Paenibacillus pasadenensis sp. nov. and *Paenibacillus barengoltzii* sp. nov., isolated from a spacecraft assembly facility

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Two novel spore-forming, Gram-positive, mesophilic, heterotrophic bacteria representing two novel species were isolated from the Jet Propulsion Laboratory Spacecraft Assembly Facility (JPL-SAF) at Pasadena, CA, USA. The incidence of similar strains was examined by screening the growing collection of isolates (~400 strains) obtained from the JPL-SAF using species-specific PCR primer sets designed from the 16S rRNA gene sequences of strains SAFN-016^T and SAFN-007^T. Phylogenetic analysis of 16S rRNA gene sequences placed these novel isolates within the genus *Paenibacillus*. Two strains, SAFN-016^T and SAFN-125, shared 98% 16S rRNA gene sequence similarity with *Paenibacillus timonensis* and 97% similarity with *Paenibacillus macerans*. Strain SAFN-007^T showed 95.2% 16S rRNA gene sequence similarity with *Paenibacillus kobensis*, its nearest phylogenetic neighbour. The results of DNA–DNA hybridization, physiological tests and biochemical analysis allowed genotypic and phenotypic differentiation of the isolates from currently recognized *Paenibacillus* species. Strain SAFN-007^T and strains SAFN-016^T and SAFN-125 are representatives of two separate novel species, for which the names *Paenibacillus pasadenensis* sp. nov. (type strain SAFN-007^T=ATCC BAA-1211^T=NBRC 101214^T) and *Paenibacillus barengoltzii* sp. nov. (type strain SAFN-016^T=ATCC BAA-1209^T=NBRC 101215^T) are proposed.

Spacecraft designed for the robotic exploration of extra-terrestrial systems potentially capable of sustaining life must be assembled in clean rooms following stringent quality-control practices that are aimed towards minimizing surface bioburden. Studies utilizing the NASA standard assay have shown that members of the spore-forming genus *Bacillus* are the most frequently isolated microbes on spacecraft and associated facility surfaces (La Duc *et al.*, 2003; Puleo *et al.*, 1977). During a survey of the Jet Propulsion Laboratory Spacecraft Assembly Facility (JPL-SAF) in December 2000, 30 strains of spore-forming microbes were systematically isolated and identified using both phenotypic tests and 16S rRNA gene sequence analysis (Fig. 1). Phylogenetic affiliations revealed that all of these strains were Gram-positive bacteria belonging to the genera *Bacillus* (23 isolates comprising nine species), *Filibacter*, *Sporosarcina*, *Paenibacillus*

and *Streptomyces*. The most abundant species was *Bacillus pumilus* (six isolates) followed by *Bacillus megaterium* (three isolates). The 16S rRNA gene sequences of two isolates, SAFN-007^T and SAFN-016^T, exhibited >95% similarity to members of the genus *Paenibacillus*. As this was the first incidence of *Paenibacillus* species reported in the JPL-SAF, both strains were further characterized using a polyphasic taxonomic approach.

The JPL-SAF consists of both classified and unclassified clean room locations. The classified portions of this facility are maintained as Class 100K clean rooms (maximum number of particles >0.5 µm in 1 cubic foot of air; ISO, 1999). Polyester swabs were used to collect samples from 200 locations at various times from both unclassified (entrance floors, ante-room and air-lock) and classified (floors, cabinet tops and air) surfaces within the JPL-SAF; specific details have been reported elsewhere (Venkateswaran *et al.*, 2001). The collected swab samples were sonicated (25 kHz) for 2 min and aliquots were either left untreated or were subjected to heat shock (80 °C for 15 min) to kill vegetative cells. Heat-shocked samples were pour-plated in tryptic soy agar (TSA) and grown at 32 °C for 2 days. Samples not

Abbreviation: JPL-SAF, Jet Propulsion Laboratory Spacecraft Assembly Facility.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of the *Paenibacillus* strains investigated in this study are given in Table 1.

Fig. 1. Phylogenetic tree of bacterial isolates from the JPL-SAF based on 16S rRNA gene sequence comparisons. Branching percentage values were determined using 1000 bootstraps. Bar, 10 changes.

subjected to heat shock were spread-plated on R2A medium and incubated at 25 °C for 7 days.

