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Bacillus aerius sp. nov., *Bacillus aerophilus* sp. nov., *Bacillus stratosphericus* sp. nov. and *Bacillus altitudinis* sp. nov., isolated from cryogenic tubes used for collecting air samples from high altitudes

S. Shivaji,[1] P. Chaturvedi,[1] K. Suresh,[1] G. S. N. Reddy,[1] P. Rajaratnam,[2] M. Wainwright,[3] J. V. Narlikar[4] and P. M. Bhargava[5]

Correspondence
S. Shivaji
shivas@ccmb.res.in

[1]Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad 500 007, India

[2]Indian Space Research Organization, Antariksha Bhavan, New BEL Road, Bangalore 500 094, India

[3]Department of Molecular Biology and Biotechnology, University of Sheffield, Fifth Court, Western Bank, Sheffield S10 2TN, UK

[4]Inter-University Centre for Astronomy and Astrophysics, Post Bag No. 4, Ganeshkhind, Pune 411 007, India

[5]Anveshna Consultancy Services, Furqan Cottage, Street No. 3, Tarnaka, Hyderabad 500 017, India

Four novel bacterial strains were isolated from cryogenic tubes used to collect air samples at altitudes of 24, 28 and 41 km. The four strains, 24K^T, 28K^T, 41KF2a^T and 41KF2b^T, were identified as members of the genus *Bacillus*. Phylogenetic analysis based on 16S rRNA gene sequences indicated that three of the strains, 24K^T, 28K^T and 41KF2a^T, are very similar to one another (>98 % sequence similarity) and show a similarity of 98–99 % with *Bacillus licheniformis* and 98 % with *Bacillus sonorensis*. DNA–DNA hybridization studies showed that strains 24K^T, 28K^T and 41KF2a^T exhibit <70 % similarity with each other and with *B. licheniformis* and *B. sonorensis*. Differences in phenotypic and chemotaxonomic characteristics between the novel strains and *B. licheniformis* and *B. sonorensis* further confirmed that these three isolates are representatives of three separate novel species. Strain 41KF2b^T showed 100 % 16S rRNA gene sequence similarity to *Bacillus pumilus*, but differed from its nearest phylogenetic neighbour in a number of phenotypic and chemotaxonomic characteristics and showed only 55 % DNA–DNA relatedness. Therefore, the four isolates represent four novel species for which the names *Bacillus aerius* sp. nov. (type strain, 24K^T=MTCC 7303^T=JCM 13348^T), *Bacillus aerophilus* sp. nov. (type strain, 28K^T=MTCC 7304^T=JCM 13347^T), *Bacillus stratosphericus* sp. nov. (type strain, 41KF2a^T=MTCC 7305^T=JCM 13349^T) and *Bacillus altitudinis* sp. nov. (type strain, 41KF2b^T=MTCC 7306^T=JCM 13350^T) are proposed.

Studies on the qualitative and quantitative distribution of micro-organisms in the upper troposphere-stratosphere (10–85 km altitude) in various parts of the Earth are important as they should help (i) in determining the role of the various atmospheric strata in the transport of micro-organisms from one part of the globe to another and (ii) to test the theory that micro-organisms might exist in space (Hoyle & Wickramasinghe, 1986, 1993, 1999) and form a part of the hundreds of tons of material that enters the atmosphere each day from space (Love & Brownlee, 1993). It is now well recognized that micro-organisms can survive the harsh conditions of the upper atmosphere and the rigours of outer space (reviewed by Bruch, 1967). Theoretical studies (Bruch, 1967) have indicated that it is also possible for

Abbreviations: DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; SEM, scanning electron microscopy; UV, ultraviolet.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains 41KF2a^T, 41KF2b^T, 24K^T and 28K^T are AJ831841–AJ831844, respectively.

The UV sensitivity of the four novel strains compared with *B. licheniformis* MTCC 429^T and *B. pumilus* MTCC 1640^T is presented in Supplementary Table S1 in IJSEM Online.

micro-organisms of an appropriate size to escape into outer space and thus be transported from one planet to another.

There have been, however, very few published studies on the quantity and nature of micro-organisms in the upper atmosphere (Bruch, 1967; Greene *et al.*, 1964; Lysenko, 1979; Rogers & Meier, 1936). These studies have used either a meteorological rocket (Lysenko, 1979) or a specially designed direct-flow sampler sent up on a balloon (Greene *et al.*, 1964; Rogers & Meier, 1936). Both bacteria and fungi have been found at altitudes of up to 85 km (Lysenko, 1979). In a recent paper, Harris *et al.* (2001) detected bacteria from stratospheric air samples collected at 41 km using scanning electron microscopy (SEM) and epifluorescence techniques. Using the same samples, Wainwright *et al.* (2003) described the presence of two bacterial species (*Bacillus simplex* and *Staphylococcus pasteurii*) and a fungus (*Engyotontium album*). In this paper, a polyphasic taxonomic approach was used to characterize four bacterial strains from cryotubes that were used to collect air at altitudes between 24 and 41 km during a balloon flight from Hyderabad, India. The four strains, 24K^T, 28K^T, 41KF2a^T and 41KF2b^T, represent four novel species of the genus *Bacillus*.

