterrestrial environments with Earth-derived biomatter.

Phenotypic characterization

Strain 34hs-1^T 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^T is presented in Table 1. This strain produced catalase, but not cytochrome oxidase, gelatinase, urease, tryptophan deaminase, lysine or ornithine decarboxylase or arginine dihydrolase. It did not show denitrification or acetoin production. Strain 34hs-1^T did not ferment glucose nor 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 thiosulfite. The carbon substrate utilization profile of 34hs-1^T, 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. fusiformis* (Table 1). Strain 34hs-1^T 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^T, are not able to grow in the absence of oxygen.

Phylogenetic characterization

The 16S rDNA sequences of all known *Firmicutes* were compared with that of 34hs-1^T. All phylogenetic analyses, based on 16S rDNA sequences, unambiguously demonstrated that 34hs-1^T 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^T. 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 belonging to rRNA group 2. Neighbour-joining, parsimony and maximum-likelihood analyses were undertaken on this subset of bacteria, using several subdomains of the 16S rDNA. In all analyses, strain 34hs-1^T was most closely related to members of the genus *Bacillus*.

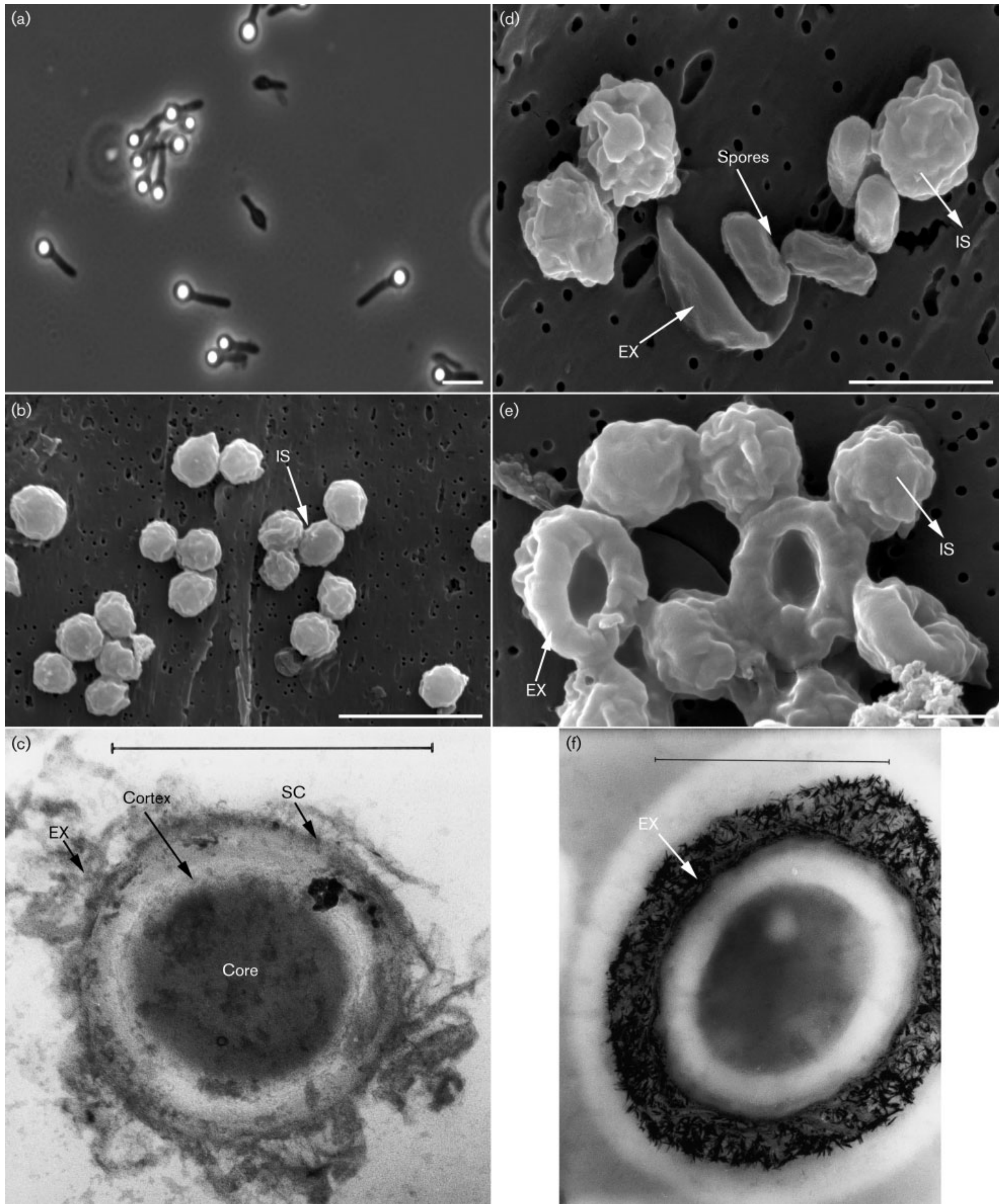

Fig. 1. Microscopic images of *Bacillus odysseyi* sp. nov. 34hs-1T. (a) Light microscopy image of sporulating vegetative cells showing terminal swelling of mother cells; bar, 5 μm. (b)–(f) Field-emission environmental SEM [b (bar, 5 μm); d (bar, 2 μm); e (bar, 1 μm)] and TEM (c, f; bars, 1 μm) micrographs of spores showing the spore surface and cross-section characteristics, respectively. (b, c) Purified spores showing intact round structure; IS, intact spore with exosporium. In (c), the exosporium (EX), spore coat (SC), cortex and spore core are shown in longitudinal section. Spores ($1{\cdot}1\times10^7$) were exposed to 0·5 Mrad gamma radiation (d) and 5 % liquid H_2O_2 for 60 min (e, f). The exosporium removed from the spore due to gamma radiation and H_2O_2 treatment is shown in (d) and (e). An electron-dense region of a H_2O_2-treated spore is shown in (f).

