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BACILLUS STRATOSPHEARICUS SP. ISOLATED FROM AIR SAMPLES OF HIGH ALTITUDE

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ABSTRACT

A novel bacterial strains were isolated from air samples at altitudes of 30 km. The strain was identified as members of the genus Bacillus. Phylogenetic analysis based on 16S rRNA gene sequences indicated that the strain indicated a similarity of 98–99% with *Bacillus licheniformis* and 98% with *Bacillus sonorensis*. Strain 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 isolate represented novel species of *Bacillus stratosphericus sp. nov*.

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INTRODUCTION

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 help in determining the role of the various atmospheric strata in the transport of microorganisms from one part of the globe to another (Hoyle & Wickramasinghe, 1986, 1993, 1999, Love & Brownlee, 1993, 1936). Both bacteria and fungi have been found at altitudes of up to 85 km Lysenko, 1979).Harris et al. (2001) and Wainwright et al. (2003) detected bacteria from stratospheric air samples collected at 41 km . In this paper, a polyphasic taxonomic approach was used to characterize bacterial strains from cryotubes that were used to collect air at altitudes between 30 and 33 km from Sadhana Pass, India. The strain representnovel species of the genus Bacillus.

Collection of air from 24 km and above

Air was collected using a cryogenic sampler at an altitude of 31 km. The samples 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 mm and then through a 0.22 mm 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 there was no growth even after 20 days,the filter was transferred to blood agar medium..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 hrs in a shaker. The liquid was then removed using sterile tubing and a syringe and filtered sequentially through 0.45 and 0.22 mm 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 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 to41 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 one isolate.

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 strain was ascertained using a Leitz

Diaplan phase-contrast microscope with an oil immersion objective (6100). The sensitivity of the culture 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 containing (l21) 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 429T, *Bacillus pumilus* MTCC 1640T and *Bacillus* sonorensis DSM 13779T were used as controls in studies related to biochemical tests, identification of fatty acids, polar lipids and DNA–DNA hybridization. The isolate was Gram-positive, rod-shaped, endospore-forming and catalase-positive bacteria with iso-C15 : 0, anteiso-C15 : 0, iso-C16 : 0, C16 : 0, C16 : 1 11 cis, iso-C17 : 0 and ant eiso-C17 : 0 as the predominant fatty acids. The lipids present includes phosphatidylethanolamine (PE), phosphatidylglycerol (PG), diphosphatidylglycerol (DPG) . The DNA G+C content of the four strains was 44–45 mol%. All these characteristics indicate that the novel strain is members of the genus *Bacillus* (see Tables 1–2).

Phylogenetic analysis

The 16S rRNA gene was amplified from genomic DNA, purified and sequenced (Shivaji et al.,2000). To ascertain the phylogenetic affiliation of the novel strain, the almost-complete 16S rRNA gene sequences of the isolate was aligned with related species of the genus *Bacillus* using CLUSTAL W (Thompson et al., 1994).Pairwise evolutionary distances were computed using theDNADIST program with the Kimura two-parameter model,as developed by Kimura (1980). Phylogenetic trees were constructed using the UPGMA and neighbour-joining treemaking algorithms of the PHYLIP package (Felsenstein,1993).

Phylogenetic analysis based on 16S rRNA gene sequence analysis indicated that strains PHR1 is closely related to *B. licheniformis* LMG 18422T (98–99 %)and *B. sonorensis* NRRL B-23154T (98 %). The neighbour-joining phylogenetic tree further confirmed that the strain was phylogenetically related to species of Bacillus and that novel isolate PHR1 form a clade with B. licheniformis LMG 18422T and B.sonorensis NRRL B-23154T, (Fig 1) However, these strains exhibit phenotypic and chemotaxonomic differences amongst themselves and from their nearest phylogenetic neighbour (Tables 1and 2), thus implying that they are different. Infact, DNA–DNA hybridization studies indicate that the relatedness between strain PHR 1 exhibit 65% DNA–DNA relatedness with *B. licheniformis* LMG 18422T and 13% relatedness with *B. sonorensis* NRRL B-23154T, respectively. Thus, based on phenotypic and chemotaxonomic (lipid and fatty acid content) differences and <70% relatedness at the DNA–DNA level, it is proposed that strain PHR1 represent novel species and proposed name is *Bacillus stratosphericus sp. nov*. It is interesting to speculate on the possible origin of the novel strain identified in this study. It is possible that the four novel strains are not contaminants carried from the Earth . By theoretical analysis of some 3.5 million organic compounds listed in Beilstein(http://www.beilstein .com)PHR1 is a gift to us from space, but other explanations, such as their terrestrial origin, cannot be ruled out.

Description of Bacillus stratosphericus sp. nov.Bacillus stratosphericus 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. 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 mg), lomefloxacin(30 mg), roxithromycin (30 mg), amikacin (30 mg), ciprofloxacin(30 mg), streptomycin (25 mg), novobiocin (30 mg),ampicillin (25 mg) and nalidixic acid (30 mg) and resistant to penicillin (10 mg), kanamycin (30 mg), co-trimoxazole (25 mg), vancomycin (30 mg), chloramphenicol (30 mg),erythromycin (15 mg), norfloxacin (10 mg), cefoperazone(75 mg), cefuroxime (30 mg), lincomycin (15 mg).

Table 1

Phenotypic and chemotaxonomic characteristics of Bacillus stratosphericus.