Collection of air from 24 km and above

The balloon used in the experiment was launched on 20 January 2001 from the National Scientific Balloon Facility of the Tata Institute of Fundamental Research at Hyderabad, India (17° 28′ 20″ N 78° 34′ 48″ E). Air samples were collected at various heights up to 41 km using a cryogenic sampler comprising a 16-probe assembly. A detailed description of the 16-probe manifold, individual probes, sterilization protocol, telemetric controls for the opening and closing of the values of the cryoprobes and the cryopump effect due to liquid neon, and all other precautions involved in the sampling process is given in Wainwright *et al.* (2003). Air was collected during the ascent of the balloon and, after collection of air at an altitude of 41 km, the balloon was parachuted back to the ground. The instrument was then tracked and recovered and the probes were stored at 5 °C until further use.

Detection of bacteria in air samples

In the first procedure, two Millipore filtration units were connected in series so that air from each of the cryoprobes passed first through a 0·45 μm and then through a 0·22 μm Millipore filter. Each of the filters (47 mm diameter) was then cut into two halves. One half was transferred directly to a nutrient agar plate [0·5 % (w/v) peptone, 0·3 % (w/v) beef extract, 0·5 % (w/v) NaCl and 1·5 % (w/v) agar] and incubated at 15 °C. If colonies did not appear after up to 20 days of incubation, the filter was transferred to blood agar plates (nutrient agar plates containing 6 % defibrinated goat blood) and incubated at 15 °C for another 20 days. The other half of the filter was stored at −70 °C for future use.

In a second procedure, attempts were made to detect bacteria that may have remained at the bottom of the cryotube or become attached to the polished walls. After all the air was expelled and filtered, the probes were injected with 100 ml sterile 0·1 M phosphate buffer and agitated for 6 h in a shaker. The liquid was then removed using sterile tubing and a syringe and filtered sequentially through 0·45 and 0·22 μm Millipore filters (47 mm diameter). Each of the filters was then cut in half and each piece was incubated in Luria–Bertani agar [1 % (w/v) tryptone, 0·5 % (w/v) yeast extract, 2·5 % (w/v) NaCl and 1·5 % (w/v) agar, pH 7·2] or on nutrient agar plates at 15 °C.

One half of the filter from the first isolation procedure which had been stored at −70 °C was brought to room temperature (25 °C) and three 5 mm^2 pieces were excised under sterile conditions. The pieces were then gold-coated and examined at 40 000 to 100 000 magnification using SEM.

Air collected from 24 to 27 km [70 l at normal temperature and pressure (NTP), 28 to 38 km (50 l at NTP) and 39 to 41 km (20 l at NTP)] did not yield any viable bacterial colonies on nutrient agar plates or blood agar plates even after 20 days at 15 °C. Even when incubated at 25 °C, the same plates did not show any colonies. It is possible that these filters contained bacteria that were non-culturable. However, attempts to detect bacteria on these filters by SEM also proved negative. Using the air sample collected at 41 km, Wainwright *et al.* (2003) had earlier identified two bacterial species (*Bacillus simplex* and *Staphylococcus pasteurii*) and a fungus (*Engyotontium album*). Attempts to detect bacteria by the rRNA gene approach were also unsuccessful as the filters did not yield any DNA.

It is possible that some bacteria may have remained at the bottom of the tube or become attached to the walls of the cryotubes and thus escaped detection. To check this possibility, cryotubes devoid of air were flushed with buffer and the buffer was then spread on media plates. This procedure yielded four isolates, 24K^T, 28K^T, 41KF2a^T and 41KF2b^T, which were isolated from tubes used to collect air at 24, 28, 41 and 41 km altitude, respectively.

Morphological, biochemical and chemotaxonomic characteristics

Morphological, growth and biochemical studies on the viable colonies were performed using standard methods (Holding & Collee, 1971; Smibert & Krieg, 1994). Nutrient agar was used for growth and maintenance of the strains and for the determination of the phenotypic and chemotaxonomic characteristics as shown in Tables 1, 2 and 3. The shape, size and motility of the strains was ascertained using a Leitz Diaplan phase-contrast microscope with an oil immersion objective (× 100). The sensitivity of the cultures to antibiotics was determined by using antibiotic discs (Himedia). Utilization of various carbon compounds as the sole carbon source was tested in mineral liquid medium

Table 1. Phenotypic and chemotaxonomic characteristics that differentiate strains 24K^T, 28K^T, 41KF2a^T, *B. licheniformis* MTCC 429^T and *B. sonorensis* DSM 13779^T