Table 1. Characteristics useful for differentiating *B. odysseyi* 34-hs1^T from related species

Strains: 1, *B. odysseyi* 34hs-1^T; 2, *B. fusiformis* NRRL NRS-350^T; 3, *B. sphaericus* DSM 28^T; 4, *B. pycnus* NRRL NRS-1691^T; 5, *B. neidei* NRRL BD-87^T; 6, *B. badius* ATCC 14574^T. NA, Not applicable; ND, not determined.

Characteristic	1	2	3	4	5	6
Substrate oxidized:						
Acetate	+	+	+	ND	ND	−
Pyruvate	+	+	+	+	−	+
α-Hydroxybutyrate	+	−	−	ND	ND	−
β-Hydroxybutyrate	+	−	−	+	−	+
Methyl pyruvate	+	+	+	ND	ND	−
L-Alanine	+	+	+	−	−	+
Glycyl L-glutamate	+	+	+	−	+	+
Adenosine	−	+	+	ND	+	+
2′-Deoxyadenosine	+	+	+	−	+	+
Inosine	+	+	+	−	+	+
AMP	+	+	+	−	+	−
UMP	+	+	+	−	+	−
Growth in 5 % NaCl	+	+	+	−	+	ND
16S rDNA sequence similarity (%)*	NA	96·4	95·7	93·1	92·4	91·5

*Similarity to 16S rDNA sequence of *B. odysseyi* 34hs-1^T.

Similarities in 16S rDNA sequence between 34hs-1^T and closely related *Bacillus* species, recognized by GenBank BLAST searches, were 95–96 %. Sequence variation of ~3·5 % was found between 34hs-1^T and *B. fusiformis* ATCC 7055^T and *B. sphaericus* DSM 28^T. A very high sequence variation (8 %) was observed between 34hs-1^T 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^T, 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. 2. The branching order of this tree 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^T. The round-spore-forming *Bacillus* group was very tightly bound phylogenetically; all members of this clade shared sequence similarities of >95 %. Strain 34hs-1^T exhibited the characteristics necessary to place it in *Bacillus* rRNA group 2. To differentiate these closely related species more accurately, DNA–DNA hybridization was performed.

