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Bacillus safensis sp. nov., isolated from spacecraft and assembly-facility surfaces

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Thirteen strains of a novel spore-forming, Gram-positive, mesophilic heterotrophic bacterium were isolated from spacecraft surfaces (Mars Odyssey Orbiter) and assembly-facility surfaces at the Jet Propulsion Laboratory in California and the Kennedy Space Center in Florida. Phylogenetic analysis of 16S rRNA gene sequences has placed these novel isolates within the genus *Bacillus*, the greatest sequence similarity (99·9 %) being found with *Bacillus pumilus*. However, these isolates share a mere 91·2 % *gyrB* sequence similarity with *Bacillus pumilus*, rendering their 16S rRNA gene-derived relatedness suspect. Furthermore, DNA–DNA hybridization showed only 54–66 % DNA relatedness between the novel isolates and strains of *B. pumilus*. rep-PCR fingerprinting and previously reported matrix-assisted laser desorption/ionization time-of-flight mass spectrometry protein profiling clearly distinguished these isolates from *B. pumilus*. Phenotypic analyses also showed some differentiation between the two genotypic groups, although the fatty acid compositions were almost identical. The polyphasic taxonomic studies revealed distinct clustering of the tested strains into two distinct species. On the basis of phenotypic characteristics and the results of phylogenetic analyses of 16S rRNA and *gyrB* gene sequences, repetitive element primer-PCR fingerprinting and DNA–DNA hybridization, the 13 isolates represent a novel species of the genus *Bacillus*, for which the name *Bacillus safensis* sp. nov. is proposed. The type strain is FO-36b^T (=ATCC BAA-1126^T=NBRC 100820^T).

Spacecraft and associated clean-room assembly-facility surfaces harbour an extremely low biomass (La Duc *et al.*, 2003; Venkateswaran *et al.*, 2001), because of stringent maintenance. However, colonization by micro-organisms specifically adapted to such facility conditions, especially those yet to be cultured and/or characterized, is of major concern to those commissioning modern-day space-related experimentation. The search for extraterrestrial life will rely heavily on validated cleaning and bioreduction strategies to ensure that terrestrial microbial contamination does not compromise the scientific integrity of such missions. It is crucial both to minimize and eradicate such microbial contaminants and to identify and characterize the recurring, prevalent micro-organisms associated with the surfaces of spacecraft and associated environments.

Studies have repeatedly shown that extremely resilient, spore-forming members of the genus *Bacillus* are the most strongly represented micro-organisms in samples collected from spacecraft and facility surfaces (La Duc *et al.*, 2003; Puleo *et al.*, 1977). The extremely oligotrophic, low-humidity, temperature-controlled conditions of spacecraft-assembly facilities appear to select for micro-organisms able to withstand such unfavourable surroundings. During monitoring of the microbial diversity of spacecraft-associated environments over a period of 5 years (1999–2004), *Bacillus pumilus* was found to be the second most dominant species among the aerobic spore-forming bacteria (the predominant species being *Bacillus licheniformis*; La Duc *et al.*, 2004a). Several of these *B. pumilus* isolates have exhibited elevated resistance to H_2O_2 (Venkateswaran *et al.*, 2001; Kempf *et al.*, 2005) and are thus considered as 'problematic' micro-organisms, since H_2O_2 is recommended for the bioreduction of spacecraft components.

In previous studies using MALDI-TOF MS (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry)

Abbreviation: MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *gyrB* gene sequences referred to in this communication are given in Table 1.

A dendrogram based on rep-PCR fingerprinting and details of DNA–DNA hybridization among *Bacillus* strains isolated from the JPL spacecraft assembly facility are available as supplementary material in IJSEM Online.

to assess similarities amongst isolates, two very distinct and consistent groups of *B. pumilus* were revealed (Dickinson *et al.*, 2004). A detailed taxonomic characterization of this group of bacteria was deemed necessary to clarify the distribution of these species in spacecraft-assembly facilities. Here, we report the results of a study in which phenotypic characteristics and the results of phylogenetic analyses of 16S rRNA and *gyrB* gene sequences, repetitive element primer (rep)-PCR fingerprinting and DNA–DNA hybridizations for several novel isolates were compared.

