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EXPERIMENTAL ARTICLES

Strain Polymorphism of the Plasmid Profiles in *Sulfobacillus* Species

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Abstract—Plasmids were discovered for the first time in strains belonging to different species of the genus *Sulfobacillus*: *S. thermosulfidooxidans, S. sibiricus, S. thermotolerans, "S. olympiadicus"*, and *S. acidophilus.* The plasmids were detected in the cells of four out of eight strains grown on a medium with ferrous iron. Adaptation to elementary sulfur was accompanied by changes in the plasmid profiles in two out of seven strains. Plasmids were detected in all the studied strains of sulfobacilli after adaptation to the pyrite–arsenopyrite ore concentrate from the Nezhdaninskoe deposit containing gold, silver, zinc, copper, and lead. No plasmids were found in *S. thermotolerans* Kr1^T after four transfers on a medium containing iron and 0.018 mM Ag^+. After adaptation of the same strain to 765 mM Zn^{2+}, only one plasmid was found in the cells, the largest among those detected earlier in this culture adapted to the Nezhdaninskoe ore concentrate. The strain *S. thermotolerans* Kr1^T, after four transfers on media with either 78 mM Cu^{2+} or 2 mM Pb^{2+}, did not contain plasmids. The presence of plasmids in the cells of sulfobacilli did not influence their resistance to the ions of the studied metals.

Key words: *Sulfobacillus* species, plasmids, energy substrate, adaptation, silver, zinc, copper, lead.

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The genus *Sulfobacillus* comprises gram-positive acidophilic bacteria which utilize ferrous iron, elemental sulfur, or reduced sulfur species as energy sources, as well as a variety of sulfide minerals in mixotrophic conditions. For constructive metabolism, sulfobacilli use carbon dioxide, yeast extract, and some organic compounds, such as carbohydrates, amino acids, and fatty acids. Presently, five species of spore-forming moderately thermophilic sulfobacilli species are known (*Sulfobacillus thermosulfidooxidans* [1], *S. acidophilus* [2], *S. sibiricus* [3], *"S. olympiadicus"* [4], and *S. benefaciens* [5]), together with one moderately thermophilic asporogenic subspecies (*S. thermosulfidooxidans* subsp. *asporogenes* [6]), and a thermotolerant species *S. thermotolerans* [7].

Sulfobacilli are found predominantly in deposits of sulfide ores. Bacteria of this genus occur also in coal deposits, hot springs, and ferrolite soils of humid subtropics characterized by high concentrations of iron compounds and low pH values [2, 8].

During oxidation of sulfide minerals, the environment is enriched with ions of heavy metals and toxic elements: Fe^{3+}, Cu^{2+}, Zn^{2+}, Ni^{2+}, As^{3+}, Pb^{2+}, and Ag^+. Resistance to metal ions in heterotrophic bacteria is usually associated with the presence of plasmids [9]. However, for the acidophilic, chemolithoautotrophic gram-negative bacterium *Acidithiobacillus ferrooxidans* which, similar to gram-positive bacteria of the genus *Sulfobacillus*, obtains energy via oxidation of sulfide minerals, chromosomal localization was demonstrated for the genes controlling resistance to mercury [10], zinc, and arsenic [11]. In the cells of *A. ferrooxidans,* plasmids were found. Notably, strains of *A. ferrooxidans* vary both in the size and number of the plasmids [12]. In some cases, adaptation to new sources of energy caused changes in both parameters [13]. Alterations in the plasmid composition were observed in *A. ferrooxidans* strain TFV-1 adapted to high Cu^{2+} concentrations [12], while in the strain MAL4-1 an increase in Cu^{2+} concentration resulted in the disappearance of plasmids [14]. Plasmid phenotypes of *A. ferrooxidans* are still undetermined. Some data suggest their role in switching the metabolism to oxidation of a new energy substrate [13]. Plasmids of bacteria of the genus *Sulfobacillus* have not been studied so far.