Strains: 1, *B. aerius* sp. nov. 24K^T; 2, *B. aerophilus* sp. nov. 28K^T; 3, *B. stratosphericus* sp. nov. 41KF2a^T; 4, *B. licheniformis* MTCC 429^T; 5, *B. sonorensis* DSM 13779^T. All strains show the following characteristics: aerobic growth, white colony colour on nutrient agar, motile, Gram-positive, rod-shaped cells, positive reactions for catalase, oxidase, β-galactosidase, amylase, aesculin hydrolysis, starch hydrolysis, nitrate to nitrite reduction and the Voges–Proskauer test, acid production from D-glucose, sucrose, D-fructose, D-lactose, D-galactose, D-mannose, D-arabinose, D-xylose and mannitol and growth between pH 6 and pH 10 and between 20 and 37 °C. All strains exhibit growth on peptone. All strains utilize D-glucose, D-melibiose, sucrose, D-fructose, D-mannose, D-xylose, mannitol, glycerol, D-ribose, D-lactose, pyruvate, lactic acid, D-maltose, L-aspargine and L-arginine as the sole carbon source, but do not utilize L-aspartic acid. All strains are negative for the following: lipase and lysine decarboxylase activities, indole production, methyl red test, growth at pH 4 and pH 11 and growth in the presence of 23·4 % NaCl. All strains are sensitive to tobramycin (15 μg), lomefloxacin (30 μg), roxithromycin (30 μg) and streptomycin (25 μg) and resistant to vancomycin (30 μg) and erythromycin (15 μg). +, Positive; −, negative; W, weakly positive; R, resistant; S, sensitive. All data are from the present study.

Characteristic	1	2	3	4	5
Colony size (mm)	3–5	5–8	3–5	5–7	3–5
Colony shape	Irregular	Irregular	Irregular	Fried egg, irregular	Irregular
Arginine decarboxylase	+	+	+	+	−
Arginine dihydrolase	−	+	−	−	+
Citrate utilization	+	+	−	+	−
Gelatinase	+	+	+	−	+
Urease	−	−	+	−	+
Tolerance of NaCl:					
11·6 %	+	+	+	+	−
17·4 %	−	−	+	−	−
Growth at:					
8 °C	+	+	+	−	−
40 °C	−	−	−	+	+
45 °C	−	−	−	−	+
pH 5·5	−	−	−	+	+
pH 10	+	+	+	−	+
Carbon source utilization:					
N-Acetylglucosamine	−	−	−	+	+
D-Arabinose	+	+	+	−	+
D-Cellobiose	−	−	+	+	+
Citric acid	+	+	−	W	−
Dulcitol	−	−	+	+	+
myo-Inositol	−	−	+	+	+
Inulin	+	+	+	−	+
Polyethylene glycol	+	+	+	−	+
D-Raffinose	−	+	+	−	+
D-Rhamnose	−	+	+	+	+
Sodium acetate	+	+	−	−	+
Sodium succinate	+	+	−	−	−
D-Sorbitol	+	+	−	−	+
L-Sorbose	+	+	+	−	−
Starch	+	+	−	+	+
Thioglycolate	+	+	+	−	−
D-Trehalose	+	+	−	−	+
Xylitol	+	+	−	−	−
Acid from D-maltose	+	+	+	+	−
Amino acid utilization:					
L-Alanine	+	+	−	−	−
L-Glycine	−	−	+	−	−
L-Lysine	+	+	−	+	+
L-Threonine	−	+	−	+	+

Table 1. cont.

Characteristic	1	2	3	4	5
L-Tryptophan	+	+	+	+	−
Antibiotic test (μg per disc):					
Amikacin (30)	R	R	S	S	S
Amoxycillin (30)	R	R	R	S	S
Ampicillin (25)	R	R	S	S	S
Cefoperazone (75)	R	R	R	S	S
Cefuroxime (30)	R	R	R	S	S
Chloramphenicol (30)	R	R	R	R	S
Ciprofloxacin (30)	R	R	S	S	S
Colistin (10)	R	R	R	S	R
Co-trimoxazole (25)	R	R	R	R	S
Kanamycin (30)	R	R	R	R	S
Lincomycin (15)	R	S	R	S	S
Nalidixic acid (30)	R	R	S	S	S
Norfloxacin (10)	R	R	R	S	R
Novobiocin (30)	S	R	S	S	S
Penicillin (10)	R	R	R	R	S
Tetracycline (30)	R	S	S	S	S
Polar lipids	PE, PG, DPG	PE, PG, DPG	PE, PG, DPG	PE, DPG	PE, DPG
DNA G+C content (mol%)	45	44	44	45	46

