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EXPERIMENTAL ARTICLES

Isolation of Bacteria of the Genus *Paenibacillus* from Soil and Springs of the Valley of Geysers (Kamchatka)

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Abstract—Strains of rod-shaped, facultatively anaerobic, spore-forming bacteria exhibiting negative Gram reaction were revealed after inoculation of soil, water, and silt samples from springs of the Valley of Geysers (Kamchatka) onto complete media at pH 5.0, 7,0, and 9.0 and cultivation at 28–30°C. The bacterial isolates differed in their phenotypic characteristics and in the G + C content of genomic DNA, which ranged from 41.1 to 53 mol %. The analysis of 16S rRNA gene nucleotide sequences of all isolated strains (GenBank EU497635–EU497641) revealed their close homologues among the known species of the genus *Paenibacillus*. At the same time, all the studied bacilli (Dg-824, Dg-904, Dg-1009, Gi-662, Gi-724, K-58, etc.) differed significantly from the nearest phylogenetic neighbors in their phenotypic characteristics, including the Gram reaction, the DNA G + C content, and a number of other determinative characteristics; therefore, they could not be assigned to previously known species.

Key words: the Valley of Geysers, gram-negative bacilli, phenotypic characteristic

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Until 1990, aerobic, gram-positive, endospore-forming bacteria were assigned to the genus *Bacillus* according to their phenotypic characteristics. Later, on the basis of phylogenetic analysis of the nucleotide sequences of genomic fragments, the genus *Bacillus* was divided into 12 genera, including the genus *Paenibacillus* [1]. Initially, this genus included aerobic or facultatively anaerobic endospore-forming microorganisms with DNA G + C content from 40 to 54 mol %, usually exhibiting a positive Gram reaction with a typical gram-positive structure of the cell wall [1]. The studies conducted over the last few years have discovered some representatives of the genus *Paenibacillus* exhibiting gram-variable or -negative staining [2–8]. The combination of gram-negative staining with the ability to form endospores is a rare phenomenon. Among the numerous publications on gram-positive, spore-forming bacteria, only a few mention endospore-forming bacteria, which are stained gram-negative at all or at some stages of growth and have a cell wall structure of either gram-positive or -negative type. For example, gram-negative nitrogen-fixing strains of *Paenibacillus borealis* form elliptical spores located terminally or subterminally [4]. Aerobic strain *Paenibacillus motobuensis* MC10T exhibits gram-negative staining but has a gram-positive type of the cell wall [2]. Vegetative cells of strain *Paenibacillus koreensis* YC300T are stained gram-positive, but the staining switches to gram-negative at the beginning of the spore formation [9]. The spore-forming strain *Bacillus* sp. NAF001 isolated from activated sludge contains gram-negative vegetative cells but some trichome fragments are stained gram-positive [10]. The ability to form endospores was revealed in a facultatively anaerobic gram-negative strain *Bacillus vireti* LMG 21834T [11], strains *Paenibacillus agaridevorans* sp. nov. and *P. agarexedens* sp. nov., nom. rev. [12], *P. wynnii* sp. nov. [13], *Alicyclobacillus sendaiensis, Ureibacillus* gen. nov. [14], *Thermobacillus xylanilyticus* sp. nov. [15], *P. hodogayensis* sp. nov. [16], *P. koleovorans* sp. nov. [17], and *Bacillus subterraneus* sp. nov. [18]. Alkaliphilic strains *Bacillus hortis* sp. nov. K13T and K38 isolated from soil in Japan formed subterminal elliptical spores and were gram-negative [19]. As a rule, publications contain results of Gram staining and only a few describe the cell ultrastructure [2, 4, 14, 18, 19].

In the course of microbiological studies of the samples of soil, water, and silt from the springs of the Valley of Geysers (Kamchatka), we obtained about 2500 microbial isolates represented by microorganisms of different taxonomic groups, including spore-forming bacteria of the genera *Bacillus, Paenibacillus, Geobacillus*, and *Brevibacillus*. Most of the isolated bacilli

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stained gram-positive with the exception of gram-negative endospore-forming isolates Dg-824, Dg-904, Dg-1009, Gi-662, Gi-691, Gi-724, Gi-733, Gi-739, K-58, and K-59.

The present work was aimed at studying phenotypic and molecular genetic characteristics of spore-forming gram-negative bacteria from the Valley of Geysers and assigning their taxonomic position.