The goal of the present work was to locate plasmids in the representatives of various species of this bacterial genus and to investigate the plasmid profiles of the strains adapted to different metal ions and energy sources.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Ten bacterial strains of the genus *Sulfobacillus* were examined, belonging to five species: *S. thermosulfidooxidans,*

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Table 1. Isolation sources of strains of different *Sulfobacillus* species and the content of the main sulfide minerals in the substrate

Type of substrate	Species	Strains	Places of isolation	Main sulfide minerals
Copper-zinc pyrite ore	*S. thermosulfidooxidans*	1269^T	Nikolaevskoe deposit, Kazakhstan	FeS_2, $CuFeS_2$, ZnS, PbS, Cu_2S, CuS, oxides
Complex sulfide ore (mine water from refuse ore)	*S. thermosulfidooxidans* subsp. *asporogenes*	41	Armanis deposit, Armenia	No data
Gold–silver–arsenic concentrate	*S. sibiricus*	N1^T	Nezhdaninskoe deposit, Russia	FeS_2 – 48%, FeAsS – 35%, ZnS, $CuFeS_2$, PbS, 123 g/t Au, 192.7 g/t Ag
Pyrrhotine gold–arsenic pyrite–arsenopyrite concentrate	S. *sibiricus*	OFO B1 B3	Olympiadinskoe deposit, zones of self-heating, Russia	FeS – 33%, FeS_2 – 12%, FeAsS – 8%, Sb_2S_3 and Sb_3S_5 – 7%, ZnS, PbS, $CuFeS_2$, 110 – 120 g/t Au, 2.5–3.5 g/t Ag
Copper-nickel ore	*Sulfobacillus* sp. (presumably a new species)	Sh8	Shanuch mine, Russia	FeS – 49.5%, $(Ni,Fe)_9S_8$ – 24.2%, $CuFeS_2$ – 0.3%, FeS_2, minerals of the violarite group
Pyrrhotine gold–arsenic pyrite–arsenopyrite concentrate	*S. thermotolerans*	Kr1^T	Olympiadinskoe deposit, Russia	FeS – 33%, FeS_2 – 12%, FeAsS – 8%, Sb_2S_3 and Sb_3S_5 – 7%, ZnS, PbS, $CuFeS_2$, 110 – 120 g/t Au, 2.5–3.5 g/t Ag
Pyrrhotine gold–arsenic pyrite–arsenopyrite concentrate	*"S. olympiadicus"*	S-5		
Coal	*S. acidophilus*	NAL^T	Coal spoil heap near Alvecote, England	FeS_2

S. sibiricus, S. thermotolerans, "S. olympiadicus", and *S. acidophilus*. Sources of isolation, substrate types, and the major sulfide minerals in the substrates are given in Table 1. Sulfobacilli strains were grown in 250-ml Erlenmeyer flasks with 100 ml of the mineral basis of the Silverman–Lundgren's medium [15] supplemented with 0.02 % yeast extract. Ferrous iron in the form of $FeSO_4 \cdot 7H_2O$ (0.0356 M Fe^{2+}), elemental sulfur (0.5%), yeast extract (0.02%), and sulfide ore concentrates from the Olympiadinskoe and Nezhdaninskoe deposits (10 g/l) were used as energy sources. The inoculum was 10% (vol/vol). The flasks were incubated on a shaker (180 rpm). The cultures were grown to the end of the exponential phase at optimal temperatures for each strain: 50°C for *S. thermosulfidooxidans* 1269^T, *S. thermosulfidooxidans* subsp. *asporogenes* 41, and *S. sibiricus* B3; 55°C for *S. sibiricus* N1^T; 48°C for *S. sibiricus* OFO; 45°C for *Sulfobacillus* sp. Sh8, *"S. olympiadicus"* S-5, *S. sibiricus* B1, and *S. acidophilus*; and 40°C for *S. thermotolerans* Kr1^T. Bacterial biomass was collected and washed according to the standard procedure [16]. The effect of ions of silver (0.02 mM) in the form of $AgNO_3$, zinc (765 mM) in the form of $ZnSO_4$, copper (30 mM) in the form of $CuSO_4 \cdot 5H_2O$, and lead (2 mM) in the form of $Pb(NO_3)_2$ on the plasmid composition of the cells was investigated for the strain *S. thermotolerans* Kr1^T adapted to each of the metals after four transfers into a medium with Fe^{2+} containing salts of these metals.

Isolation of plasmid DNA was performed by a modified technique described in [12]. According to the modified procedure, along with 2 mg/ml of lysozyme (Sigma, United States), 0.5 mg/ml of proteinase K (Sigma, United States) was added to the solution 1 for cell lysis.