The bacterial strains examined in this study are shown in Table 1. A total of 13 strains of this novel micro-organism were isolated from spacecraft and assembly-facility surfaces by standard swabbing procedures (Anonymous, 1980; La Duc *et al.*, 2004b) at various times and locations. Comparative analyses were performed with these newly described strains, four *B. pumilus* strains isolated from spacecraft-assembly facilities or International Space Station hardware and two strains purchased from the American Type Culture Collection (Manassas, VA, USA), including the *B. pumilus* type strain ATCC 7061^T. All strains were maintained in trypticase soy agar (TSA) stabs at room temperature for short-term analysis and in glycerol at −80 °C for long-term storage. Liquid cultures were grown in trypticase soy broth (TSB; Becton Dickinson) at 32 °C with vigorous aerobic shaking for an appropriate period of time.

Cellular morphology and motility were examined by phase-contrast microscopy. Gram staining was performed using the modified Hucker method (Smibert & Krieg, 1994). A nutrient sporulation medium rich in divalent cations was used to produce spores, as described previously (Schaeffer *et al.*, 1965; Nicholson & Setlow, 1990). The refractile nature of the spores was examined by using phase-contrast microscopy (Olympus BX-60). The optimum NaCl concentration for growth was determined in salt-free TSB supplemented with 0, 5·0, 7·5, 10·0, 12·5 and 15·0 % (w/v) NaCl, and cultures were monitored for growth for 3 weeks with incubation at 32 °C. Optimum and limiting temperatures were determined by growing cells at 4, 10, 37, 50 and 55 °C on TSA for 3 weeks. Routine biochemical tests were carried out using commercially available API kits [API 20NE, API 20E, API 50 CH (assimilation), API 50 CH (acid production) and API ZYM], which were utilized according to the instructions of the manufacturer (bioMérieux). Nutritional requirements for growth were assessed using Davis minimum medium (Becton Dickinson). Casein hydrolysis, starch hydrolysis and the production of lipase (hydrolysis of Tweens 20, 40 and 80) were tested as described by Smibert & Krieg (1994). Whole-cell fatty acid methyl ester profiles for the novel isolates and reference strains were determined using the MIDI system (Sasser, 1990). The DNA G+C content was determined by using the HPLC method of Tamaoka & Komagata (1984).

Table 1. Strains investigated in this study and their sources of isolation

JPL-SAF, Jet Propulsion Laboratory spacecraft-assembly facility; KSC, Kennedy Space Center.

Strain	GenBank accession number		Source, location and year of isolation
	16S rRNA gene	*gyrB* gene	
***Bacillus safensis* sp. nov.**			
FO-36b^T (=ATCC BAA-1126^T=NBRC 100820^T)	AF234854	AY167867	Clean-room air particulate, JPL-SAF, 1999
FO-33	AF234851	AY167868	Clean-room air particulate, JPL-SAF, 1999
SAFN-001 (=ATCC BAA-1128=NBRC 100821)	AY167886	AY167877	Entrance floor, JPL-SAF, 2001
SAFN-027	AY167884	AY167876	Anteroom, JPL-SAF, 2001
SAFN-036	AY167881	AY167873	Clean-room floor, JPL-SAF, 2001
SAFN-037	AY167880	AY167872	Clean-room floor, JPL-SAF, 2001
KL-052 (=ATCC BAA-1129=NBRC 100822)	AY030327	AY167878	Clean-room cabinet top, JPL-SAF, 2001
51-3C (=ATCC BAA-1127=NBRC 100823)	AF526907	–	Mars Odyssey spacecraft surface, 2002
81-4C	AF526903	–	Mars Odyssey assembly-facility floor, KSC, 2002
82-2C	AF526902	–	Mars Odyssey assembly-facility floor, KSC, 2002
84-1C	AF526898	–	Mars Odyssey assembly-facility floor, KSC, 2002
84-3C	AF526896	–	Mars Odyssey assembly-facility floor, KSC, 2002
84-4C	AF526895	–	Mars Odyssey assembly-facility floor, KSC, 2002
Bacillus pumilus			
ATCC 7061^T	AY876289	AY167869	Reference strain
ATCC 27142	AY876287	AY167870	Reference strain
SAFN-029	AY167883	AY167875	Clean-room airlock, JPL-SAF, 2001
SAFN-034	AY167882	AY167874	Clean-room airlock, JPL-SAF, 2001
SAFR-032	AY167879	AY167871	Clean-room airlock, JPL-SAF, 2001
0105342-2	AY876228	–	International Space Station hardware, 2001