MATERIALS AND METHODS

Physiological and biochemical characteristics of the isolates were studied by standard methods [20, 21]. Morphological features of strains were investigated using an Axioskop 40 light microscope (Carl Zeiss, Germany) and a Hitachi H-600 electron microscope (Japan).

Ability of the strains to grow at different temperature and pH values was studied using LB medium made of trypton (Difco, United States), 10 g/l; yeast extract (Difco, United States), 5 g/l; and NaCl, 10 g/l. Growth was assayed by measuring the optical density (OD) of cell suspensions at 550 nm with an SF-46 spectrophotometer (Russia). Each experiment was carried out in triplicate; the averaged values were used.

Antibiotic resistance of the strains was tested by using the disc method [22]. Discs (NITsF, St. Petersburg, Russia) contained (μg/disc): rifampicin, 5; penicillin, oxacillin, ampicillin, and gentamycin, 10 each; oleandomycin, erythromycin, and lincomycin, 15 each; streptomycin, neomycin, kanamycin, monomycin, tetracycline, levomycetin, and ristomycin, 30 each; carbenicillin, 100 each; and polymyxin, 300 U.

Determination of the DNA G + C content. High-molecular-weight DNA was isolated from microbial cells by the Marmur method [23]. The G + C content was determined using a formula adapted to the solutions with low ionic force: $(G + C) = 2.08\ T_m - 106.4$. DNA from *Escherichia coli* B was used as an internal standard.

Analysis of nucleotide sequences of the PCR products corresponding to the 16S rRNA gene. Total DNA from pure cultures was isolated by method described in [24]. The 16S rRNA gene fragment was PCR-amplified using 16S rRNA primers specific to eubacteria: 5'-AGAGTTTGATCCTGGCTCAG-3' and 5'-CGGCTACCTTGTTACGACTT-3'. Nucleotide sequences of the PCR products were determined by the Sanger method using a BigDye 3.1 Terminator Cycle Sequencing Kit and an automatic ABI 3130x1 DNA genetic analyzer (Applied Biosystems, United States) at the Interinstitute Center for DNA Sequencing, Siberian Branch, Russian Academy of Sciences (Novosibirsk) (GenBank accession no. EU497635–EU497641).

Phylogenetic analysis was performed with the aid of the MEGA (version 4) software package [25] and GenBank database (http://www.ncbi.nlm.nih.gov) as a source for DNA sequences of closely related species.

RESULTS AND DISCUSSION

Isolation of the pure cultures. The samples of soil, water, and silt were collected in August 2004 and 2006 from the sites of the Valley of Geysers (Kamchatka), which were far removed from each other and differed in pH, temperature, floristic diversity, and other characteristics. Gram-negative, spore-forming bacteria were isolated after plating the samples onto fish nutrient agar (FNA) (pH 5.0, 7.0, and 9.0) and incubation at 28–30°C.

Morphological characteristics of cells and colonies. Cells of strains Dg-824, Dg-904, Dg-1009, Gi-662, Gi-691, Gi-733, Gi-739, Gi-724, K-58, and K-59 were motile regular rods (0.4–0.8 × 2–5 μm) with binary cell fission. Bacteria could be stained gram-negative at all stages of growth. Colonies of strains Gi-733, Gi-739, Gi-724, Gi-662, Gi-691, K-58, and K-59 grown on FNA at 30°C for 24–48 h were transparent or semitransparent, compact, small, and whitish or yellowish. Strains Dg-824, Dg-904, and Dg-1009 formed sprawling, flat colonies of irregular shape with a complicated surface structure (Fig. 1).

Ultrastructure of the cells. Electron microscopic examination of the studied strains revealed that all endospores' formation stages and envelopes during the stages were typical of spore-forming bacteria. Spores of strains Dg-824, Dg-904, Gi-733, and Gi-739 were characterized by the presence of additional surface structures shaped like longitudinal ridges, the number and exact form of structures depending on the strain (Fig. 2). Similar structures have been earlier revealed in the gram-negative microorganism *Paenibacillus borealis* [4] and in strain *P. motobuensis* MC10^T, which was characterized by gram-negative staining and a gram-positive structure of cell wall [2]. In the cells of the studied strains, elliptic endospores were located terminally or subterminally along the cell axis (strains Gi-662, Gi-691, Dg-904, Dg-1009, K-58, and K-59) or at an angle to it (strains Dg-824, Gi-733, Gi-739, and Gi-724). Sporangium was enlarged in spore-forming cells of all the studied strains, which is well revealed by phase contrast microscopy (Figs. 2a–2c).