Plasmid profile analysis. The DNA solution (10 μl) was heated for 2 min in a water bath at 100°C to remove the open circular plasmid DNA. The buffer for exonuclease *Exo*III (1 μl × 10) (Fermentas, Lithuania) and 1 μl of *Exo*III (40 U/μl) (SibEnzyme, Russia) were added. Processing aimed at destruction of linear plasmid DNA was carried on for 2 h at 37°C. Plasmid profiles of the sulfobacilli strains were analyzed according to the standard technique by electrophoresis on 1% agarose gel (agarose, type 1, universal, with a low EEO, Panreac, Spain) in TAE buffer at pH 8.0 and 90 V direct voltage. Fragments of phage λ DNA cut with the *Hind*III restriction endonuclease, and in some cases DNA of

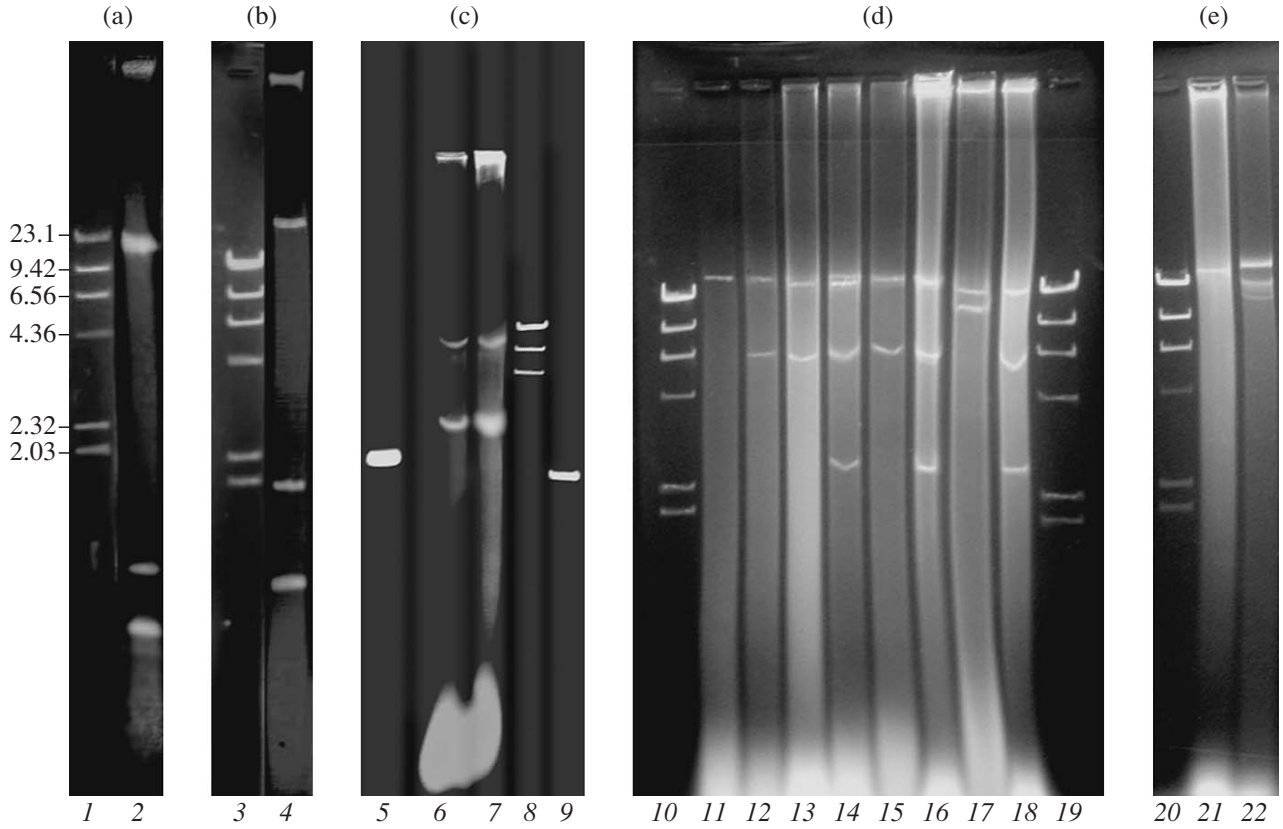

Fig. 1. Plasmid profiles of strains belonging to different *Sulfobacillus* species, grown on a medium with ferrous iron or adapted to other sources of energy: *2*, *S. thermosulfidooxidans* subsp. *asporogenes* 41 on iron; *4, Sulfobacillus* sp. Sh8 on iron; *6*, *S. sibiricus* $N1^T$ on iron; *7*, *S. sibiricus* $N1^T$ on sulfur; *11*, *S. sibiricus* B1 on iron; *12*, *S. sibiricus* B1 on sulfur; *13*, *S. sibiricus* B1 on concentrate; *14*, *S. sibiricus* OFO on sulfur; *15*, *S. sibiricus* OFO on yeast extract; *16*, *S. sibiricus* OFO on concentrate; *17*, *S. sibiricus* $N1^T$ on concentrate; *18*, *S. thermotolerans* $Kr1^T$ on concentrate; *21*, *S. thermosulfidooxidans* 1269^T on concentrate; *22*, *"S. olympiadicus"* S-5 on concentrate; 1, 3, 8, 10, 19, 20 phage λ DNA, split with restriction endonuclease *Hind*III; *5*, DNA of pBR322 treated with *Exo*III; *9*, DNA of pUC19 split with restriction endonuclease *Hind*III. Sizes of the DNA fragments in kbp are set out next to lane *1*.

