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SHORT COMMUNICATIONS

Investigation of the Phylogenetic Position of Aerobic, Moderately Thermophilic Bacteria Oxidizing Fe^{2+}, S^0, and Sulfide Minerals and Affiliated to the Genus *Sulfobacillus*

G. I. Karavaiko, T. P. Tourova, I. A. Tsaplina, and T. I. Bogdanova

Institute of Microbiology, Russian Academy of Sciences, pr. 60-letiya Oktyabrya 7, k. 2, Moscow, 117811 Russia

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At present, the genus *Sulfobacillus* includes three species of chemolithotrophic, acidophilic, moderately thermophilic or mesophilic bacteria. These are the type species of the genus *S. thermosulfidooxidans* (strains VKM B-1269 = DSM 9293^T and BC1) [1, 2], *S. acidophilus* (strains NAL^T and ALV) [2], and *S. disulfidooxidans* (strain SD-11^T) [3]. Based on their phenotypic properties, two additional strains, 41 and K1, were assigned as subspecies of *S. thermosulfidooxidans*, and named *S. thermosulfidooxidans* subsp. "*asporogenes*" [4] and *S. thermosulfidooxidans* subsp. "*thermotolerans*" [5], respectively. Several unidentified strains (C-MT1, YTH2, and YTH1) were also assigned to the genus *Sulfobacillus* based on 16S rDNA sequence data [6, 7]. Phylogenetic analysis demonstrated that *Sulfobacillus* clusters with species of the genus *Alicyclobacillus* [3, 8].

The aim of the present work was to unravel phylogenetic relationships of various sulfobacilli by determining their DNA base compositions, DNA hybridization levels, and 16S rDNA sequences.

Sulfobacilli were grown on Manning [9] and Brierley [10] media. DNA was isolated according to the Marmur procedure [11]. The DNA base composition was determined by thermal denaturation [12]. The DNA–DNA homology was determined by the optical reassociation method according to De Ley *et al.* [13]. Amplification and sequencing of 16S rDNA were performed according to Lane [14].

The 16S rDNA sequences of strains VKM B 1269, 41, and K1 have been deposited in GenBank under the accession numbers AF137501, AF137503 and AF137502, respectively.

The rooted phylogenetic tree was constructed using the programs of the TREECON package [15].

The results of DNA–DNA hybridization demonstrated an intraspecies level of relatedness of strain 41 to *S. thermosulfidooxidans* 1269 (89%). Other *Sulfobacillus* strains studied were related to each other at least at the interspecies level of DNA homology (7–37%). The homology level with *Alicyclobacillus* representatives was within the range of experimental error (Table 1).

To determine phylogenetic relationships of *Sulfobacillus* and *Alicyclobacillus* strains, partial sequences (about 500 nucleotides) of their 16S rRNA genes were determined, corresponding to positions 90 to 650 of the *E. coli* numbering. The phylogenetic analysis of these sequences involved reference strains of the *Sulfobacillus–Alicyclobacillus* group (see figure).

The 16S rDNA sequence of the strain *S. thermosulfidooxidans* 1269 showed a high degree of similarity (99.7%, difference within experimental error) to the 16S rDNA sequence of the synonym strain DSM 9293

Table 1. Base compositions and homology levels of DNAs of sulfobacilli

Strain	G+C, mol %	DNA homology, %				
		S. acidophilus NAL	*S. disulfidooxidans* SD-11	*S. thermosulfidooxidans*		
				1269	41	K1
S. acidophilus, strain NAL [2]	57.1	100				
S. disulfidooxinads, strain SD-11 [3]	52.7	26	100			
S. thermosulfidooxidans, strain 1269	47.5	7	21	100		
S. thermosulfidooxidans subsp. *asporogenes*, strain 41	46.5	7	23	89	100	
S. thermosulfidooxidans subsp. *thermotolerans*, strain K1	48.1	5	22	37	31	100
A. acidocaldarius, strain 24	61.6	3	5	3	3	3