The temperature range for the strain growth was 20–45 (Gi-724, Gi-733, Gi-739, Dg-824, Gi-904, and Gi-662), 20–42 (Gi-691, K-58, and K-59), and 20–55°C (Dg-1009). The optimum growth temperature was 30–42 (Gi-724, Gi-733, and Gi-739), 30–37 (Gi-691, Dg-824, and Dg-1009), 30 (Gi-662, K-58, and K-59), and 37°C (Gi-904) (Table 1). It should be noted that all the strains retained their viability after heating of the spore suspensions at 80°C for 10 min.

Strain growth at different pH. No growth of the studied strains was observed at pH 3.0 and 4.0 for 72 h. The pH range suitable for growth was 5.0–8.0 (Gi-691, Gi-724, Gi-733, and Gi-739), 5.0–9.0 (Gi-662),

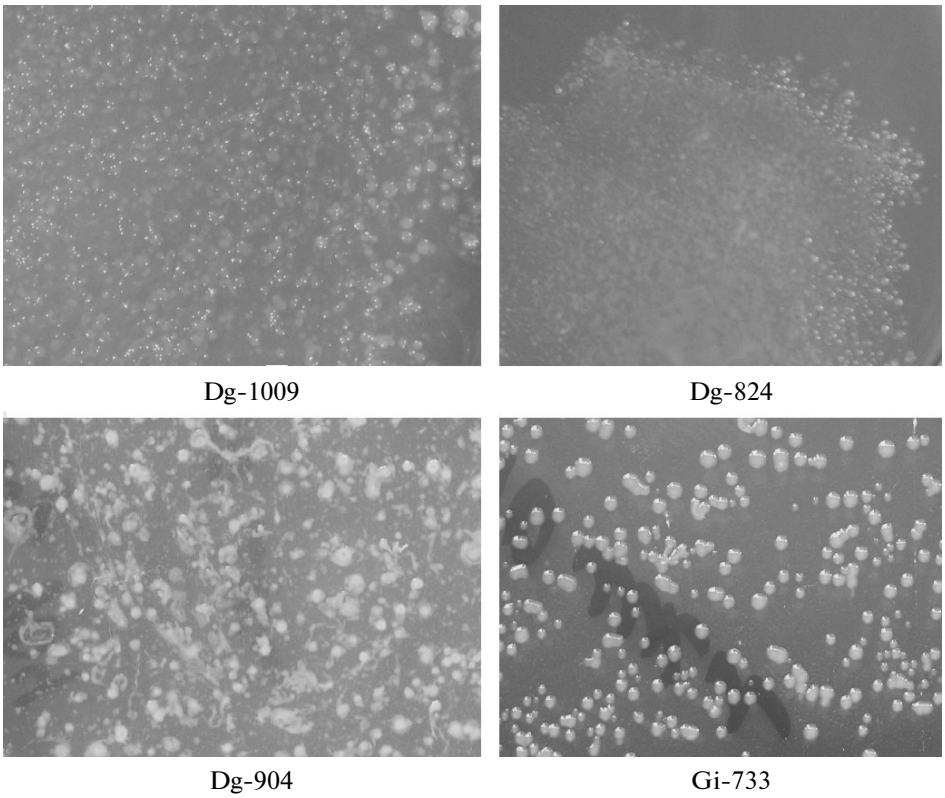

Fig. 1. Colony morphology of gram-negative, spore-forming bacteria isolated from the Valley of Geysers. Colonies of strains Dg-1009, Dg-824, and Dg-904 are represented by a fragment of a single giant colony with nonuniform surface; in the case of strain Gi-733, the image contains many small, round, separate colonies.

5.0–10.0 (Gi-904 and Dg-1009), and 5.0–12.0 (Gi-824). Strains K-58 and K-59 were isolated from the mud-bath of the Valley of Geysers at pH 3.5; however, optimal pH for their growth was 7.0–8.0, minimal pH was 6.0, and maximal pH was up to 11.0. The optimum pH for other strains was also near neutral, except for strain Dg-824 which grew actively at pH from 7.0 to 9.0 and strain Dg-1009 which retained high growth rate at pH from 5.0 to 7.0 (Table 1).