Characteristic	1	2	3
Colony size (mm)	3–5	5–7	3–5
Colony shape	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	−	+	+
L-Tryptophan	+	+	−
Antibiotic test (µg per disc):			
Amikacin (30)	S	S	S
Amoxycillin (30)	R	S	S
Ampicillin (25)	S	S	S
Cefoperazone (75)	R	S	S
Cefuroxime (30)	R	S	S
Chloramphenicol (30)	R	R	S

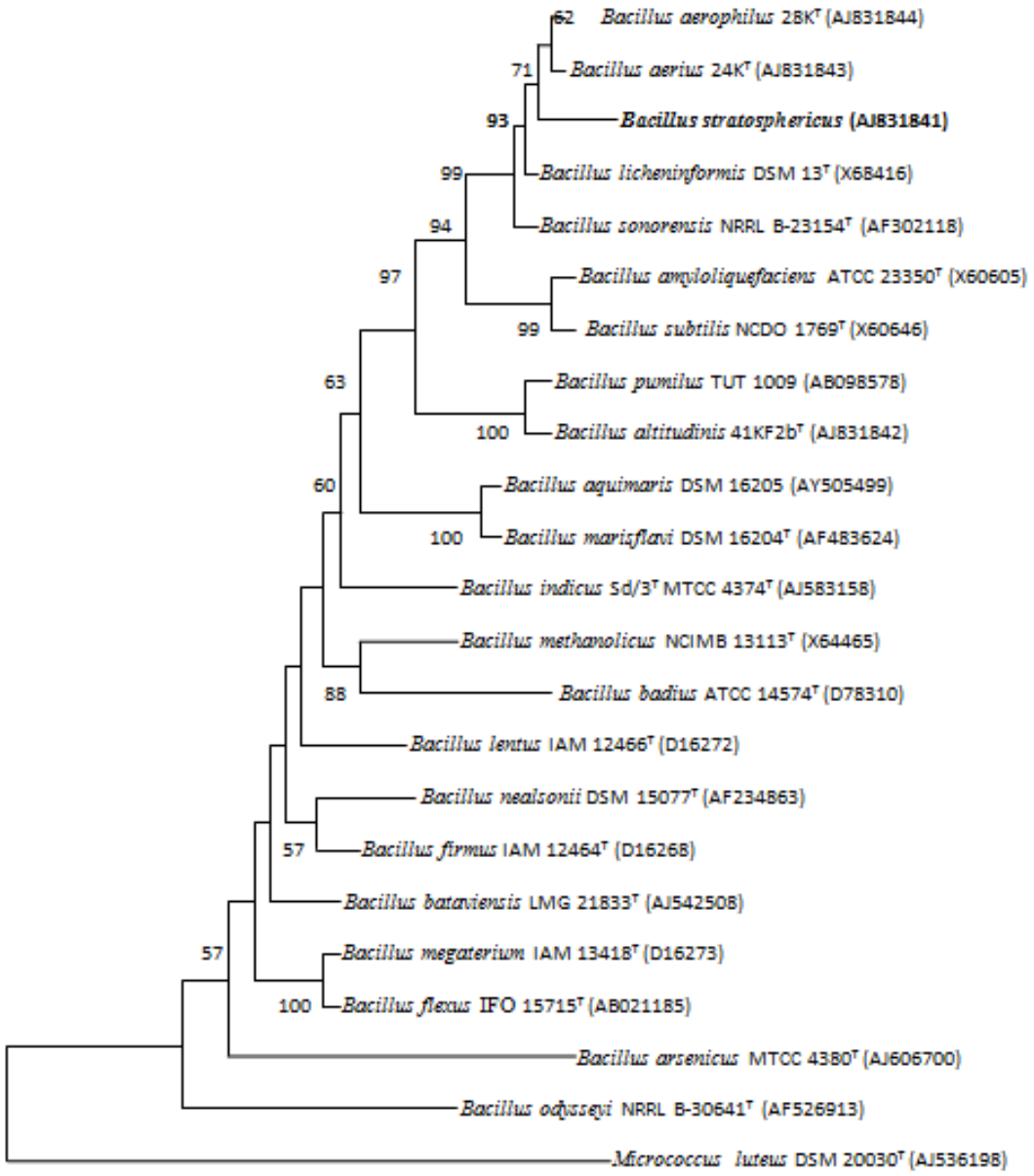
Ciprofloxacin (30)	S	S	S
Colistin (10)	R	S	R
Co-trimoxazole (25)	R	R	S
Kanamycin (30)	R	R	S
Lincomycin (15)	R	S	S
Nalidixic acid (30)	S	S	S
Norfloxacin (10)	R	S	R
Novobiocin (30)	S	S	S
Penicillin (10)	R	R	S
Tetracycline (30)	S	S	S
Polar lipids	PE, PG, DPG	PE, DPG	PE, DPG
DNA G+C content (mol %)	44	45	46

Table 2

Fatty acid methyl ester composition of Bacillus stratosphericus, Bacillus licheniformis and Bacillus pumilus.

Fatty acid methyl ester	1	2	3
iso-$C_{14:0}$	-	0.4	-
iso-$C_{15:0}$	57.0	34.8	29.73
anteiso-$C_{15:0}$	32.8	26.5	46:52
$C_{15:1}$	-	-	-
iso-$C_{16:0}$	0.6	2.6	2.48
$C_{16:0}$	0.9	3.4	2.7
$C_{16:1}$ 9 *cis*	-	-	-
$C_{16:1}$ 11 *cis*	1.8	2.1	5.35
iso-$C_{17:0}$	4.9	15.6	13.26
anteiso-$C_{17:0}$	1.1	9.2	-
$C_{18:0}$	-	0.4	-
$C_{18:1}$	-	-	-

Table 3
Phylogenetic tree for Bacillus Stratosphericus



CONCLUSION

The above isolated microbe Bacillus stratospheicus can be expoited for the production of microbial cell batteries which can easily serve as a alternative for meeting the never ending demand of electricity.

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