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SHORT COMMUNICATIONS

Electron Donors at Oxidative Phosphorylation in Bacteria of the Genus *Sulfobacillus*

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Bacteria of the genus *Sulfobacillus*, as a part of microbial associations, leach sulfide ores of nonferrous and precious metals; they are extremely acidophilic mixotrophic chemolithotrophs [1, 2]. Investigations into the metabolism of this group of microorganisms revealed that sulfobacilli obtain carbon from CO_2 and organic compounds and extract energy via substrate-linked [3] and electron transport phosphorylation [4–6]. In sulfobacilli, substrate-level phosphorylation during sugar catabolism occurs under both mixotrophic and heterotrophic conditions via the Embden–Meyerhof–Parnas and Entner–Doudoroff pathways [1, 3, 4]. It was established that Fe(II) is a source of energy and an electron donor for the electron transport system (ETS) of the thermotolerant strain *S. thermotolerans* Kr1, and of the moderate thermophiles *S. sibiricus* N1 and *S. thermosulfidooxidans* BC1 [4–6]. Growth on media with organic substrates was observed as well [2, 4, 7, 8]. The fermentation products of the cultures of sulfobacilli grown under active aeration were not detected under either mixotrophic or organoheterotrophic conditions. It was previously demonstrated that NaN_3 and KCN inhibit respiration of cells of sulfobacilli [5, our unpublished data]. Under optimal mixotrophic conditions, active dehydrogenases that supply reducing equivalents to the ETS are involved in the glucose metabolism. However, it is still unknown whether, under mixotrophic conditions, in the presence of Fe(II), glucose or yeast extract act as electron donors and energy sources for oxidative phosphorylation. The purpose of this work was to investigate this question.

Our previous studies of growing cultures of sulfobacilli showed that the mineral substrate (Fe(II)) and organic compound (glucose), added to the medium individually or as part of a mixture, are utilized with the resulting formation of the intracellular ATP pool, the accumulation dynamics and level of which depend on the nutrition mode. The ATP pool reaches its maximum under mixotrophic conditions, whereas, under hetero- and autotrophic conditions, it is lower [7, 8]. Our task was to determine the rates of ATP synthesis resulting due to ETS functioning during the oxidation of organic and/or inorganic substrates by cell suspensions, to study the inhibition of ATP synthesis by the ATPase inhibitor dicyclohexylcarbodiimide (DCCD), as well as to determine the rates of cell respiration on various substrates.

The objects of this study were two *Sulfobacillus* species, *S. thermotolerans* Kr1^T (VKM B-2339^T = DSM 17362^T) and *S. sibiricus* N1^T (VKM B-2380^T = DSM 17363^T), with temperature optima at 40 and 55°C, respectively, isolated as predominant strains in the course of bioleaching of gold-containing concentrates [9, 10]. Strains Kr1 and N1 were grown under mixotrophic conditions: 9K mineral medium base [7] was supplemented with Fe(II) (in the form of $FeSO_4 \cdot 7H_2O$) and glucose to concentrations of 40 and 1.1 mM, respectively, as energy sources, as well as with 0.02% of yeast extract. The cultures were grown in 2.5-liter flasks with 2 l of the medium under intense aeration (1.0 volume of air/volume of medium per min). The amount of inoculum was 10 vol %. Mid-exponential growth phase cells were harvested by centrifugation (T-23 Janetzki, Germany, 25°C) at 8000 *g* for 30 min, washed three times with the salt base of 9K medium (pH 1.7) that did not contain any energy source, resuspended in a solution of the same composition, and incubated for 1 h at optimal temperatures on a rotor shaker (180 rpm) in 25-ml conical flasks with 4.0 ml of the medium (1.15–1.5 mg protein/ml) in order to decrease the endogenous concentration of intracellular ATP. The incubation mixture was then supplemented with substrates (total volume of the medium was 4 ml). Under mixotrophic conditions, the mixture was supplemented with the following compounds: (1) Fe(II) (to the final concentration of 40 mM) and glucose (2 mM); (2) Fe(II) and yeast extract (0.03%); and (3) Fe(II), glucose, and yeast extract in the aforementioned concentrations. In order to create autotrophic or heterotrophic conditions, Fe(II) or glucose and/or yeast extract,