Resistance to increased concentrations of NaCl. Eight out of ten strains grew at a NaCl concentration up to 2.0%, six strains grew at salt concentrations up to 3.0%, and none of the strains were able to grow at 5% NaCl (Table 1). Strains K-58 and K-59 were the most sensitive to salt concentration, and their growth was inhibited at 1.0% NaCl, which is understandable, since they were isolated from water samples with low concentration of sodium ions (7 mg/ml as compared with 68–575 mg/ml in other samples).

Biochemical properties. All strains were catalase-, oxidase-, and esculin-positive; showed a positive reaction in the methyl red (MR) test; exhibited no lecithinase, lipolytic, phenylalanine deaminase, and arginine decarboxylase activity; were unable to hydrolyze casein and utilize acetate, citrate, and sorbitol; did not form indole; grew in the presence of 0.001% of lysozyme; and did not grow at 5°C. All strains except for Dg-904 and Dg-824 possessed hemolytic properties. The distinctive characteristics of the strains by which the strains could be divided into separate groups are summarized in Table 2. Some resemblance in the phenotypic characteristics was observed between strains Gi-733, Gi-739, and Gi-724, between strains Gi-691 and Gi-662, and between strains K-58 and K-59, as well as between strains Dg-824 and Dg-1009, confirming the nonrandomness of their corresponding phylogenetic similarity. Strain Dg-904 differed considerably in its properties from all other strains studied.

The antibiotic resistance of the strains. The studied strains were highly sensitive to neomycin, gentamycin, erythromycin, and ristomycin, as well as levomycetin (except for Gi-739) and rifampicin (except for Dg-824). They were resistant to ampicillin, polymyxin, and penicillin (except for Dg-1009 and Dg-904). Although all the strains inhabited sites free of anthropogenic impact, surprisingly about half of them possessed multiple resistance to antibiotics (Table 3).

Analysis of the G + C content of DNA. The data on the G + C content of chromosomal DNA of the studied strains were within the range known for bacteria of the genus *Paenibacillus* (41.1–53.0 mol %). No correlation was revealed between the phenotypic properties of the strains and the G + C content of DNA (Table 2).