containing (l^{-1}) 1 g ammonium chloride, 0·075 g dipotassium hydrogen phosphate, 1·45 g calcium chloride, 30·0 g sodium chloride, 0·075 g magnesium chloride, 0·75 g potassium chloride and 0·028 g ferrous sulphate, supplemented with 0·2 % of the filter-sterilized carbon source. Fatty acid methyl esters were prepared from cells grown at 25 °C to late exponential phase in nutrient broth according to the method of Sato & Murata (1988) and analysed as described by Kiran *et al.* (2004). The modified method of Bligh & Dyer (1959) was performed to extract polar lipids and molybdenum blue reagent was used to detect lipids containing phosphate esters. The isolation of DNA and estimation of DNA G+C content (mol%) was carried out according to Shivaji *et al.* (2005). DNA–DNA hybridization was performed by the membrane filter method of Tourova & Antonov (1987), as described by Shivaji *et al.* (1992). *Bacillus licheniformis* MTCC 429^T, *Bacillus pumilus* MTCC 1640^T and *Bacillus sonorensis* DSM 13779^T were used as controls in studies related to biochemical tests, identification of fatty acids, polar lipids and DNA–DNA hybridization.

All four novel isolates are Gram-positive, rod-shaped, endospore-forming and catalase-positive bacteria with iso-$C_{15:0}$, anteiso-$C_{15:0}$, iso-$C_{16:0}$, $C_{16:0}$, $C_{16:1}$ 11 *cis*, iso-$C_{17:0}$ and anteiso-$C_{17:0}$ as the predominant fatty acids. The lipids present include phosphatidylethanolamine (PE), phosphatidylglycerol (PG), diphosphatidylglycerol (DPG) and two unknown phospholipids as found in *B. licheniformis* MTCC 429^T. The DNA G+C content of the four strains was 44–45 mol%. All these characteristics indicate that the four novel strains are members of the genus *Bacillus* (see Tables 1–3).

Sensitivity to UV radiation

Cultures were grown to an OD_{660} of 1·0 at 30 °C in nutrient broth and harvested at 6000 r.p.m. for 5 min at 5 °C and the resulting pellet was suspended in 10 ml phosphate buffer, pH 7·2. A 100 μl sample of the culture was spread on nutrient agar plates. The plates were exposed to a UV lamp (UV-B, 15 W × 4; Sankyo Denki) with the lids open in a laminar flow hood for different time intervals. At the end of the exposure, the plates were incubated at 30 °C and the colony count was determined after 3 days. The UV intensity was determined using a UV monitor (RX003; UVI Tech). Cultures of *B. licheniformis* and *B. pumilus* type strains were used as controls in these experiments. The results indicate that the four novel strains were more UV-resistant than *B. licheniformis* MTCC 429^T and *B. pumilus* MTCC 1640^T (see Supplementary Table S1 in IJSEM Online).

Phylogenetic analysis

The 16S rRNA gene was amplified from genomic DNA, purified and sequenced as described earlier (Shivaji *et al.*, 2000). To ascertain the phylogenetic affiliation of the novel strains, the almost-complete 16S rRNA gene sequences of the four isolates were aligned with related species of the genus *Bacillus* using CLUSTAL W (Thompson *et al.*, 1994). Pairwise evolutionary distances were computed using the DNADIST program with the Kimura two-parameter model, as developed by Kimura (1980). Phylogenetic trees were constructed using the UPGMA and neighbour-joining tree-making algorithms of the PHYLIP package (Felsenstein, 1993). Stability among the clades of the phylogenetic tree was assessed by taking 1000 replicates and analysing the

Table 2. Phenotypic and chemotaxonomic differences between *B. altitudinis* sp. nov. 41KF2b^T and *B. pumilus* MTCC 1640^T

Both strains show the following characteristics: aerobic growth, white colony colour; motile, Gram-positive and rod-shaped cells; positive for catalase, oxidase, β-galactosidase and amylase activities; positive for aesculin hydrolysis; negative for reduction of nitrate to nitrite and indole production; positive for lysine decarboxylase and phenylalanine deaminase activities; do not produce gas from D-glucose; acid production from D-glucose, D-arabinose, mannitol and D-xylose; tolerance to 2 % NaCl; growth between pH 6 and pH 8; growth between 20 and 40 °C; growth on peptone agar; ability to utilize sucrose, D-trehalose, starch and *N*-acetylgalactosamine; inability to utilize citric acid, sodium acetate, sodium formate, sodium succinate, cellulose, L-alanine, L-threonine, L-lysine and L-arginine; sensitive to norfloxacin (10 μg), penicillin (10 μg), cefoperazone (75 μg), cefuroxime (30 μg), cephotaxime (10 μg), kanamycin (30 μg), co-trimoxazole (25 μg), tetracycline (30 μg), streptomycin (25 μg) and nalidixic acid (30 μg), and resistant to colistin (10 μg). +, Positive; −, negative; R, resistant; S, sensitive. All data are from the present study.