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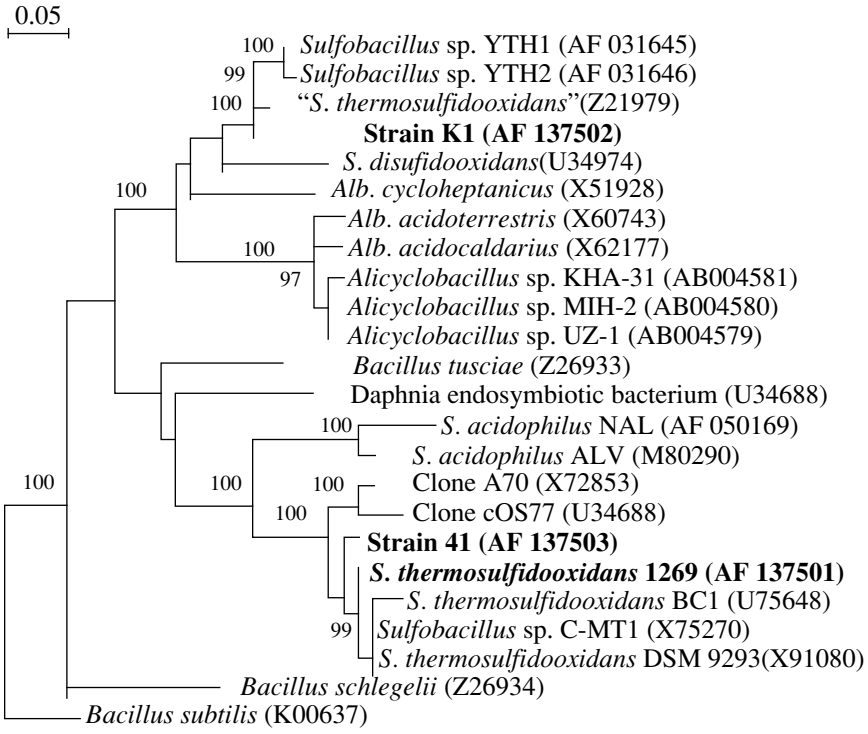

Phylogenetic tree of the *Sulfobacillus–Alicyclobacillus* group. Space bar represents 5 nucleotide substitutions per 100 nucleotides. Figures show statistical significance of the branching order determined by bootstrap analysis of 100 alternative trees; values lesser than 95% are not shown.

determined by Durand [16] (accession number X91080), and a low degree of similarity (87.7%) to the 16S rDNA sequence that we earlier ascribed to strain 1269 (accession number Z21979) [8]. At the same time, the latter sequence proved to be highly similar to the 16S rDNA sequence of strain K1 (99.7%, difference within experimental error). Thus, there was an error in our previous publication [8], most likely reflecting the occasional contamination of the culture of strain 1269 with strain K1.

The newly determined 16S rDNA sequence of strain *S. thermosulfidooxidans* 1269 exhibited a high level of similarity (98.5%) to the sequence of strain 41. This was in agreement with the high (intraspecies) level of DNA–DNA homology between these strains (Table 1). Thus, strain 41 indeed belongs to the type species of the genus *Sulfobacillus*, and is an asporogenous variant of this species. The existence of such variants has been demonstrated in other bacterial species as well [17].

High levels of similarity were also revealed between the type strain *S. thermosulfidooxidans* 1269 and the strain *S. thermosulfidooxidans* BC1 described by Norris *et al.* [2] and the strain *Sulfobacillus* sp. C-MT1 described by Goebel and Stackebrandt [6] (98.8 and 99.7%, respectively). Thus, the above-mentioned strains (VKM B-1269 = DSM 9293, 41, BC1, and C-MT1) form a coherent subcluster related (93.1–94.9%) to another subcluster that includes the strains *S. acidophilus* ALV and NAL^T [2]. This conclusion is in agreement with the clusterization pattern revealed by other authors [2].

Strain K1, described as *S. thermosulfidooxidans* sub. "*asporogenes*" [5] and strain SD-11, described as *S. disulfidooxidans* [3], exhibited a 87.7% similarity with strain 1269 and a 99.7% similarity between each other and thus belonged to another cluster that also contains the species of the genus *Alicyclobacillus* and the unidentified moderately thermophilic isolates YTH1 and YTH2 [7] (see figure).