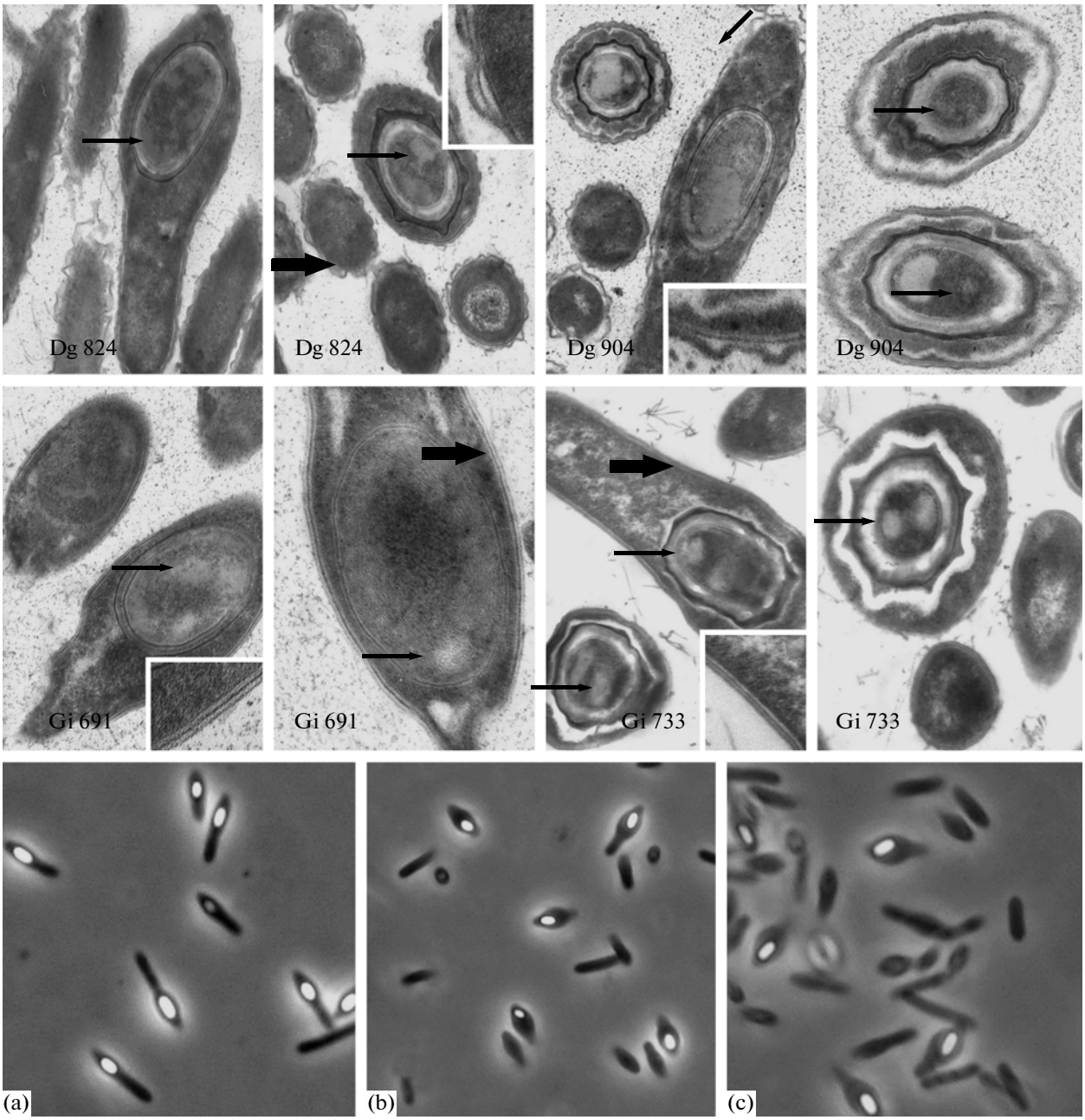

Fig. 2. Ultrathin cell sections of strains Dg-824, Dg-904 (×50000), Gi-691 (×60000), and Gi733 (×50000) at different stages of sporulation. The magnified fragments of bacterial cell wall are given in rectangular inserts (×120000). Phase contrast images of sporulating cells: (a) spores are located along the cell axis; (b) and (c) spores are located either along the cell axis or at angle to it (strains Gi-733 and Gi-739, respectively) (×2500).

Phylogenetic analysis. Comparative analysis of 16S rRNA gene nucleotide sequences of isolates Dg-824, Dg-904, Dg-1009, Gi-662, Gi-691, Gi-733, Gi-739, Gi-724, K-58, and K-59 revealed their close homology to the members of the genus *Paenibacillus*. Nucleotide sequences determined for some isolates (***K-58*** and K-59; ***Gi-724***, Gi-733, and Gi-739; and Gi-662 and ***Gi-691***) were identical; therefore, only one of the sequences (marked in bold italics) was submitted to GenBank and used for phylogenetic analysis. Comparative phylogenetic analysis was performed using sets of over 70 16S rRNA gene sequences known for representatives of the genus *Paenibacillus*. A typical phylogenetic tree constructed using a limited set of characteristic representatives of this genus with the highest homology to studied isolates is given in Fig. 3. The tree was generated by the neighbor-joining method; the trees constructed by using criteria of maximum parsimony, minimal evolution, and UPGMA algorithm had the same topology .

Table 1. The temperature, pH, and NaCl concentration ranges for growth of gram-negative, spore-forming bacteria isolated from the Valley of Geysers

Strain	Growth range						NaCl concentration (%)/strain growth			
	Temperature (°C)			pH						
	min	opt	max	min	opt	max	0.5	1.0	2.0	3.0
Dg-824	20	30–37	45	5.0	7.0–9.0	12.0	+	+	+	–
Dg-904	20	37	45	5.0	6.0–8.0	10.0	+	+	+	+
Dg-1009	20	30–37	55	5.0	5.0–7.0	10.0	+	+	+	+
Gi-662	20	30	45	5.0	6.0–7.0	9.0	+	+	+	–
Gi-691	20	30–37	42	5.0	6.0–7.0	8.0	+	+	+	+
Gi-724	20	30–42	45	5.0	6.0–7.0	8.0	+	+	+	+
Gi-733	20	30–42	45	5.0	6.0–7.0	8.0	+	+	+	+
Gi-739	20	30–42	45	5.0	6.0–7.0	8.0	+	+	+	+
K-58	20	30	42	6.0	7.0–8.0	11.0	+	–	–	–
K-59	20	30	42	6.0	7.0–8.0	11.0	+	–	–	–

Designations: (+) and (–) stand for "growth" and "no growth," respectively; min, opt, and max indicate minimal, optimal, and maximal values of the studied factors, respectively.