pBR322, treated with *Exo*III, or pUC19 DNA split with *Hind*III were used as molecular mass standards.

This work features results of 2–3 experiments in two repeats.

RESULTS AND DISCUSSION

Plasmid profiles and their number in strains of various species of the genus *Sulfobacillus*, grown on a medium with ferrous iron as an energy source or adapted to other energy substrates, are shown in Figs. 1 and 2 and Table 2. Out of eight strains grown on the medium with ferrous iron, plasmids were found in four. The strains differed in the number of plasmids in cells and their size.

Out of nine strains of *Sulfobacillus* adapted to elemental sulfur, plasmids were detected in five (Table 2).

The maximum number of strains (8 out of 8) containing plasmids was observed in different *Sulfobacillus* species after adaptation to the ore concentrate from the Nezhdaninskoe deposit. In some strains (*S. thermosulfidooxidans* 1269^T, *S. sibiricus* B3, *S. thermotolerans* $Kr1^T$, *"S. olympiadicus"* S-5), the plasmids were found only in cultures adapted to this concentrate (Fig. 1e, lane *21*; Fig. 2, lane *2*; Fig. 1d, lane *18*; Fig. 1e, lane *22*, respectively).

Since not all the sulfobacilli strains grown on iron-containing medium have plasmids, their presence in the cells is not related to the ability to use this source of energy. Switching the metabolism to oxidation of sulfur instead of iron did not induce appearance of plasmids in strains *S. thermosulfidooxidans* 1269^T, *S. sibiricus* B3, *S. thermotolerans* $Kr1^T$, and *"S. olympiadicus"* S-5. Adaptation to the ore concentrate from the Nezhdaninskoe deposit was accompanied either by selection of the plasmid-bearing clones from a population heterogeneous with respect to the plasmid content, or by an increase in the number of plasmids due to a higher rate of their replication. Pyrite is the major sulfide mineral of the ore found in the Nezhdaninskoe deposit. Adaptation of *S. thermotolerans* $Kr1^T$ to two types of pyrite was not followed by an emergence of plasmids [16].

The Nezhdaninskoe ore concentrate, apart from a high content of silver, up to 200 g/t (Table 1), contains

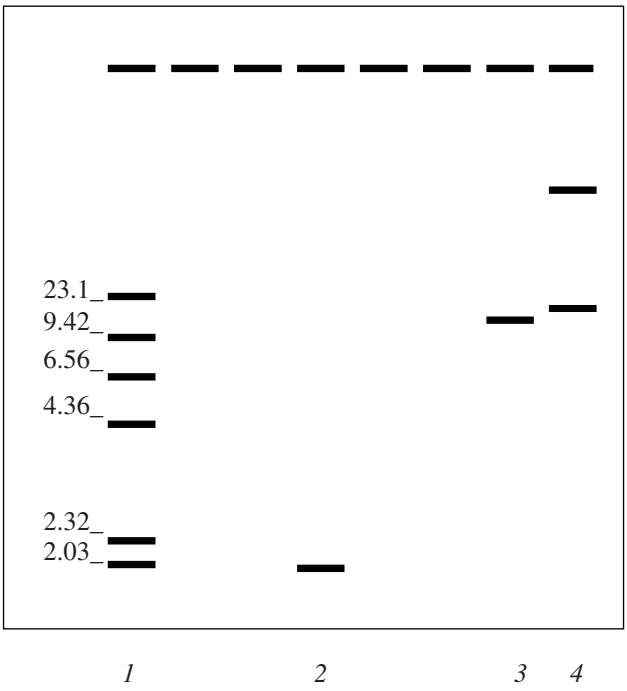

Fig. 2. Schematic representation of the plasmid profiles of the following strains: *S. sibiricus* B3 on concentrate (*2*); *S. acidophilus* on sulfur (*3*); *S. thermosulfidooxidans* subsp. *asporogenes* 41 on sulfur (*4*). *1*, DNA of phage λ, split with restriction endonuclease *Hind*III. Sizes of the DNA fragments in kbp are set out next to stripe *1*.