DNA–DNA hybridization

DNA–DNA hybridization was performed between 34hs-1^T and round-spore-forming *Bacillus* and *Sporosarcina* species

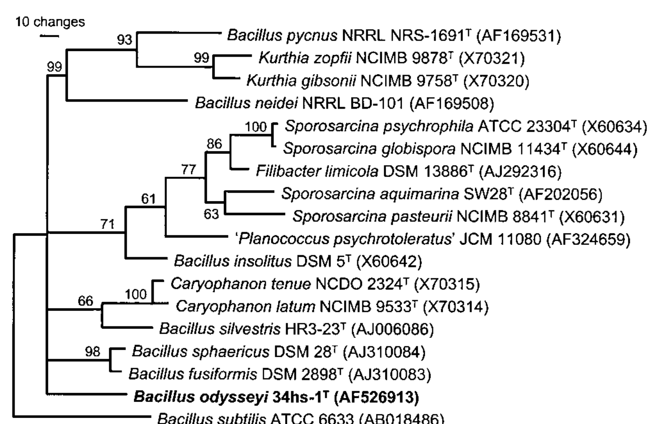

Fig. 2. Phylogenetic tree of round-spore-forming *Bacillus* and other species closely related to strain 34hs-1^T based on maximum-parsimony analysis of 16S rDNA nucleotide sequences. Strain numbers and GenBank accession numbers are shown. Numbers above lines are percentage bootstrap values of 500 replications of that branch of the tree. Bar, 10 changes.

(Table 2). None of the *Bacillus* species that showed very high 16S rDNA sequence similarities (~96 %) exhibited >70 % DNA–DNA reassociation values with 34hs-1^T, i.e. the cut-off value required to place strains within the same species (Wayne *et al.*, 1987). In particular, the hybridization value between 34hs-1^T and *B. silvestris* NRRL B-23336^T was only 17 %, whereas their 16S rDNA sequences were 96·4 % similar. Also, strain 34hs-1^T and *B. sphaericus* NRRL BD-113 showed 17 % DNA–DNA hybridization, but ~97 % 16S rDNA sequence similarity. Based on DNA–DNA reassociation values, strain 34hs-1^T represents a novel *Bacillus* species.

Table 2. DNA–DNA hybridization between *B. odysseyi* sp. nov. 34hs-1^T and related species

Values are means of at least two determinations; ND, not determined.

Strain	Similarity (%) to labelled DNA from:			
	1	2	3	7
1. *B. odysseyi* 34hs-1^T	100	18	17	17
2. *B. fusiformis* ATCC 7055^T	23	100	17	15
3. *B. silvestris* NRRL B-23336^T	17	18	100	15
4. *B. pycnus* NRRL NRS-1691^T	11	ND	ND	11
5. '*B. aminovorans*' NRRL NRS-341	10	ND	ND	16
6. *B. neidei* NRRL BD-101	7	ND	ND	15
7. *B. sphaericus* NRRL BD-113	7	ND	ND	100
8. *Sporosarcina aquimarina* SAFN-008	7	ND	ND	8

Description of *Bacillus odysseyi* sp. nov.

Bacillus odysseyi (o.dys.se′yi. L. n. *Odyssea* the Odyssey; N.L. gen. n. *odysseyi* pertaining to the Mars Odyssey spacecraft, from which the organism was isolated).

Cells are rod-shaped, 4–5 μm in length, 1 μm in diameter and motile. Gram-positive and aerobic. Forms terminal endospores. Spores show an additional exosporium layer. Colonies on TSA are round, smooth, flat with entire edges and beige in colour. Sodium ions are not essential for growth; growth occurs in 0–5 % NaCl. 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. Prefers pyruvate, amino acids, purine or pyrimidine bases and related compounds as carbon and energy sources. Catalase-positive, but does not produce gelatinase, arginine dihydrolase, lysine or ornithine decarboxylase, lipase, amylase or alginase. Does not produce H_2S from thiosulfite and is not involved in denitrification.

The type strain, strain 34hs-1^T (=ATCC PTA-4993^T= NRRL B-30641^T=NBRC 100172^T), was isolated from the surface of the Mars Odyssey spacecraft.

ACKNOWLEDGEMENTS

We thank M. Lubarsky and W. Nicholson for technical assistance and K. Buxbaum, R. Kern and T. Luchik for valuable advice and encouragement. We acknowledge J. Edens, P. Koen and J. Kulleck for assistance in performing the electron microscopy and M. Wiedeman for gamma radiation analyses. We are grateful for various USDA strains as a gift from A. Rooney. The research described in this publication was carried out at the Jet Propulsion Laboratory, California Institute of Technology, under a contract with the National Aeronautics and Space Administration.

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