Note: None of the strains grew at 5% NaCl and at 15°C.

Strains Gi-724, Gi-733, and Gi-739 with similar phenotypic characteristics were phylogenetically close (over 99% homology) to the earlier described species *P. azoreducens* (AJ272249, *Paenibacillus* sp. CMI) [26] but differed in a number of properties, such as Gram staining, presence of oxidase activity, ability to reduce nitrate to nitrite and to hydrolyze rhamnose, inability to utilize D-arabinose and grow at 50°C, formation of colorless colonies, and different pH and temperature ranges for cell growth (Tables 1 and 2). **Strains Gi-662** and **Gi-691** with similar 16S rRNA gene sequences showed over 99% homology with gram-positive bacterium *P. amylolyticus* (X60606) [27], but differed from it in other essential characteristics, like gram-negative staining, inability to reduce nitrates, hydrolysis of esculin but not of casein or gelatin, presence of oxidase and urease activities, acid production from rhamnose and lactose, and ability to grow at 40°C and in the presence of 0.001% lysozyme. **Strain Dg-824** showed over 99% homology in 16S rRNA gene sequence with *P. lautus* (AB073188) but differed in fundamental phenotypic characteristics, including Gram staining; DNA G + C content; ability to hydrolyze gelatin, starch, and sorbitol; oxidase activity; production of acetyl methyl carbinol; and others (Tables 1 and 2). Phenotypic characteristics of **strain Dg-904,** such as the DNA G + C content; Gram staining; hydrolysis of casein, gelatin, and starch; production of indole; and cell growth at pH 5.7 also differed from those of phylogenetically homologous (over 99%) strain *P. alvei* (AJ320491) (Tables 1 and 2).

Isolate **Dg-1009** showed high homology (over 98%) with strain *P. cookii* (AJ250317) isolated from volcanic soils of an active fumarole at a temperature of 30–60°C (Antarctica, region of geothermal activity, scoria cone of the Lucifer Hill crater) [28]. These strains were similar in many phenotypic characteristics. However, **strains Dg-1009** and *P. cookii* differed in DNA G + C content (41.1 and 51.6 mol %, respectively). The strains were gram-negative at all stages of cultivation, were unable to utilize starch and produce acid from