Characteristic	*B. altitudinis* sp. nov. 41KF2b^T	*B. pumilus* MTCC 1640^T
Growth at:		
pH 5	+	−
8 °C	+	−
45 °C	+	−
Hydrolysis of:		
Casein	−	+
Gelatin	+	−
Starch	+	−
Utilization of citrate	−	+
Lipase	−	+
Degradation of tyrosine	+	−
Voges–Proskauer test	−	+
Carbon source utilization:		
N-Acetylglucosamine	+	−
D-Arabinose	+	−
D-Cellobiose	+	−
Dulcitol	+	−
Glycerol	+	−
myo-Inositol	+	−
Inulin	+	−
Polyethylene glycol	+	−
Pyruvate	+	−
D-Raffinose	−	+
D-Rhamnose	+	−
D-Sorbitol	+	−
L-Sorbose	+	−
Thioglycolate	+	−
Amino acid utilization:		
L-Glycine	+	−
L-Tyrosine	+	−
Antibiotics (μg per disc):		
Amikacin (30)	R	S
Amoxycillin (30)	S	R
Ampicillin (25)	R	S
Ciprofloxacin (30)	R	S
Lincomycin (15)	R	S
Novobiocin (30)	R	S
Polar lipids	PE, PG, DPG	PE, DPG
DNA G+C content (mol%)	43	45

dataset using the SEQBOOT, DNADIST, NEIGHBOR and CONSENSE programs from the PHYLIP package.

Phylogenetic analysis based on 16S rRNA gene sequence analysis indicated that strains 24K^T, 28K^T and 41KF2a^T are closely related to one another (>98 % gene sequence similarity) and to *B. licheniformis* LMG 18422^T (98–99 %) and *B. sonorensis* NRRL B-23154^T (98 %). Strain 41KF2b^T shows 100 % sequence similarity with *B. pumilus* TUT 1009. The neighbour-joining phylogenetic tree further confirmed that the strains are phylogenetically related to species of *Bacillus* and that novel isolates 24K^T, 28K^T and 41KF2a^T form a clade with *B. licheniformis* LMG 18422^T and *B. sonorensis* NRRL B-23154^T, whereas strain 41KF2b^T forms a separate clade with *B. pumilus* TUT 1009 (Fig. 1). However, these strains exhibit phenotypic and chemotaxonomic differences amongst themselves and from their nearest

Table 3. Fatty acid content of strains 24K^T, 28K^T, 41KF2a^T, 41KF2b^T, *B. licheniformis* MTCC 429^T and *B. pumilus* MTCC 1640^T

Strains: 1, *B. aerius* sp. nov. 24K^T; 2, *B. aerophilus* sp. nov. 28K^T; 3, *B. stratosphericus* sp. nov. 41KF2a^T; 4, *B. altitudinis* sp. nov. 41KF2b^T; 5, *B. licheniformis* MTCC 429^T; 6, *B. pumilus* MTCC1640^T. All cultures were grown to late exponential phase in nutrient broth at 25 °C and the cell pellets were used for fatty acid analysis. Values are percentages of total fatty acids. −, Fatty acid not detected.

Fatty acid methyl ester	1	2	3	4	5	6
iso-$C_{14:0}$	1·2	0·6	0·4	−	0·4	−
iso-$C_{15:0}$	18·6	18·0	14·2	57·0	34·8	29·73
anteiso-$C_{15:0}$	18·0	40·2	23·6	32·8	26·5	46·52
$C_{15:1}$	−	0·9	−	−	−	−
iso-$C_{16:0}$	5·5	2·5	3·9	0·6	2·6	2·48
$C_{16:0}$	2·8	3·6	5·5	0·9	3·4	2·7
$C_{16:1}$ 9 *cis*	5·8	−	2·0	−	−	−
$C_{16:1}$ 11 *cis*	5·6	10·1	10·4	1·8	2·1	5·35
iso-$C_{17:0}$	25·0	19·3	29·8	4·9	15·6	13·26
anteiso-$C_{17:0}$	10·1	2·0	4·8	1·1	9·2	−
$C_{18:0}$	1·7	1·1	1·5	−	0·4	−
$C_{18:1}$	1·3	1·1	1·5	−	−	−