The bacterial strains analysed in this study are shown in Table 1. A set of related type strains were received as gifts from the Agricultural Research Service Culture Collection (<http://nrrl.ncaur.usda.gov>) or purchased from the Swedish Culture Collection (CCUG), University of Göteborg, Sweden, and used as reference strains. All isolates were maintained in TSA stabs (Becton Dickinson) at room temperature for short-term analysis and in glycerol at –80 °C for long-term storage. Liquid cultures were grown in tryptic soy broth (TSB) (Becton Dickinson) and incubated at 32 °C with vigorous aerobic shaking for an appropriate period of time. Representative strains have been deposited in the American Type Culture Collection (ATCC) and the National Institute of Technology and Evaluation, Biological Resource Center (NBRC), Japan (Table 1).

Cellular morphology, motility, Gram-staining and the refractile nature of the endospores were examined by phase-contrast microscopy. A nutrient sporulation medium was used to produce spores as described previously (La Duc *et al.*, 2003; Nicholson & Setlow, 1990; Schaeffer *et al.*, 1965). Routine biochemical tests were carried out using commercially available API kits (API 20NE and API 20E), inoculated according to the manufacturer's instructions (bioMérieux). Metabolic profiling was performed using the Biolog 96-well microplate test for the oxidation of 95 different carbon sources (Biolog).

Phenotypic characteristics of the novel isolates SAFN-007^T, SAFN-016^T and SAFN-125 and other closely related *Paenibacillus* species are presented in Table 2. Strain SAFN-007^T did not reduce nitrate, was positive for Voges–Proskauer tests, liquefied gelatin and assimilated arabinose, glucose, maltose, mannose, malate, mannitol and *N*-acetylglucosamine. However, strains SAFN-016^T and SAFN-125 did not assimilate any of the carbon substrates tested apart from gluconate. Strain SAFN-007^T did not assimilate gluconate as a sole carbon source. The Biolog-based carbon substrate profiles of the novel strains did not match those of any of the *Bacillus* or *Paenibacillus* species provided in the manufacturer's database. The phenotypic characteristics of *Paenibacillus kobensis*, the closest neighbour of strain SAFN-007^T according to 16S rRNA gene sequences, differed from those of strain SAFN-007^T as regards nitrate reduction, urea hydrolysis, malate assimilation and acid production from sucrose and L-arabinose (Kanzawa *et al.*, 1995) (Table 2). These differences strongly support the conclusion of the phylogenetic analysis that strain SAFN-007^T represents a novel species in the genus *Paenibacillus*. When strains SAFN-016^T and SAFN-125 were tested, no significant colouration of tetrazolium dye was seen. This was in agreement with API test strips in which the majority of carbon substrates tested were not assimilated by either of these strains (Table 2). Significant characteristic phenotype differences between strains SAFN-016^T and SAFN-125 and *Paenibacillus timonensis* CCUG 48216^T are nitrate reduction, acid production from sugars and the assimilation of several carbohydrates,

Table 1. Source and isolation of strains used in this study

Strain	16S rRNA gene sequence accession no.	Source
<i>Paenibacillus amylolyticus</i> NRRL NRS-290 ^T	D85396	Soil
SAFN-022b	AY167825	Clean room floor, JPL-SAF, 2000
<i>Paenibacillus barengoltzii</i> SAFN-016 ^T (= ATCC BAA-1209 ^T = NBRC 101215 ^T)	AY167814	Clean room floor, JPL-SAF, 2000
SAFN-125 (= ATCC BAA-1210)	DQ124699	Clean room floor, JPL-SAF, 2001
<i>Paenibacillus illinoisensis</i> NRRL NRS-1356 ^T	D85397	Soil
SAFN-035	AY167800	Clean room floor, JPL-SAF, 2000
<i>Paenibacillus macerans</i> NRRL B-172 ^T	X57306	Unknown
<i>Paenibacillus pabuli</i> NRRL NRS-924 ^T	AB045094	Unknown
SAFN-068	DQ124700	Clean room floor, JPL-SAF, 2001
<i>Paenibacillus pasadenensis</i> SAFN-007 ^T (= ATCC BAA-1211 ^T = NBRC 101214 ^T)	AY167820	Clean room floor, JPL-SAF, 2000
<i>Paenibacillus polymyxa</i> NRRL B-4317 ^T	AJ320493	Unknown
<i>Paenibacillus timonensis</i> CCUG 48216 ^T	AY323610	Blood, France, 2002
<i>Stenotrophomonas maltophilia</i> SAFR-173	DQ124701	Clean room floor, JPL-SAF, 2001

as shown in Table 2. Phenotypic characteristics of the novel *Paenibacillus* species are given in the species descriptions.