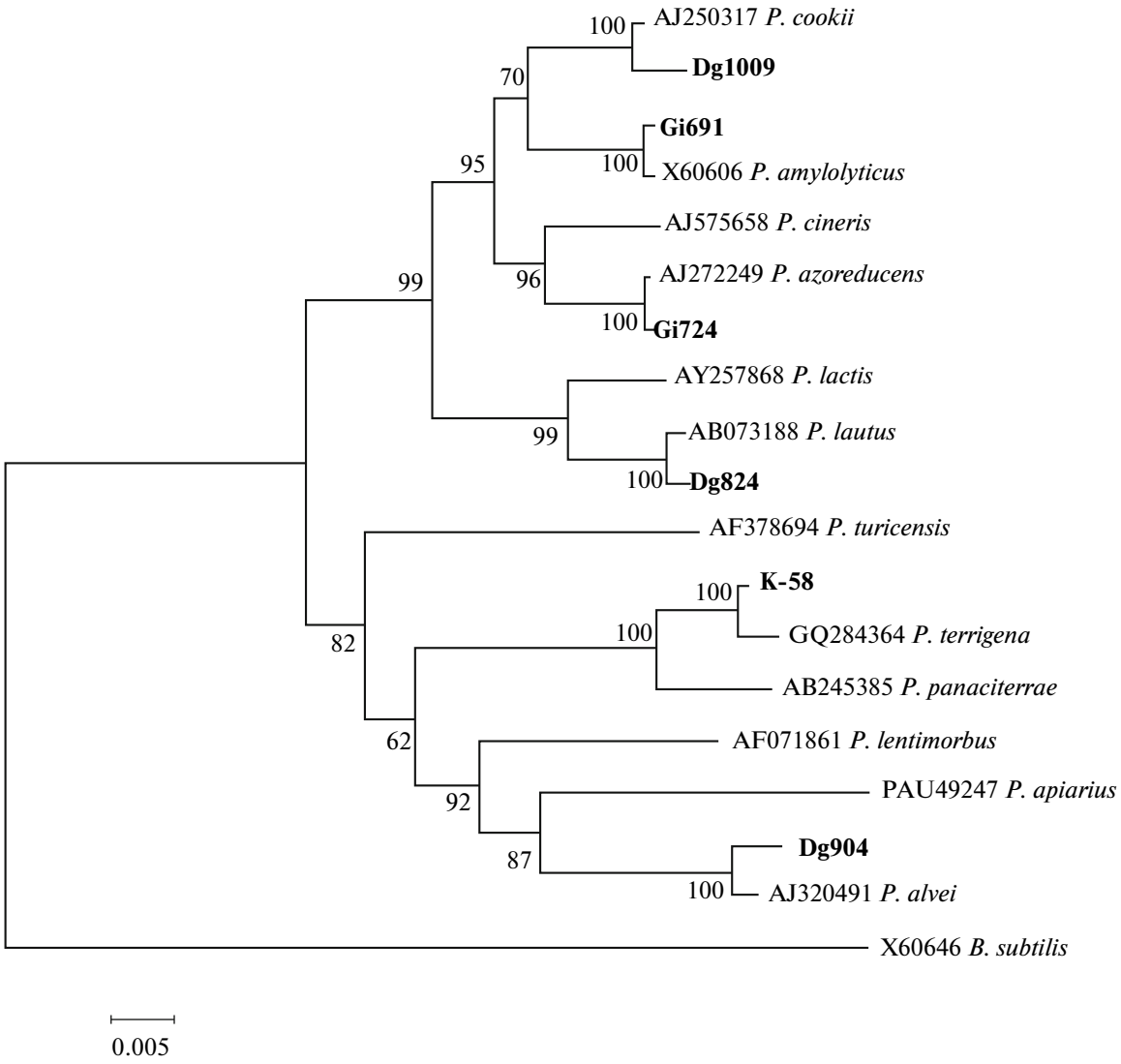

Fig. 3. Phylogenetic tree of nucleotide sequences of the 16S rRNA gene fragments of eubacteria of the genus *Paenibacillus* constructed by the neighbor-joining method. Species of eubacteria and the GenBank numbers are shown. Indices of support for cladistic groups are given at the nodes. Names of the studied strains are shown in bold.

D-arabinose, and did not grow in the presence of 5% NaCl.

Strains K-58 and **K-59** showed over 99% homology with gram-positive strain *P. terrigena* (GQ284364). Isolates K-58 and K-59 were characterized by low biochemical activity and were able to grow at alkaline pH. They differed from *P. terrigena* in Gram staining, the Voges-Proskauer and the nitrate reduction tests, and DNA G + C content; did not produce acid from arabinose and maltose (in the case of K-59); formed colorless small colonies; and were characterized by different requirements for temperature, NaCl concentration, and pH (did not grow at pH 5.7).

Thus, the analysis of nucleotide sequences of 16S rRNA gene of gram-negative, endospore-forming bacteria isolated from the Valley of Geysers (Dg-824, Dg-904, Dg-1009, Gi-662, Gi-691, Gi-733, Gi-739, Gi-724, K-58, and K-59) revealed their close homologues among the known species of the genus *Paenibacillus* (the bootstrap test support indices for corresponding phylogeny nodes were 100). At the same time, all isolates differed considerably in phenotypic characteristics from their nearest homologues; precise taxonomic positioning of them will probably require additional studies.

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Table 2. Differentiation of species *P. amylolyticus* (X60606 = NRRL NRS-290T), *P. chibensis* (D85395 = NRRL B142T), *P. latus* (AB073188 = NRRL NRS-666T), *P. azoreducens* (*Paenibacillus* sp. CM1, AJ272249 = DSM 13822), *P. alvei* (DSM 29T AJ320491 = LMG13253T = IFO3343T), *P. terrigena* (GQ284364 = strain PCWCS12), *P. cookii* (AJ250317 = *Bacterium* LMG 18419 = CIP 108110T) and gram-negative, spore-forming strains isolated from the Valley of Geysers