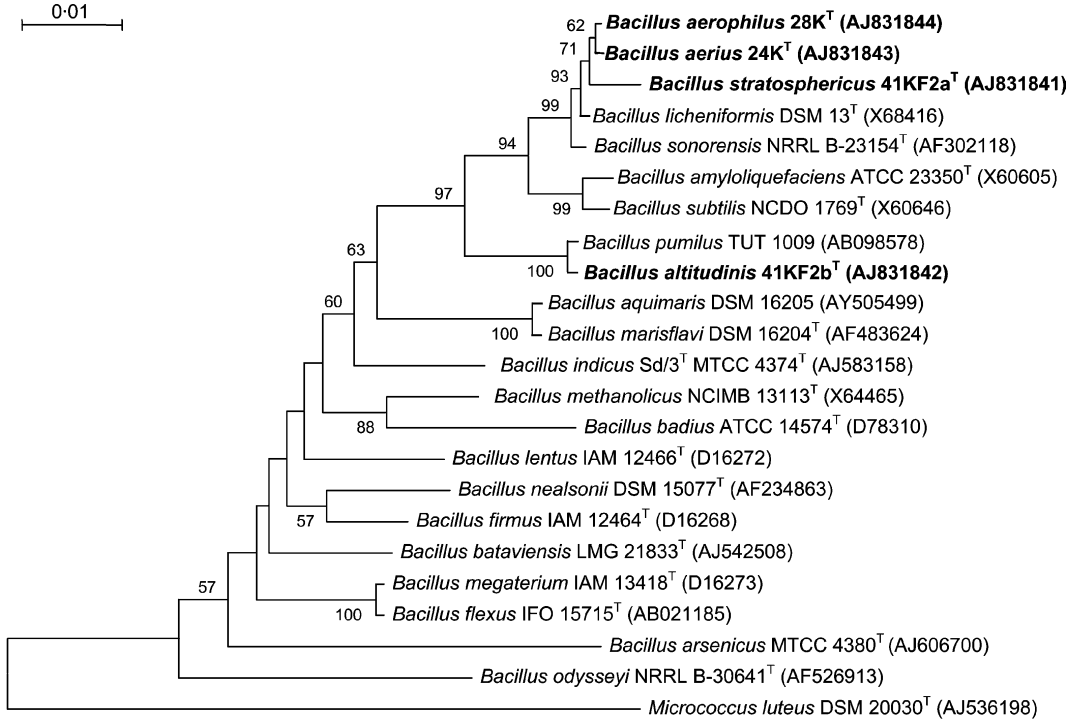

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences (1440 bases) showing the phylogenetic relationship between *B. aerius* sp. nov. 24K^T, *B. aerophilus* sp. nov. 28K^T, *B. stratosphericus* sp. nov. 41KF2a^T and *B. altitudinis* sp. nov. 41KF2b^T and closely related species of *Bacillus*. *Micrococcus luteus* DSM 20030^T was used as an outgroup in the tree. Bootstrap values (expressed as percentages of 1000 replications) >50 % are given at nodes. Bar, 1 substitution per 100 nucleotides.

phylogenetic neighbour (Tables 1, 2 and 3 and Supplementary Table S1), thus implying that they are different. In fact, DNA–DNA hybridization studies indicate that the relatedness between strains 24K^T and 28K^T is 39 %, with 42 % relatedness between strains 24K^T and 41KF2a^T and 10 % between strains 28K^T and 41KF2a^T. Further, strains 24K^T, 28K^T and 41KF2a^T exhibit 37, 39 and 65 % DNA–DNA relatedness with *B. licheniformis* LMG 18422^T and 25, 8 and 13 % relatedness with *B. sonorensis* NRRL B-23154^T, respectively. Strain 41KF2b^T exhibits 55 % relatedness with *B. pumilus* TUT 1009. Thus, based on phenotypic and chemotaxonomic (lipid and fatty acid content) differences and <70 % relatedness at the DNA–DNA level, it is proposed that strains 24K^T, 28K^T, 41KF2a^T and 41KF2b^T represent novel species and the names *Bacillus aerius* sp. nov., *Bacillus aerophilus* sp. nov., *Bacillus stratosphericus* sp. nov. and *Bacillus altitudinis* sp. nov. respectively, are proposed.

It is interesting to speculate on the possible origin of the four novel strains identified in this study. It is possible that the four novel strains are not contaminants carried from the Earth during the balloon flight or subsequent processing of the samples. By theoretical analysis of some 3·5 million organic compounds listed in Beilstein (http://www.beilstein.com), it has recently been argued (Morowitz *et al.*, 2000) that, if carbon- and water-based life exists anywhere else, it would have the same basic pattern of intermediary metabolism that is found in living organisms on our planet. This would make it likely that prokaryotes that may have evolved elsewhere would be similar to those found here. It is therefore tempting to speculate that strains 24K^T, 28K^T, 41KF2a^T and 41KF2b^T are a gift to us from space, but other explanations, such as their terrestrial origin, cannot be ruled out.