To purify DNA, cells were grown overnight in TSB at 30 °C and harvested by centrifugation. Pellets were resuspended in TE buffer (pH 8.0) supplemented with 1 % Triton X-100 and boiled to destroy cell walls. The 16S rRNA gene was PCR-amplified with universal primer sets as previously described (La Duc *et al.*, 2004), purified on Qiagen filtration columns and sequenced. The identity of a given PCR product was verified by bidirectional sequencing. The phylogenetic relationships of the organisms investigated in this study were determined by comparison of individual 16S rRNA gene sequences with other sequences in public databases using the BLAST algorithm (Altschul *et al.*, 1990). Evolutionary trees were constructed with PAUP software, following maximum parsimony parameters (Swofford, 1990). Alignment gaps, primer regions for PCR amplification and unidentified base positions were not taken into consideration for the calculations. The robustness of the topology of the phylogenetic trees was evaluated by a bootstrap analysis with 1000 replications. GenBank accession numbers for the 16S rRNA gene sequences analysed in this study are shown in Table 1.

A phylogenetic tree based on 16S rRNA gene sequences (Fig. 2) showed that the novel isolates clustered with members of the genus *Paenibacillus*, the nearest neighbours being *P. kobensis* DSM 10249^T for strain SAFN-007^T and *Paenibacillus timonensis* CCUG 48216^T for strains SAFN-016^T and SAFN-0125. Strain SAFN-007^T showed only 95.2 % 16S rRNA gene sequence similarity with *P. kobensis*

DSM 10249^T. Strains SAFN-016^T and SAFN-125 exhibited 98 % 16S rRNA gene sequence similarities with *P. timonensis* CCUG 48216^T and 97 % with *Paenibacillus macerans* NRRL B-172^T and shared 99.8 % sequence similarity with each other. The phylogenetic distances between strain SAFN-007^T and its nearest neighbours in the genus *Paenibacillus* suggest that this strain represents a novel species (Wayne, 1988). Since strains SAFN-016^T and SAFN-125 are so closely related to their nearest neighbours on the basis of 16S rRNA gene sequences, further study was necessary to confirm their taxonomic position. Gene sequence similarities between the two novel *Paenibacillus* species and the 18 other members of the genus *Paenibacillus* ranged from 92 to 98 %. The two novel species showed only 86–87 % gene sequence similarity to *Bacillus subtilis*.

PCR primer sets specific for strains SAFN-007^T and SAFN-016^T were designed to screen the large collection of bacteria (~400 strains) isolated from the JPL-SAF for similar strains. The 16S rRNA gene sequences from both novel strains, SAFN-016^T and SAFN-007^T, were compared with those of their closest related *Paenibacillus* species. Species-specific primers were designed for each group and tested against DNA extracts from both the novel strains and the type strains listed in Table 1. Isolates similar to strain SAFN-007^T were screened for by using primers 172Fb (5'-CCAGATACGCGATCTTC-3') and 649R (5'-GTTTCCCGTGCGAC-TTGG-3'). SAFN-016^T group isolates were selected for using primers 173Fa (5'-CCGATACGCAAGTCTC-3') and 1161Rb (5'-CTAGAGTGCCCAACCTACT-3'). The PCR conditions were as follows: denaturation for 1 min at 95 °C, annealing for 2 min at 55 °C and elongation

Table 2. Phenotypic characterization of the novel *Paenibacillus* strains and closely related species

Strains: 1, Strain SAFN-007^T; 2, *P. kobensis* IFO 15729^T; 3, *P. pabuli* NRRL NRS-924^T; 4, strain SAFN-016^T; 5, strain SAFN-125; 6, *P. timonensis* CCUG 48216^T; 7, *P. amylocticus* NRRL B-14945^T; 8, *P. illinoisensis* NRRL NRS-1356^T; 9, *P. macerans* NRRL B-172^T; 10, *P. polymyxa* NRRL B-4317^T. All data are from this study unless otherwise indicated. All strains were Gram-positive rods, motile, spore-forming and positive for the production of catalase and oxidase. All strains hydrolysed ONPG and aesculin. None of the strains produced H₂S, indole or tryptophan. ND, Not determined; W, weak reaction.