Parameter	*P. amylolyticus*	Gi-662	Gi-691	*P. chibensis*	*P. cookii*	Dg-1009	*P. terrigena*	K-58	*P. lautus*	Dg-824	*P. azoreducens*	Gi-724	Gi-739	*P. alvei*	Dg-904
Gram staining	+	−	−	+	v	−	+	−	+	−	v	−	−	+	−
Anaerobic growth	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+
Colony pigmentation	−	−	−	+	+	w	+	−	v	−	+	−	−	−	+
Oxidase	−	+	+	−	+	+	+	+	−	+	−	+	+	nd	+
Urease	−	+	+	−	−	−	−	−	nd	−	−	−	−	nd	−
Nitrate reduction	+	−	−	+	+	+	+	−	+	+	−	+	+	−	−
Production of: indole	−	−	−	−	−	−	nd	−	−	−	−	−	−	+	−
acetyl methyl carbinol	−	−	−	−	+	+	+	−	−	+	−	−	−	+	+
Hydrolysis of: casein	w	−	−	−	w	−	nd	−	−	−	−	−	−	+	−
gelatin	+	−	−	−	v	−	−	−	+	−	+	+	+	+	−
starch	+	+	+	+	+	−	+	−	+	−	+	+	−	+	−
Growth at 5% NaCl	−	−	−	−	+	−	−	−	v	−	−	−	−	v	−
0.001% lysozyme	−	+	+	+	nd	+	+	+	+	+	+	+	+	+	+
40°C	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+
50°C	−	−	−	+	−	+	−	−	−	−	+	−	−	−	−
pH 5.7	+	+	+	+	+	+	+	−	+	+	+	+	+	−	+
Acid formation from: *D*-arabinose	+	+	+	+	-	+	-	-	+	+	+	−	−	−	−
lactose	−	+	+	−	+	+	−	+	−	+	+	+	+	−	−
mannitol	+	+	+	+	−	−	−	−	+	+	+	+	+	−	−
rhamnose	−	+	+	−	−	−	−	−	−	+	−	+	+	−	−
D-xylose	+	+	+	+	+	+	−	+	+	+	+	+	+	−	−
galactose	+	+	+	+	+	+	−	+	+	−	+	+	+	nd	+
glycerol	+	+	+	−	w	+	−	+	+	+	+	+	+	+	+
inositol	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
D-maltose	+	+	+	+	+	+	w	+	+	+	+	+	+	+	+
esculin	−	+	+	−	+	+	+	+	+	+	nd	+	+	+	+
Optimal temperature for cell growth (°C)	37	30	30–37	37	40	30–37	25	30	28–30	30–37	37	30–42	30–42	28	37
DNA G + C content (mol %)	46–47	49.6	49.6	53.0	51.6	41.1	48.1	45.4	49–50	45.6	47–46.8	43.5	47.0	45– 47	42.9

Designations: (+), (−), v, and w stand for positive, negative, variable, and slight positive reaction, respectively; nd signifies “no data”.

Table 3. Antibiotic sensitivity of gram-negative spore-forming bacteria isolated from the Valley of Geysers

Strain	Antibiotics/zones of cell lysis or growth suppression (mm)																
	β-lactams				Aminoglycosides					Macrolides		Others					
	Penicillin	Oxacillin	Ampicillin	Carbenicillin	Streptomycin	Neomycin	Kanamycin	Monomycin	Gentamycin	Oleandomycin	Erythromycin	Lincomycin	Tetracycline	Levomycetin	Polymyxin	Ristomycin	Rifampicin
Dg-904	10	17	0	0	25	12	0	0	23	25	35	0	30	35	0	22	26
Dg-824	0	0	0	0	0	15	15	22	23	0	15	0	0	14	0	15	0
Dg-1009	14	15	0	27	20	18	15	25	28	13	27	10	10	19	9	17	12
Gi-662	0	12	0	15	24	25	18	25	30	30	30	11	24	30	0	18	20
Gi-691	0	0	0	18	25	24	18	30	28	30	27	10	30	34	0	17	17
Gi-724	0	9	0	25	0	13	0	0	23	0	20	8	0	20	0	18	13
Gi-733	0	0	0	20	0	14	0	0	25	12	23	10	0	14	0	20	15
Gi-739	0	12	0	20	0	12	0	0	19	11	20	0	0	9	0	17	10
K-58	0	0	0	16	21	24	0	13	16	30	23	10	26	24	0	24	24
K-59	0	12	0	22	24	20	0	16	30	30	26	16	24	25	0	20	20

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