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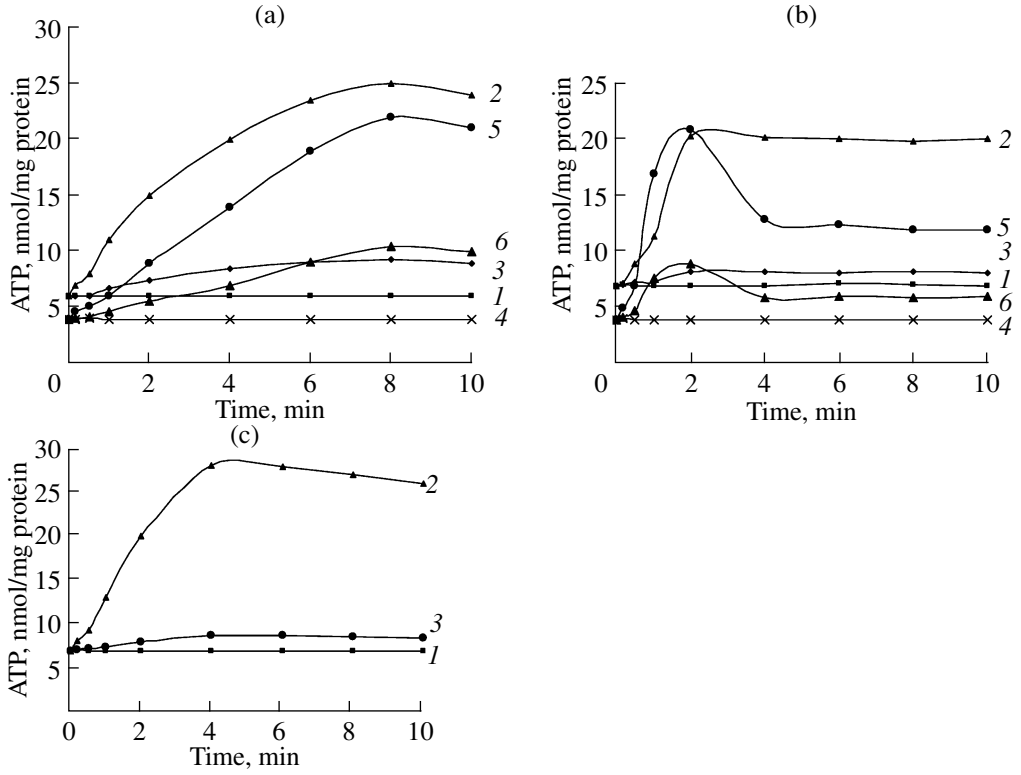

Production of ATP by the *Sulfobacillus* washed cells. (a) *S. thermotolerans* Kr1: endogenous level (*1*); the mixture of Fe(II) and glucose added (*2*); the mixture of DCC, Fe(II), and glucose added (*3*); *S. sibiricus* N1: endogenous level (*4*); the mixture of Fe(II) and glucose added (*5*); the mixture of DCC, Fe(II), and glucose added (*6*); (b) *S. thermotolerans* Kr1: endogenous level (*1*); the mixture of Fe(II) and yeast extract added (*2*); the mixture of DCC, Fe(II), and yeast extract added (*3*); *S. sibiricus* N1: endogenous level (*4*); the mixture of Fe(II) and yeast extract added (*5*); the mixture of DCC, Fe(II), and yeast extract added (*6*); (c) *S. thermotolerans* Kr1: endogenous level (*1*); the mixture of Fe(II), glucose, and yeast extract added (*2*); the mixture of DCC, Fe(II), glucose, and yeast extract added (*3*).

respectively, were added to the incubation mixture. After the mixture was supplemented with the substrates, 200-μl samples were taken immediately (zero time) and after 10 s, 30 s, 1, 2, 4, 6, 8, and 10 min. In order to inhibit ATPase, dicyclohexylcarbodiimide (200 μM) was added 10 min before the addition of the substrate(s); the samples were then taken as described above. ATP was extracted with 200 μl dimethyl sulfoxide added to 200 μl of each sample. The ATP concentration in the sample was determined by the modified bioluminescent method [7] with the Microlum ATP reagent based on the soluble glowworm luciferase (Lumtek, Russia). The respiration rates were determined using an LP-9 polarograph (Laboratorni pristroje Praha, Czech Republic) at room temperature. The obtained data are presented in the paper with the deduction of endogenous respiration. The figures and table show the results of typical experiments performed in five replicates.