Characteristic	1	2*	3	4	5	6	7	8	9	10
Nitrate to nitrite	–	+	+	+	+	–	–	–	+	+
Arginine dihydrolase	W	–	–	–	–	–	–	–	–	–
Gelatinase	+	–	–	–	–	–	–	–	+	+
Assimilation of:										
N-Acetylglucosamine	+	+	+	–	–	+	–	+	–	–
Arabinose	+	+	+	–	–	+	+	+	+	+
Gluconate	–	–	+	+	+	+	+	+	+	+
Glucose	+	+	+	–	–	+	+	+	+	+
Malate	+	–	–	–	–	–	–	–	–	–
Maltose	+	+	+	–	+/–	+	+	+	+	–
Mannitol	+	–	+	–	–	–	+	+	+	+
Mannose	+	–	+	–	–	+	+	–	+	+
Acetoin production	+	+	+	–	–	ND	ND	ND	ND	+
Acid production from:										
Amylose	–	–	–	–	–	+	ND	ND	ND	–
Arabinose	–	+	–	–	–	+	ND	ND	ND	–
Mannitol	–	–	–	–	–	–	ND	ND	ND	+
Melibiose	–	–	–	–	–	+	ND	ND	ND	–
Rhamnose	–	–	–	–	–	+	ND	ND	ND	–
Sucrose	–	+	–	–	–	–	ND	ND	ND	–

*Data from: a, Kanzawa *et al.* (1995); b, Smerda *et al.* (2005); c, Dasman *et al.* (2002).

for 3 min at 72 °C for 32 cycles using a thermal cycler (MJ Research).

Three additional strains produced PCR-amplified products with either the SAFN-016^T- or SAFN-007^T-specific primer sets. Strain SAFN-068 (isolated in March 2001) yielded a 477 bp amplicon specific for strain SAFN-007^T. Strains SAFN-125 (isolated June 2001) and SAFR-173 (isolated September 2001) exhibited 988 bp amplicons specific for strain SAFN-016^T. Although the PCR-amplified products from strains SAFN-068 and SAFR-173 were faint in comparison with that of strain SAFN-125 when visualized on agarose gels, the full-length 16S rRNA genes of all three isolates were amplified and sequenced. Strain SAFN-068 was identified as *Paenibacillus pabuli* based on 16S rRNA gene sequence similarity (99.7%), API 20NE, 20E and Biolog analyses. Strain SAFR-173 was found to be closely related to *Stenotrophomonas maltophilia* (98.2 % gene sequence similarity). As the most closely related species to strain SAFN-173

was Gram-negative, phenotypic characteristics were not determined. This confirmed that strains exhibiting faint bands were not the intended sequence targets, but rather were the result of non-specific amplification. Strain SAFN-125 was the only isolate found that closely matched the 16S rRNA gene sequence of strain SAFN-016^T (99.8 % similarity). Species-specific PCR screening did not reveal any additional strains for the SAFN-007^T isolate.

For DNA–DNA hybridization analysis, 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 following standard procedures (Johnson, 1981). DNA–DNA hybridization was conducted by microplate hybridization methods (Ezaki *et al.*, 1989) with photobiotin labelling and colorimetric detection, using 1,2-phenylene-diamine (Sigma) as the substrate and streptavidin–peroxidase conjugate (Boehringer Mannheim) as the colorimetric substrate (Satomi *et al.*, 1997). Since the 16S rRNA gene sequences of strains SAFN-016^T and SAFN-125 showed high similarity values with *P. timonensis* (98 %) and *P. macerans* (97 %), type strains of several *Paenibacillus* were included and a DNA–DNA hybridization study was performed. Due to the very low gene sequence similarity revealed between strain SAFN-007^T and *P. kobensis*, DNA–DNA hybridization was deemed to be unnecessary. The results of this study are given in Table 3. Strain SAFN-016^T exhibited ~38 % DNA–DNA relatedness with *P. timonensis* CCUG 48216^T and *P. macerans* NRRL B-172^T. This strongly supports the claim that the isolates represent two novel species within the genus *Paenibacillus*.

Although phenotypic characterization differentiated these novel species from their most closely related phylogenetic neighbours, a metabolic fingerprint based on Biolog results was not available in the manufacturer's database to identify the novel bacterial isolates. Of the genotypic analyses performed, 16S rRNA gene sequence analysis unambiguously discriminated strain SAFN-007^T as representing a novel *Paenibacillus* species, but DNA–DNA hybridization was required to describe strains SAFN-016^T and SAFN-125. Based on the results of the polyphasic study described above, it is suggested that the three strains isolated from the space-craft assembly facilities represent two novel species within the genus *Paenibacillus*. The names *Paenibacillus pasadenensis* sp. nov. and *Paenibacillus barengoltzii* sp. nov. are proposed for strain SAFN-007^T and strains SAFN-016^T and SAFN-125, respectively.

