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EXPERIMENTAL ARTICLES

The Dependence of Intracellular ATP Level on the Nutrition Mode of the Acidophilic Bacteria *Sulfobacillus thermotolerans* and *Alicyclobacillus tolerans*

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Abstract—The dynamics of the ATP pool in the aerobic spore-forming acidothermophilic mixotrophic bacteria *Sulfobacillus thermotolerans* Kr1^T and *Alicyclobacillus tolerans* K1^T were studied in the course of their chemolithoheterotrophic, chemoorganoheterotrophic, and chemolithoautotrophic growth. It was established that, during mixotrophic growth, the maximum ATP concentrations in the cells of *S. thermotolerans* Kr1 and *A. tolerans* K1 were 3.8 and 0.6 nmol/mg protein, respectively. The ATP concentrations in sulfobacilli and alicyclobacilli during organotrophic growth were 2.2 and 3.1 nmol/mg protein, respectively. In the cells of the obligately heterotrophic bacterium *Alicyclobacillus cycloheptanicus* 4006^T, the maximum ATP concentration was several times higher and reached 12.3 nmol/mg protein. During lithotrophic growth, the maximum values of the ATP concentration in the cells of *S. thermotolerans* Kr1 and *A. tolerans* K1 were 0.3 and <0.1 nmol/mg protein, respectively; in the cells of the autotrophic bacterium *Acidithiobacillus ferrooxidans* TFBk, the ATP content was about 60–300 times higher (17.0 nmol/mg protein). It is concluded that low ATP content is among the possible causes of growth cessation of *S. thermotolerans* Kr1 and *A. tolerans* K1 under auto- and heterotrophic conditions after several culture transfers.

Key words: ATP pool, sulfobacilli, alicyclobacilli, chemolithoautotrophic, chemoorganoheterotrophic, and mixotrophic growth.

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Bacteria of the genus *Sulfobacillus* represent a group of chemolithotrophic microorganisms affiliated with the family *Alicyclobacillaceae* on the basis of the analysis of their 16S rRNA gene sequences, the fatty acid compositions of their membranes, the nature of their menaquinones, and some other phenotypic characteristics. The common property of the majority of sulfobacilli is their ability to grow stably only under mixotrophic conditions [1]; only some of them can grow stably under auto- or heterotrophic conditions [2]. The list of bacteria which grow at the expense of mixotrophic nutrition includes representatives of carboxydobacteria, as well as hydrogen-oxidizing and sulfate-reducing bacteria, nitrifiers, thiobacilli, etc. Unlike sulfobacilli, the majority of them can grow stably under lithotrophic and organotrophic conditions [3].

S. thermotolerans Kr1, representing a novel species of sulfobacilli, oxidizes Fe (II), elemental sulfur, and sulfide minerals; it utilizes yeast extract and some sugars and amino acids in low concentrations as energy sources and electron donors [4]. *S. thermotolerans* Kr1 is a mixotroph; however, its chemolithoautotrophic properties are more pronounced than chemoorganoheterotrophic. Another strain of sulfobacilli, *S. thermosulfidooxidans* subsp. *thermotolerans* K1, was recently reclassified by us. On the basis of its phenotypic and chemotaxonomic properties, it was assigned to the species *Alicyclobacillus tolerans*. Strain K1 is able to oxidize elemental sulfur, sulfide minerals, and, to a lesser extent, Fe(II) [5]. It is also a mixotroph; however, its chemoorganoheterotrophic properties are more pronounced than those of strain Kr1.