The novel isolates were Gram-positive, spore-forming rods and were aerobic, motile and oxidase- and catalase-positive and so demonstrated several morphological and physiological characteristics typical of members of the genus *Bacillus* (Claus & Berkeley, 1986); this indicated that all of these isolates belonged to this genus or related genera. The Biolog identification system indicated that the novel isolates were most similar to *B. pumilus* (Dickinson *et al.*, 2004). Additionally, 174 phenotypic tests were performed to study the phenotype of the novel isolates. Two phenotypic characteristics, acid production from inositol and utilization of inositol, allowed the discrimination of the novel isolates from *B. pumilus* (Table 2). Additional phenotypic characteristics (production of acid phosphatase, Tween 80 hydrolysis, casein hydrolysis in litmus milk and utilization of raffinose) were also useful for separating these two bacterial groups, though responses to various substrates were variable among strains. Further details regarding physiological and phenotypic characteristics are discussed in the species description. The major fatty acids of the novel isolates were $C_{15:0}$ iso (50·4–56·7 %), $C_{15:0}$ anteiso (23·3–25·2 %), $C_{17:0}$ iso (4·52–6·93 %) and $C_{17:0}$ anteiso (3·71–4·69 %). All of the novel isolates and the five *B. pumilus* strains tested in this study had similar fatty acid methyl ester profiles. The DNA G+C contents of novel isolates FO-36b^T, KL052, SAFN001 and 51-3C were 41·0, 41·0, 41·4 and 41·2 mol%, respectively. The G+C contents of *B. pumilus* strains ATCC 7061^T and SAFR032 were 40·2 and 39·5 mol%, respectively.

For the phylogenetic and genetic analyses, genomic DNA was extracted using standard methods (Johnson, 1981; Sambrook *et al.*, 1989) from strains cultured in TSB supplemented with 2 % glycine. The 16S rRNA and *gyrB* genes were PCR-amplified with the universal primer sets described by Weisburg *et al.* (1991) and Yamamoto & Harayama (1995), respectively, and sequenced. The identity of a given PCR product was verified by bidirectional sequencing analysis. The phylogenetic relationships of the micro-organisms examined in this study were determined by comparing individual 16S rRNA or *gyrB* gene sequences with sequences in the public databases using the BLAST algorithm (Altschul *et al.*, 1990). The multiple alignment calculation of nucleotide substitution rates (K_{nuc} values), described by Kimura (1980), and the construction of phylogenetic trees by the neighbour-joining method (Saitou & Nei, 1987) were performed using the CLUSTAL W computer program (Thompson *et al.*, 1994). Alignment gaps, primer regions for PCR amplification and unidentified base positions were not taken into consideration in the calculations. The topological robustness of the phylogenetic trees was evaluated by a bootstrap analysis involving 1000 replications. The GenBank nucleotide accession numbers for the 16S rRNA and *gyrB* gene sequences are shown in Table 1.

Table 2. Phenotypic differentiation between the novel isolates (*B. safensis* sp. nov.) and *B. pumilus* strains

Diagnostic traits were determined from a total of 174 biochemical tests. API tests (20NE, 20E, 50 CH, 50 CH assimilation, ZYM), tests of NaCl tolerance etc. were used to generate these results. Details of these tests are given in the text.