Description of *Paenibacillus pasadenensis* sp. nov.

Paenibacillus pasadenensis (pa.sa.den.en'sis. N.L. masc. adj. *pasadenensis* referring to Pasadena, the city in which the JPL-SAF is located).

Cells are Gram-positive rods, 0.5–0.8 × 3.0–5.0 µm in size and motile by means of peritrichous flagella. Ellipsoidal spores are formed in swollen sporangia. Colonies are flat,

Fig. 2. Phylogenetic tree of novel *Paenibacillus* isolates from the JPL-SAF based on 16S rRNA gene sequence similarity. Branching values determined using 1000 bootstraps. Bar, 10 changes.

smooth, circular, entire and brownish yellow. No soluble pigment is produced on nutrient agar. Catalase and oxidase tests are positive. Acetylmethylcarbinol is produced (as determined by the Voges–Proskauer reaction). Hydrogen

sulfide and indole are not produced. Nitrate is not reduced to nitrite. Gelatin is liquefied, aesculin is hydrolysed and β -galactosidase is produced. Growth occurs in the presence of 2 % NaCl and 0.001 % lysozyme. Growth is inhibited by

Table 3. DNA–DNA hybridization among *Paenibacillus* strains isolated from JPL-SAF

ND, Not determined.

Strain	DNA–DNA reassociation value (%)					
	1	2	3	4	5	6
1. <i>Paenibacillus pasadenensis</i> SAFN-007 ^T	100	18	ND	ND	6	8
2. <i>Paenibacillus barengoltzii</i> SAFN-016 ^T	10	100	89	34	10	9
3. <i>Paenibacillus barengoltzii</i> SAFN-125	ND	88	100	37	ND	ND
4. <i>P. timonensis</i> CCUG 48216 ^T	ND	36	38	100	ND	ND
5. <i>P. amylolyticus</i> SAFN-022b	7	10	ND	ND	100	26
6. <i>P. illinoisensis</i> SAFN-035	8	11	ND	ND	39	100
<i>P. amylolyticus</i> NRRL B-14945 ^T	10	12	ND	ND	88	24
<i>P. illinoisensis</i> NRRL NRS-1356 ^T	6	15	ND	ND	47	88
<i>P. macerans</i> NRRL B-172 ^T	12	38	ND	ND	4	7
<i>P. pabuli</i> NRRL NRS-924 ^T	7	11	ND	ND	50	25
<i>P. polymyxa</i> NRRL B-4317 ^T	7	7	ND	ND	8	7

3 % NaCl. Utilizes α -cyclodextrin, D-cellobiose, D-fructose, maltose, D-melibiose, methyl β -D-glucoside, D-ribose, pyruvic acid, L-alanyl glycine and L-serine. Acid is not produced from D-glucose.

The type strain, SAFN-007^T (= ATCC BAA-1211^T = NBRC 101214^T), was isolated from the entrance floor of the JPL-SAF, Pasadena, CA, USA.

Description of *Paenibacillus barengoltzii* sp. nov.

Paenibacillus barengoltzii (ba.ren.gol'tzi.i. N.L. gen. n. *barengoltzii* referring to Jack Barengoltz, a well-known American physicist and NASA planetary protection scientist).

Cells are Gram-positive rods, 0.5–0.8 × 3.0–5.0 µm in size, strictly aerobic and motile by means of peritrichous flagella. Ellipsoidal spores are formed in swollen sporangia. Colonies are flat, smooth, circular, entire and brownish yellow. No soluble pigment is produced on nutrient agar. Catalase and oxidase tests are positive. Acetylmethylcarbinol is produced (as determined by the Voges–Proskauer reaction). Hydrogen sulfide and indole are not produced. Nitrate is reduced to nitrite. Gelatin is not liquefied, aesculin is hydrolysed and β -galactosidase is produced. Growth occurs between 10 and 50 °C and at pH 4.5–9.0. Optimum growth occurs at 37 °C and at pH 7.0. Growth occurs in the presence of 2 % NaCl and 0.001 % lysozyme. Growth is inhibited by 5 % NaCl. Of the carbon substrates tested, only gluconate is utilized. Acid is not produced from D-glucose.

The type strain, SAFN-016^T (= ATCC BAA-1209^T = NBRC 101215^T) was isolated from clean room floors of the JPL-SAF, Pasadena, CA, USA. Strain SAFN-125 (= ATCC BAA-1210) is a reference strain.

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