It was established that, in the suspensions of *S. sibiricus* and *S. thermotolerans* cells incubated under autotrophic conditions in the presence of *Fe(II)*, the ATP concentration increased 3.5- to 5-fold as compared to the endogenous level and reached 12.3–

Respiration rates of the cell suspensions of *S. thermotolerans* Kr1^T and *S. sibiricus* N1^T during oxidation of Fe(II) and/or organic substrates

Substrates	Fe(II)	Glu*	Y. E.*	Glu + Y. E.	Fe(II) + Glu	Fe(II) + Y. E.	Fe(II) + Glu + Y. E.
Respiration rate, nmol O_2/(min mg protein)	*S. thermotolerans* Kr1						
	571.6	310.0	270.0	580.0	685.1	657.4	733.4
	S. sibiricus N1						
	520.0	220.0	295.5	400.5	620.7	643.1	690.2

Note: *, yeast extract or glucose.

15.2 nmol/mg protein. Incubation of the cell suspensions with the ATPase inhibitor DCCD resulted in a 53–78% decrease in ATP production. In the presence of *Fe(II)*, the cell suspensions demonstrated high respiration rates (see the table). Under heterotrophic conditions, the *glucose-dependent* ATP synthesis by the cell suspensions of strains Kr1 and N1 was 10.3–11.5 nmol/mg protein and was inhibited by the inhibitor by 82–93%. The respiration rate on glucose was 220–310 nmol O_2/(min mg protein) (see the table). Similar values of cell respiration and ATP synthesis rates, as well as of the level of inhibition of ATP production were obtained upon addition of yeast extract into the incubation mixture.

Iron-glucose-dependent ATP synthesis up to the maximum level of 22–25 nmol/mg protein (figure, a) was observed when, *glucose*, together with *Fe(II)*, was glucose, was the oxidation substrates for cells of sulfobacilli. In the case of *S. thermotolerans* cell suspensions, the maximum rate of ATP synthesis and the highest level of its inhibition by DCCD observed 10 s after the addition of the substrates were 6 nmol ATP/(min mg protein) and 100%, respectively. Similar results (4.1 nmol ATP/(min mg protein) and 85.4%, respectively) were obtained for the *S. sibiricus* cell suspensions. In the incubation medium with *iron* and *yeast extract*, the ATP-concentration increased rapidly up to its maximum (20–21 nmol ATP/mg protein) (figure, b). Within the first 10 s after the addition of the substrates, the rates of ATP synthesis in the cell suspensions of strains Kr1 and N1 were 1.8 and 5.9 nmol ATP/(min mg protein), respectively; the maximum level of inhibition of ATP synthesis by DCCD detected in the cell suspensions of *S. thermotolerans* and *S. sibiricus* was 100 and 80%, respectively. An increase in the rate of oxygen uptake by the cell suspensions from 520.0–571.6 (on iron) up to 620.7–685.1 nmol O_2/(min mg protein) (see the table) suggests that sulfobacilli utilize organic compounds as an electron donor and energy source in the presence of Fe(II).

The highest levels of ATP synthesis (26 and 28 nmol/mg protein) were observed in the cell suspensions of *S. sibiricus* and *S. thermotolerans*, respectively, incubated in the presence of a mixture of two organic and one inorganic substrates (as is the case of growing cultures). In the experimental variant with *Fe(II), glucose*, and *yeast extract*, the maximum respiration rate of cells of sulfobacilli was observed as well (see the table). The data on ATP synthesis in the *S. thermotolerans* cell suspensions during the oxidation of the above-mentioned substrate mixture (figure, c) in the presence of DCCD indicate a 91% decrease in ATP production within the first 10 s after the addition of oxidation substrates; the rate of ATP synthesis at this time interval in the absence of inhibitor was 6.5 nmol ATP/(min mg protein). A similar dependency was observed under the same incubation conditions in the experiment with the *S. sibiricus* cell suspension. Nevertheless, it should be noted that, despite the intensification of the ATP synthesis and high respiration rates of both cultures during growth on organic substrate(s) and Fe(II), the level of inhibition of ATP synthesis was lower in all experiments with washed cells incubated at 55°C (as compared to that observed at 40°C), probably due to some side effects, such as the complex effects exerted by both high temperature and extreme acidophilic conditions (pH 1.7) on DCCD in the presence of heavy metal ions (Fe(II)). Unfortunately, there is no published information on the inhibitor resistance to extreme conditions.

On the whole, the capacity for mixotrophic growth is common among many facultative chemolithoautotrophs and chemolithoheterotrophs (hydrogen-oxidizing, nitrifying, and colorless sulfur bacteria) [11]. The results of our study demonstrated that members of the genus *Sulfobacillus* may be considered facultative chemolithoautotrophs that simultaneously utilize CO_2 and organic compounds as carbon sources [4, 9, 12], and both inorganic and organic compounds as electron donors and the sources of energy generated via the ETS functioning. This illustrates the broad metabolic potential of the *Sulfobacillus* species inhabiting the changing natural and technogenic ecosystems.

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