Reaction	*B. safensis* sp. nov.		*B. pumilus*	
	FO-36b^T	Twelve other strains*	ATCC 7061^T	Five other strains*
Acid phosphatase				
Lipase (Tween 80)	−	17	+	100
Casein hydrolysis in litmus milk	−	17	+	100
Acid production from:				
Inositol	+	100	−	0
Methyl α-D-glucopyranoside	+	100	−	80
Maltose	+	100	−	80
D-Turanose	+	100	−	80
Utilization of:				
Inositol	+	100	−	0
Methyl α-D-glucopyranoside	+	100	−	80
Melibiose	+	100	−	80
Raffinose	+	100	−	20
Maltose	+	100	−	80
D-Turanose	+	100	−	80

*Percentage of strains that gave a positive reaction.

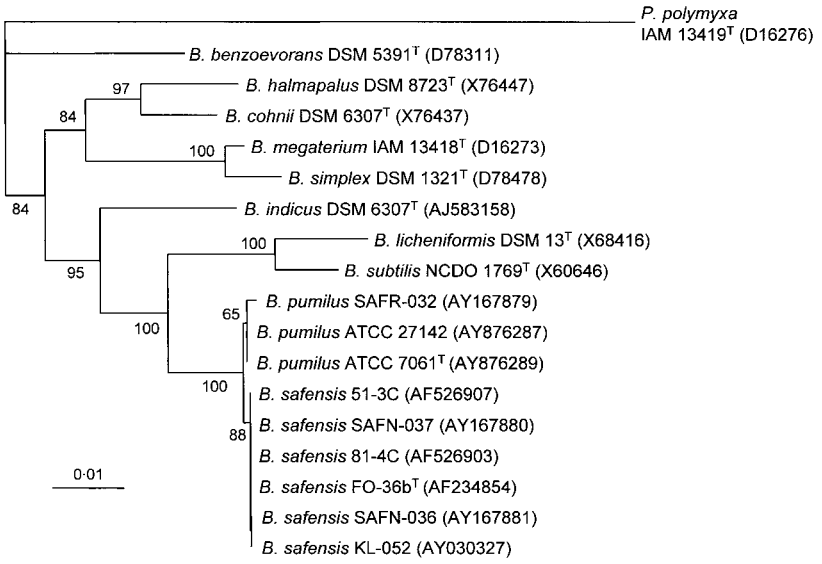

Fig. 1. Phylogenetic tree of members of the genus *Bacillus*, based on 16S rRNA gene sequences. The tree was constructed using the neighbour-joining method, and genetic distances were computed by using Kimura's model. Numbers at nodes indicate percentages of occurrence in 1000 bootstrapped trees. *Paenibacillus polymyxa* IAM 13419^T was used as the outgroup. Accession numbers are given in parentheses. Bar, genetic distance of 0·01.

rep-PCRs were carried out according to the manufacturers' instructions (Bacterial Barcodes); the detailed protocol of the DiversiLab System LabChip kit used can be found elsewhere (http://www.bacbarcodes.com/diversilab/35-0018-00rev6%20-LabChip.pdf). Analysis of PCR-fragment banding patterns and dendrogram construction were performed following the method of de Bruijn (1992). DNA–DNA hybridization was studied by using microplate hybridization methods (Ezaki *et al.*, 1989) with photobiotin labelling and colorimetric detection, with 1,2-phenylenediamine (Sigma) as the substrate and a streptavidin–peroxidase conjugate (Boehringer Mannheim) as the colorimetric enzyme (Satomi *et al.*, 1997).

Sequences of 1·4 kb of the 16S rRNA genes (covering base positions 44–1471; *Escherichia coli* numbering) and the 1·1 kb of the *gyrB* genes (covering base positions 316–1472; *E. coli* numbering) were used for phylogenetic analyses. The sequence similarity of 16S rRNA genes among the novel isolates was greater than 99·9 %. A phylogenetic tree based on the 16S rRNA gene (Fig. 1) showed that the novel isolates clustered with members of the genus *Bacillus*, the nearest neighbour being *B. pumilus* (99·9 % sequence similarity). Since several reports have been published showing that strains with >99 % 16S rRNA gene sequence similarity may not belong to the same species (La Duc *et al.*, 2004c; Satomi *et al.*, 2002; Venkateswaran *et al.*, 1999; Stackebrandt & Goebel, 1994), comparative *gyrB* gene sequence analyses were carried out. As has been observed in previous studies (La Duc *et al.*, 2004b) *gyrB* gene sequence-based phylogenetic topology proved more highly discriminative, grouping these strains monophyletically in a cluster separate from *B. pumilus*, clearly delineating them as a distinct species (Fig. 2). The sequence similarity values required to separate species on the basis of the *gyrB* gene vary according to the genus (Venkateswaran *et al.*, 1999; Satomi *et al.*, 2002, 2003, 2004). Additional reputable genetic analyses are therefore necessary to confirm the novelty of these isolates. On the