The ATP pool in the cells is the result of the dynamic equilibrium between the processes of its accumulation and utilization [6]. It is well known from the literature that, usually (if not always), the level of ATP in bacterial cells grown under physiologically different conditions reflects the true relationship between the energy-releasing and energy-consuming reactions. It is believed that, if the growth rate is limited by the energy input (as usually is the case with chemolithoautotrophic

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bacteria), the coupling of ATP production and utilization is maximal, irrespective of the presence or absence of a positive feedback between the energy-releasing and energy-consuming reactions (growth control) [7]. Defects in the energy source catabolism, which have been previously detected in sulfobacilli (the lack of activity of pentose phosphate pathway enzymes under autotrophic and heterotrophic conditions, as well as the lack of activity of pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase, and isocitrate lyase, observed irrespective of the nutrition type), may result in an impaired energy supply for cells and cause the cessation of litho- or organotrophic growth after several culture transfers [1, 8]. In connection with the considerations mentioned above, of considerable interest is investigation into the bioenergetics of acidophilic oligotrophic sulfobacilli and alicyclobacilli with a mixotrophic mode of nutrition.

In this work, we studied ATP concentrations in the cells of *S. thermotolerans* Kr1^T and *A. tolerans* K1^T in order to find out why litho- and organotrophic growth of these mixotrophs ceases after several culture transfers. For comparison, the obligate autotroph *Acidithiobacillus ferrooxidans* TFBk and the heterotroph *A. cycloheptanicus* 4006^T were also studied.

MATERIALS AND METHODS

Organisms and cultivation conditions. In our study, we used *Sulfobacillus thermotolerans* Kr1^T (=VKM B-2339 = DSM 17362) and *Alicyclobacillus tolerans* K1^T (=VKM B-2304 = DSM 16297), bacteria that oxidize Fe^{2+}, as well as reduced sulfur compounds and some organic compounds. Strain Kr1 was isolated from the concentrate pulp during the processing of gold–pyrite–arsenic ore in Eastern Siberia [4]; strain K1, from a sample taken in the Kurgashinkan lead–zinc deposit [9]. The physiological and biochemical properties of these bacteria were described previously [4, 5]. The autotrophic bacterium *Acidithiobacillus* (*At.*) *ferrooxidans* TFBk [10] and the obligately heterotrophic bacterium *Alicyclobacillus cycloheptanicus* DSM 4006^T (=ATCC 49029) were used for comparison.

Strains *S. thermotolerans* Kr1 and *At. ferrooxidans* TFBk were grown on a modified 9K mineral medium containing (g/l): $(NH_4)_2SO_4$, 3.0; KCl, 0.1; KH_2PO_4, 0.5; $MgSO_4 \times 7H_2O$, 0.5; and $Ca(NO_3)_2 \times 4H_2O$, 0.01 [4]. Strain *A. tolerans* K1 was grown on Manning medium containing (g/l): $(NH_4)_2SO_4$, 6.0; KCl, 0.2; KH_2PO_4, 0.2; $MgSO_4 \times 7H_2O$, 1.0; and $Ca(NO_3)_2 \times 4H_2O$, 0.02 [5]. In order to cultivate these bacteria under autotrophic conditions, both media were supplemented with $FeSO_4 \times 7H_20$ to concentrations of 1.5, 2–2.5, and 7 g/l of Fe^{2+} for *A. tolerans* K1, *S. thermotolerans* Kr1, and *At. ferrooxidans* TFBk, respectively. For mixotrophic growth of *S. thermotolerans* Kr1 and *A. tolerans* K1 on 9K and Manning media, a mixture of glucose (0.02%) and yeast extract (0.02%) was used in combination with ferrous iron in the aforementioned concentrations. The medium for *A. tolerans* K1 cultivation was additionally supplemented with 2 mM $Na_2S_2O_3$. For chemoorganoheterotrophic cultivation, media with glucose and yeast extract were used. Strain *A. cycloheptanicus* 4006 was grown on BAC medium containing (g/l): $CaCl_2 \times 2H_2O$, 0.4; $MgSO_4 \times 7H_2O$,1.0; $(NH_4)_2SO_4$, 0.4; KH_2PO_4, 6.0; yeast extract, 10.0; and glucose, 10.0 [5]. The medium acidities were adjusted with 10 N H_2SO_4 to pH 1.8, 2.5, 1.9–2.0, and 4.0 for *At. ferrooxidans* TFBk, *A. tolerans* K1, *S. thermotolerans* Kr1, and *A. cycloheptanicus* 4006, respectively.