In Table 2, the main phenotypic properties of alicyclobacilli and sulfobacilli are compared. Sulfobacilli are represented by aerobic gram-positive spore-forming (except strain 41) rods. They are mixotrophs (chemolithooligotrophs oxidizing Fe^{2+}, S^0, and sulfide minerals) [1–5]. Strains 1269, 41, and K fail to show stable growth either under heterotrophic of autotrophic conditions [20, 21]. Cellular lipids of sulfobacilli contain ω-cyclohexanoic acids; during growth under heterotrophic conditions, the content of these acids reaches 58–69% of the total fatty acids [18].

According to their phenotypic properties, sulfobacilli can be divided into two groups, one of which includes moderately thermophilic strains (1269, 41, BC-1, NAL, and ALV). The other group contains strains that are virtually mesophilic (K1 and SD-11). Strains of the second group are distinguished by greater cell size and some of their physiological properties. For example, strains of the first group can completely oxi-

Table 2. Morphological and physiological characteristics of the bacteria of the genera *Sulfobacillus* and *Alicyclobacillus*

Characteristic	*Sulfobacillus*						*Alicyclobacillus*		
	S. thermosulfidooxidans				*S. acidophilus* ALV [2]	*S. disulfidooxidans* SD-11 [3]	*A. acidocaldarius* (DSM 446) [19]	*A. acidoterrestris* (DSM 3922) [19]	*A. cycloheptanicus* (DSM 4006) [19]
	VKM B-1269 = DSM 9293 [1, 18]	subsp. *asporogenes*, 41 [4, 18]	subsp. *thermotolerans*, K-1 [5, 18]	BC1 [2]					
Cell size, μm	Rods 0.6–0.8 × 1.0–3.0	Rods 0.5–0.9 × 2.0–4.0	Rods 0.9–0.1 × 3.0–6.0	Rods 0.6–0.9 × 1.5–3.5	Rods 0.5–0.8 × 3.0–5.0	Rods 0.6–1.0 × 1.0–6.0	Rods 0.7–0.8 × 2.0–3.0	Rods 0.6–0.8 × 2.9–4.3	Rods 0.35–0.55× 2.5–4.5
Growth pH range (optimum)	1.5–5.5 (1.7–2.4)	1.5–3.9 (1.6–1.8)	1.5–5.0 (2.0–2.7)	(1.8)	(2.0)	(1.5–2.5)	2.0–6.0	2.2–5.8	3.0–5.5 (3.5–4.5)
Growth temperature range, °C (optimum)	20–60 (50–55)	30–50 (45–50)	5–55 (38–42)	(45–50)	(45–50)	4–40(35)	45–70	35–55 (42–53)	40–53(48)
G+C, mol %	47.2–47.5	45.5–46.5	48.1–49.3	48.2	55–57	53	60.3	52.2	55.6
Genome size, Da	3.7×10^9	3.0×10^9	3.9×10^9	ND	4.0×10^9	3.0×10^9	ND	ND	ND
Fatty acid composition	ω-cyclohexylundecanoic, ω-cyclohexyltridecanoic, ω-cyclohexyl-α-hydroxyundecanoic.			ND	ND	ω-cyclohexanoic fatty acids	Gopanoids, sulfonolipids, menaquinone MK-7; ω-cyclohexylundecanoic and ω-cyclohexyltridecanoic acids	ω-cyclohexanoic fatty acids, menaquinone MK-6	ω-cycloheptylundecanoic, ω-cycloheptyltridecanoic, ω-cycloheptyl-α-hydroxyundecanoic acids, menaquinones MK-6, MK-7, MK-9, sulfonolipids
Mineral substrates	Fe^{2+}, S^0 and sulfide minerals						No	No	No

Note: ND means "no data."

dize Fe^{2+}, S^0, and sulfide minerals if the concentration of yeast extract or other organic compounds does not exceed 0.5 g/l. Strains of the second group are only capable of partial oxidation of mineral substrates [3, 5] and grow with higher concentrations of organic substrates (up to 1.0 and 2.5 g/l, respectively).

Bacteria of the genus *Alicyclobacillus* are aerobic and moderately thermophilic acidophilic heterotrophs [19]. They are less acidophilic than sulfobacilli and do not oxidize mineral substrates. Lipids of alicyclobacilli also contain ω-cyclohexanoic acids.

Our data on the phenotypic heterogeneity of sulfobacilli are in agreement with the data of phylogenetic analysis. In future studies, the taxonomic position of the strains belonging to the second group and their affiliation with the genus *Sulfobacillus* should be reconsidered.

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