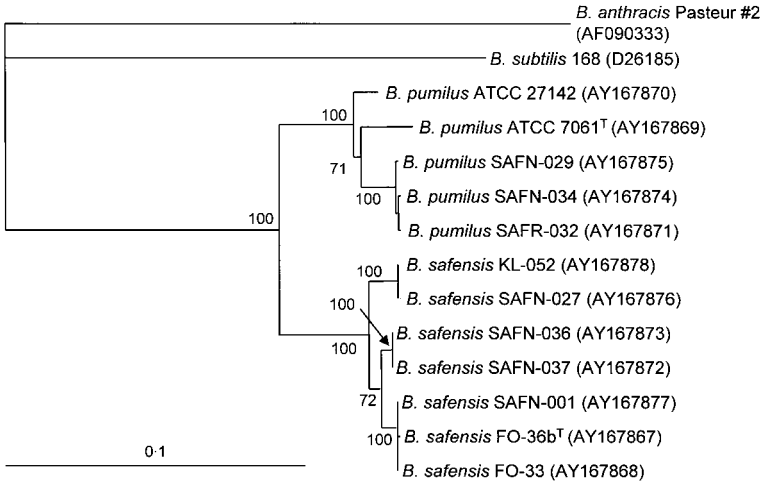

Fig. 2. Phylogenetic tree of the novel isolates and *B. pumilus*, based on *gyrB* gene sequences. The tree was constructed using the neighbour-joining method, and genetic distances were computed by using Kimura's model. Numbers at nodes indicate percentages of occurrence in 1000 bootstrapped trees. *Bacillus anthracis* Pasteur #2 and *Bacillus subtilis* 168 served as the outgroup. Accession numbers are given in parentheses. Bar, genetic distance of 0·1.

basis of the results of rep-PCR fingerprinting (Supplementary Fig. S1 available in IJSEM Online), all of the novel isolates are grouped together in their own cluster, separate from the distinct cluster formed by previously described *B. pumilus* strains, supporting the *gyrB* analysis and previous MALDI-TOF MS results (Dickinson *et al.*, 2004). The most notable difference between the protein profiles of the *B. pumilus* type strain (ATCC 7061^T) and the proposed FO-36b^T strain group is the presence of a peak at 7620 Da, found only in the FO-36b^T group strains (Dickinson *et al.*, 2004). Another difference between the two groups is that the FO-36b^T group forms a tight cluster in terms of MALDI-TOF MS protein profiling. rep-PCR fingerprinting is able to resolve down to, and perhaps beyond, the species level. It has been used in recent taxonomic studies to evaluate DNA relatedness among bacterial strains (Thompson *et al.*, 2003). The rep-PCR results generated in our work clearly distinguish the novel isolates from *B. pumilus* and, when coupled with supporting MALDI-TOF MS profiles (Dickinson *et al.*, 2004), strongly suggest that these isolates should be recognized as belonging to a distinct genotype. To confirm the conclusions generated from the results of the phylogenetic analyses, DNA–DNA hybridization was performed (Supplementary Table S1 available in IJSEM Online). DNA–DNA hybridization revealed that the 13 novel isolates were all closely related (80–99 % DNA relatedness values), suggesting that they belong to the same species, but exhibited lower levels of hybridization with *B. pumilus* strains (54–66 %). This strongly supports the claim that these isolates represent a novel species within the genus *Bacillus* (Wayne *et al.*, 1987).