To obtain the inoculum for further cultivation under various conditions, the cultures of *S. thermotolerans* Kr1 and *A. tolerans* K1 were grown under mixotrophic conditions on the above-specified mineral media with yeast extract and ferrous iron at 38 and 40°C, respectively. Strains *At. ferrooxidans* TFBk and *A. cycloheptanicus* 4006 were grown on mineral (9K) and complex (BAC) media at 28 and 45°C, respectively. The bacteria were cultivated in 250-ml Erlenmeyer flasks containing 100 ml of medium at constant agitation on a rotor shaker (180 rpm). The cultures were tested for purity by plating aliquots onto nutrient agar and agarized BAC medium or 9K medium with Fe^{2+} and yeast extract, as well as by microscopic examination of specimens under a LyumamI-1 light microscope (LOMO, Russia) equipped with a phase-contrast device. The strains were kept physiologically active by successive transfers to liquid media, except *A. cycloheptanicus* 4006, which was subcultured on agarized BAC medium.

Bacterial growth was assessed from the protein content determined by the Lowry method; the iron content was determined by titration with the complexon Trilon B; the glucose content was determined by the anthranone method [11].

Methods of ATP extraction and analysis. ATP was extracted from the cells during the culture growth with dimethyl sulfoxide (DMSO) as follows: cells from 50-ml samples were harvested by centrifugation (T-23 Janetzki, Germany) at 5000 *g* for 20 min; the sediment was resuspended in 1 ml of distilled water and supplemented with 1 ml of DMSO; then the mixture was thoroughly agitated on a shaker for two minutes and put into a refrigerator. Immediately before the ATP concentration was measured, 3 mg of dry ATP reactant was dissolved in 1 ml of deionized water. To determine ATP concentration, 20 μl of the reactant solution was supplemented with 1 ml of 0.1 M Tris–acetate buffer (pH 7.8) and 100 μl of the DMSO extract of bacterial cells. To prepare 100 ml of 0.1 M Tris–acetate buffer (pH 7.8), 25 ml of 0.4 M Tris buffer was supplemented with 33 ml of a 0.2 N solution of glacial acetic acid; then, distilled water was added so as to bring the solution to the required volume. The buffer also contained 10 mM $MgSO_4 \times 7H_2O$ and 2 mM EDTA. The ATP concentration in the sample was determined by the modified bioluminescent method [12, 13] with the Microlum ATP reactant based on the soluble glowworm luciferase