also zinc, copper, antimony, and lead. Silver is among the most toxic elements for microorganisms. Could the presence of plasmids in the cells of the cultures adapted to this substrate be related to improvement of resistance to silver in the course of adaptation? After four transfers of the strain *S. thermotolerans* $Kr1^T$ on a medium containing 0.018 mM Ag^+ (as $AgNO_3$) and ferrous iron as the source of energy, no plasmids were detected in the cells. The growth rate decreased with each passage. Disappearance of plasmids was observed in *A. ferrooxidans* MAL4 during adaptation to 20–30 g/l of Cu^{2+} [14]. The authors explained this result as inhibition of plasmid replication by copper ions. Silver ions probably have a similar effect on replication of plasmid DNA in *S. thermotolerans* $Kr1^T$.

Experiments on adaptation of *S. thermotolerans* $Kr1^T$ to 78 mM Cu^{2+} ($CuSO_4$) or 2 mM Pb^{2+} ($Pb(NO_3)_2$) on media with ferrous iron as an energy source did not reveal plasmids. Transfers of *S. thermotolerans* $Kr1^T$ on a medium containing 765 mM Zn^{2+} ($ZnSO_4$) and ferrous iron as an energy source resulted in emergence of one plasmid, the largest of those detected earlier during transfers of strain $Kr1^T$ on medium with the ore concentrate from the Nezhdaninskoe deposit (Fig. 1d, lane *18*).

Our knowledge of *A. ferrooxidans*, is still incomplete, although this is the most thoroughly studied species of acidophilic chemolithoautotrophic bacteria producing energy via oxidation of ferrous iron, sulfide minerals, elemental sulfur, or reduced sulfur species, and highly resistant to ions of heavy and toxic elements. Plasmid phenotypes in *A. ferrooxidans* are still unknown. They are believed to be cryptic. The genes of resistance to mercury and arsenic are localized on the chromosomal DNA [9, 10]. Some approaches to unveiling the role of plasmids for *A. ferrooxidans* were considered in [11, 12]. It was demonstrated that the number and electrophoretic mobility of the plasmids varied with the change of energy substrates. It, however,

Table 2. The number of plasmids found in the cells of strains belonging to different *Sulfobacillus* species grown on a medium with ferrous iron or adapted to other sources of energy

Species	Strains	Number of plasmids on media		
		with ferrous iron	with elemental sulfur	with ore concentrate from the Nezhdaninskoe deposit
S. thermosulfidooxidans	1269^T	0	0	1
S. thermosulfidooxidans subsp. *asporogenes*	41	3	2	1
S. sibiricus	$N1^T$	2	2	2
	OFO*	No data	4	3
	B1	1	2	2
	B3	0	0	1
Sulfobacillus sp. nov.	Sh8	3	No data	No data
S. thermotolerans	$Kr1^T$**	0	0	3
"S. olympiadicus"	S-5	0	0	3
S. acidophilus	NAL^T	No data	1	1

Notes: * Two plasmids were found on medium with yeast extract.
** One plasmid was found on medium with yeast extract.

remained unclear whether this phenomenon was characteristic of *A. ferrooxidans* alone, or applied also to other microorganisms inhabiting similar econiches with high concentrations of metal ions and frequent change of energy substrates.

The representatives of the genus *Sulfobacillus* were used as objects in the present research. The presence of plasmids in the cells of sulfobacilli was demonstrated for the first time. Similar to *A. ferrooxidans*, their number changed with adaptation to new oxidation substrates. Our results clearly demonstrate that the changes in the plasmid profile are not related to increased resistance to the ions of silver, copper, or lead. The fact that some strains of sulfobacilli resistant to metal ions have plasmids, while others do not, confirms that this parameter it is not determined by plasmids in either *Sulfobacillus* species or *A. ferrooxidans*. Localization of resistance genes in the chromosomes probably ensures more rapid and flexible regulation of this parameter in response to increased concentrations of metal ions that may result from the oxidation of sulfide minerals in the course of both natural and anthropogenic processes, which have been the source for isolation of a number of species and strains of sulfobacilli used in the present work. In other acidophilic chemolithoautotrophic microorganisms, including archaea of the genus *Ferroplasma*, which have not been studied in this respect, chromosomal DNA should also be expected to contain the genes of resistance to ions of heavy and toxic metals.

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