The results from biochemical characterizations, *gyrB* gene sequence analysis, DNA hybridization, rep-PCR profiling and MALDI-TOF MS protein profiling strongly indicated that the 13 novel strains isolated from spacecraft-assembly facilities represent a novel bacterial species within the genus *Bacillus*, although fatty acid methyl ester analysis and 16S rRNA gene sequence analysis failed to discriminate between the tested isolates and *B. pumilus*. On the basis of the data from the polyphasic studies described above, the 13 isolates represent a novel species of the genus *Bacillus*, for which the name *Bacillus safensis* sp. nov. is proposed.

Description of *Bacillus safensis* sp. nov.

Bacillus safensis [sa.fen′sis. N.L. masc. adj. *safensis* arbitrarily derived from SAF (the spacecraft-assembly facility at the Jet Propulsion Laboratory, Pasadena, CA, USA), from where the organism was first isolated].

Cells are mesophilic, aerobic, chemoheterotrophic, Gram-positive, spore-forming rods that are motile by means of polar flagella. Cells are 0·5–0·7 μm in diameter and 1·0–1·2 μm in length. Growth occurs at 0–10 % (w/v) NaCl and at pH 5·6. Growth occurs at 10–50 °C (optimum, 30–37 °C) but not at 4 or 55 °C. Colonies are round, undulate, dull white, non-luminescent and have irregular margins on TSA plates incubated at 32 °C for 24 h. Oxidase, catalase, β-galactosidase, β-glucosidase, alkaline phosphatase, naphthol-AS-BI-phosphatase and esterase are produced, but H_2S, indole, amylase, agarase, lecithinase, DNase, urease, leucine arylamidase, cystine arylamidase, valine arylamidase, trypsin, α-galactosidase, *N*-acetyl-β-glucosamidase, α-fucosidase, tryptophan deaminase, phenylalanine deaminase, arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase are not. Cells do not reduce nitrate, but do hydrolyse gelatin, aesculin and RNA. Casein hydrolysis varies among strains. Voges–Proskauer test is positive. Growth occurs on agar plates supplemented with 1 % glycine and ox gall, but does not occur in 0·0001 % lysozyme broth. Negative for gas production from D-glucose. Acid is produced from D-glucose, glycerol, L-arabinose, ribose, D-xylose, galactose, fructose, mannose, inositol, mannitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, *N*-acetylglucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, sucrose, trehalose, D-turanose and D-tagatose, but not from erythritol, D-arabinose, L-xylose, adonitol, methyl β-D-xylopyranoside, sorbose, rhamnose, dulcitol, sorbitol, inulin, melezitose, raffinose, starch, glycogen, xylitol, D-lyxose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate or 5-ketogluconate. Reactions for lactose, melibiose and gentiobiose vary among strains. Citrate, malate, D-glucose, glycerol, L-arabinose, ribose, D-xylose, galactose, fructose, mannose, inositol, mannitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, *N*-acetylglucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, sucrose, trehalose, gentiobiose, D-turanose, D-tagatose, gluconate, lactate, L-aspartate and L-glutamate are readily utilized as energy sources. Erythritol, D-arabinose, L-xylose, adonitol, methyl β-D-xylopyranoside, sorbose, dulcitol, sorbitol, inulin, lactose, melezitose, starch, glycogen, xylitol, D-lyxose, D-fucose, L-fucose, D-arabitol, L-arabitol, 2-ketogluconate, 5-ketogluconate, capric acid, adipic acid, phenylacetic acid, propionate and glycine are not utilized as energy sources. Rhamnose utilization varies among strains. The DNA G+C content is 41·0–41·4 mol%. The chain composition of the whole-cell fatty acids is primarily $C_{15:0}$ iso, $C_{15:0}$ anteiso, $C_{17:0}$ iso and $C_{17:0}$ anteiso.

The type strain, FO-36b^T (=ATCC BAA-1126^T=NBRC 100820^T), was isolated from the spacecraft-assembly facility of the Jet Propulsion Laboratory, Pasadena, CA, USA. Strains SAFN-001 (=ATCC BAA-1128=NBRC 100821), KL-052 (=ATCC BAA-1129=NBRC 100822) and 51-3C (=ATCC BAA-1127=NBRC 100823) are reference strains.

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