The possible Earth origin of micro-organisms in extra-terrestrial samples is further supported by data indicating routine meteorite exchanges between Earth and Mars (Gladman *et al.*, 1996), the probability that bacterial spores could survive interplanetary transfer (Mileikowsky *et al.*, 2000; Nicholson *et al.*, 2000) and the likelihood that certain bacterial species are resistant to current sterilization protocols and are therefore carried by spacecraft from Earth. In fact, with this in mind, robotic spacecraft used to search for life on other planets are assembled in clean rooms with controlled air circulation and are sterilized using a number of methods, including hydrogen peroxide vapour and UV radiation (Chung *et al.*, 2000). Despite these stringent sterilization protocols, a number of microbial species have

been isolated from the NASA Jet Propulsion Laboratories Spacecraft Assembly Facility (JPL-SAF) (Venkateswaran *et al.*, 2001) and from the Mars Odyssey spacecraft and its encapsulation facility (La Duc *et al.*, 2003). The predominant isolates belonged to the spore-forming genus *Bacillus* and were closely related to *B. pumilus* (Link *et al.*, 2004; Venkateswaran *et al.*, 2001). Many of these strains of *B. pumilus* were significantly more UV-resistant than previously isolated strains and one of these strains exhibited the highest degree of UV-resistance when compared with all known *Bacillus* species (Link *et al.*, 2004). These studies also led to the discovery of two novel species of *Bacillus*, *Bacillus nealsonii*, isolated from a spacecraft assembly facility, and *Bacillus odysseyi*, which were resistant to UV radiation (La Duc *et al.*, 2004; Venkateswaran *et al.*, 2003). Therefore, the possibility that the four novel strains, 24K^T, 28K^T, 41KF2a^T and 41KF2b^T, which are endospore-forming *Bacillus* species, escaped the stringent sterilization protocols (which included acetone wash, ethanol rinse, autoclaving and baking at 150 °C for 1 h) used to sterilize the cryotubes cannot be ruled out. The greater UV-resistance observed in the four novel strains compared with strains of *B. licheniformis* and *B. pumilus* (Supplementary Table S1 in IJSEM Online) may be related to their isolation from altitudes >24 km where UV radiation is likely to be more intense than on the ground. Strains 24K^T, 28K^T, 41KF2a^T and 41KF2b^T, which differ from their nearest phylogenetic neighbours *B. licheniformis*, *B. sonorensis* and *B. pumilus*, are proposed as representatives of four novel species of *Bacillus*.

Description of *Bacillus aerius* sp. nov.

Bacillus aerius (ae′ri.us. L. masc. adj. *aerius* pertaining to the air, aerial).

Colonies on nutrient agar are white, irregular, raised and 3–5 mm in diameter. Growth occurs at 8–37 °C, but not at 40 °C. Growth occurs between pH 6 and 10, but not at pH 4 or pH 11. Tolerates up to 11·6 % NaCl. Resistant to UV radiation. Grows on peptone. Positive for arginine decarboxylase activity and negative for arginine dihydrolase activity. Produces acid from a number of substrates and utilizes a number of sugars, amino acids and other carbon compounds as sole carbon sources (Table 1). The type strain is sensitive to tobramycin (15 μg), lomefloxacin (30 μg), roxithromycin (30 μg), streptomycin (25 μg) and novobiocin (30 μg) and resistant to penicillin (10 μg), nalidixic acid (30 μg), ampicillin (25 μg), kanamycin (30 μg), colistin (10 μg), novobiocin (30 μg), co-trimoxazole (25 μg), vancomycin (30 μg), tetracycline (30 μg), chloramphenicol (30 μg), erythromycin (15 μg), norfloxacin (10 μg), ciprofloxacin (30 μg), lincomycin (15 μg), cefoperazone (75 μg), amikacin (30 μg), cefuroxime (30 μg) and amoxycillin (30 μg). The major fatty acids are iso-$C_{17:0}$, iso-$C_{15:0}$, anteiso-$C_{15:0}$, anteiso-$C_{17:0}$, iso $C_{16:0}$, $C_{16:1}$ 9 *cis* and $C_{16:1}$ 11 *cis* (Table 3). The lipids present are PE, PG, DPG and two unknown phospholipids. The DNA G+C content is 45 mol%.

The type strain, 24K^T (=MTCC 7303^T=JCM 13348^T), was isolated from cryogenic tubes used for collecting air samples from high altitudes.

Description of *Bacillus aerophilus* sp. nov.

Bacillus aerophilus (ae.ro.phi′lus. Gr. n. *aêr* air; Gr. adj. *philos* loving; N.L. masc. adj. *aerophilus* air-loving).

Colonies on nutrient agar are white, irregular, raised and 5–8 mm in diameter. Growth occurs at 8–37 °C, but not at 40 °C. Growth occurs between pH 6 and pH 10, but not at pH 5 or pH 11. Tolerates up to 11·6 % NaCl. Resistant to UV radiation. Grows on peptone. Positive for arginine decarboxylase and arginine dihydrolase activities. Produces acid from a number of substrates and utilizes a number of sugars, amino acids and other carbon compounds as sole carbon sources (Table 1). The type strain is sensitive to tobramycin (15 μg), lomefloxacin (30 μg), roxithromycin (30 μg), lincomycin (15 μg), tetracycline (30 μg) and streptomycin (25 μg) and resistant to amikacin (30 μg), ampicillin (25 μg), nalidixic acid (30 μg), penicillin (10 μg), kanamycin (30 μg), co-trimoxazole (25 μg), vancomycin (30 μg), chloramphenicol (30 μg), erythromycin (15 μg), norfloxacin (10 μg), colistin (10 μg), novobiocin (30 μg), ciprofloxacin (30 μg) cefoperazone (75 μg), cefuroxime (30 μg) and amoxycillin (30 μg). The major fatty acids are anteiso-$C_{15:0}$, iso-$C_{17:0}$, iso-$C_{15:0}$ and $C_{16:1}$ 11 *cis* and (Table 3). The lipids present are PE, PG, DPG and two unknown phospholipids. The DNA G+C content is 44 mol%.

The type strain, 28K^T (=MTCC 7304^T=JCM 13347^T), was isolated from cryogenic tubes used for collecting air samples from high altitudes.

Description of *Bacillus stratosphericus* sp. nov.

Bacillus stratosphericus (stra.to.sphe.ri′cus. N.L. fem. n. *stratosphera* stratosphere; L. suff. -*icus* adjectival suffix used with the sense of belonging to; N.L. masc. adj. *stratosphericus* belonging to the stratosphere).

Colonies on nutrient agar are white, irregular, raised and 3–5 mm in diameter. Growth occurs between 8 and 37 °C, but not at 40 °C. Growth occurs between pH 6 and pH 10, but not at pH 5 or pH 11. Tolerates up to 17·4 % NaCl. Resistant to UV radiation. Shows growth on peptone. Positive for arginine decarboxylase activity and negative for arginine dihydrolase activity. Produces acid from a number of substrates and utilizes a number of sugars, amino acids and other carbon compounds as sole carbon sources (Table 1). Sensitive to tobramycin (15 μg), lomefloxacin (30 μg), roxithromycin (30 μg), amikacin (30 μg), ciprofloxacin (30 μg), streptomycin (25 μg), novobiocin (30 μg), ampicillin (25 μg) and nalidixic acid (30 μg) and resistant to penicillin (10 μg), kanamycin (30 μg), co-trimoxazole (25 μg), vancomycin (30 μg), chloramphenicol (30 μg), erythromycin (15 μg), norfloxacin (10 μg), cefoperazone (75 μg), cefuroxime (30 μg), lincomycin (15 μg), colistin

(10 μg) and amoxycillin (30 μg). The major fatty acids are iso-$C_{17:0}$, anteiso-$C_{15:0}$, iso-$C_{15:0}$, $C_{16:1}$ 11 *cis* and $C_{16:0}$ (Table 3). The lipids present are PE, PG, DPG and two unknown phospholipids. The DNA G+C content is 44 mol%.

The type strain, 41KF2a^T (=MTCC 7305^T=JCM 13349^T), was isolated from cryogenic tubes used for collecting air samples from high altitudes.

Description of *Bacillus altitudinis* sp. nov.

Bacillus altitudinis (al.ti′tu.di.nis. L. fem. n. *altitudo* altitude; L. fem. gen. n. *altitudinis* of altitude).

Colonies on nutrient agar are white, convex with a regular margin and 2–3 mm in diameter. Growth occurs between 8 and 45 °C and at pH 5–8. Tolerates up to 2 % NaCl. Degrades tyrosine, but tests negative for casein hydrolysis, urease and phenylalanine deaminase activities, reduction of nitrate to nitrite, utilization of citrate and the Voges–Proskauer test. Produces acid from mannitol. Utilizes D-trehalose, starch, *N*-acetylglucosamine, D-rhamnose, D-cellobiose, D-sorbitol, dulcitol, *myo*-inositol, *N*-acetylgalactosamine, L-glycine, L-threonine and L-lysine as sole carbon sources. Sensitive to norfloxacin (10 μg), penicillin (10 μg), cefoperazone (75 μg), cefuroxime (30 μg), kanamycin (30 μg), co-trimoxazole (25 μg), tetracycline (30 μg), nalidixic acid (30 μg) and amoxycillin (30 μg), but resistant to amikacin (30 μg), ciprofloxacin (30 μg), lincomycin (15 μg), novobiocin (30 μg) and ampicillin (25 μg). Major fatty acids are iso-$C_{15:0}$, anteiso-$C_{15:0}$ and iso-$C_{17:0}$ (Table 3). The lipids present are PE, PG, DPG and two unknown phospholipids. The DNA G+C content is 43 mol%.

The type strain, 41KF2b^T (=MTCC 7306^T=JCM 13350^T), was isolated from cryogenic tubes used for collecting air samples from high altitudes.

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