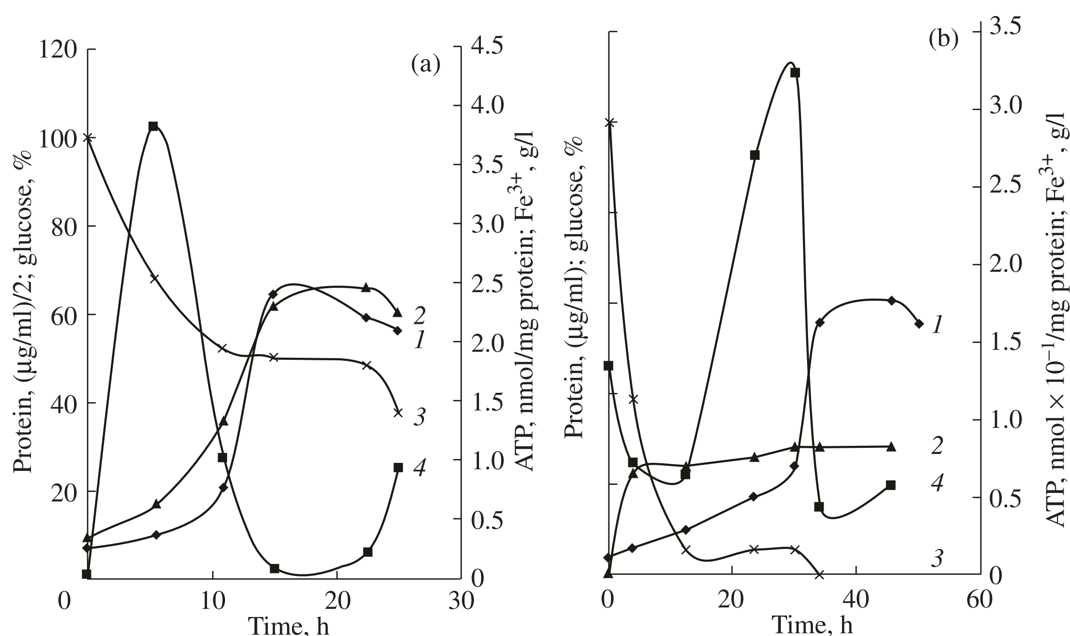

Fig. 1. Mixotrophic growth of strains (a) *S. thermotolerans* Kr1 and (b) *A. tolerans* K1 on the medium with ferrous iron, glucose, and yeast extract: (*1*) protein; (*2*) Fe^{2+} oxidation; (*3*) glucose utilization, % of the initial content; (*4*) changes in the ATP concentration.

(Lumtek, Russia). The sensitivity of this method allows the ATP concentration to be determined even at a low cell density in the sample (weak growth of the culture in question). The luminescence intensity was measured with an RTF 20046 luminometer (Germany) equipped with a recorder, at a voltage ranging from 300 μV to 100 mV (1 μV = 5×10^6 quanta/s; Hastings–Weber light standard). The ATP concentration was determined using the calibration curves which were obtained for each series of measurements using standard ATP solutions (Sigma, United States) in DMSO with concentrations ranging from 10^{-4} to 10^{-7} M. The experiments were performed in at least five replicates; typical results are presented.

RESULTS

Mixotrophic growth. When grown under mixotrophic conditions on the medium containing glucose, yeast extract, and iron, strains *A. tolerans* K1 and *S. thermotolerans* Kr1 differed in the lag phase durations and specific rates of exponential growth, 0.17 and 0.25 h^{-1}, respectively (Figs. 1a and 1b; curves00 *1*). Strain *S. thermotolerans* Kr1 simultaneously oxidized the mineral and organic substrates only at the initial stage of growth (Fig. 1a, curves *2* and *3*). The rate of iron oxidation was 140–250 mg/h, depending on the growth phase. The peak rate of glucose utilization was 13 mg/h. Strain Kr1 completely oxidized iron in 22 h of cultivation and utilized 63% of the glucose added to the medium in 25 h. Strain K1 oxidized only 40% of the ferrous iron added to the medium, at a rate of 30 mg/h (Fig. 1b, curve *2*); however, it utilized glucose more completely than strain Kr1 (Fig. 1b, curve *3*) and consumed its entire amount in 34 h at a maximum rate of 17.6 mg/h.

The ATP content in the cells of both cultures correlated with the rate of substrate consumption (for strain K1, primarily glucose), as well as with the growth phase. The ATP concentration in strain Kr1 cells ranged from a maximum value of 3.8 nmol/mg protein (before the culture growth accelerated) to 0.1 nmol/mg protein (at the beginning of the stationary phase) (Fig. 1a, curve *4*). The second increase in the ATP level after reaching the stationary phase (after the exhaustion of the iron pool) is probably associated with the resumption of active glucose consumption. The results of microscopic examinations demonstrated that, during this period, the majority of cells in the population retained the morphological properties typical of vegetative cells; however, some cells began to produced spore.

The ATP concentration in the *A. tolerans* K1 cells was minimal after inoculation and increased to 0.3 nmol/mg protein in parallel to the energy substrate consumption before the culture growth accelerated (Fig. 1b, curve *4*). At the onset of the stationary phase, the ATP content decreased. By the end of the culture growth, the morphology of most cells remained unchanged; however, some forespores were detected.

Organotrophic growth. Strain *S. thermotolerans* Kr1 grew on the medium with glucose and yeast extract at a specific rate of 0.17 h^{-1} (Fig. 2a, curve *1*). The two-

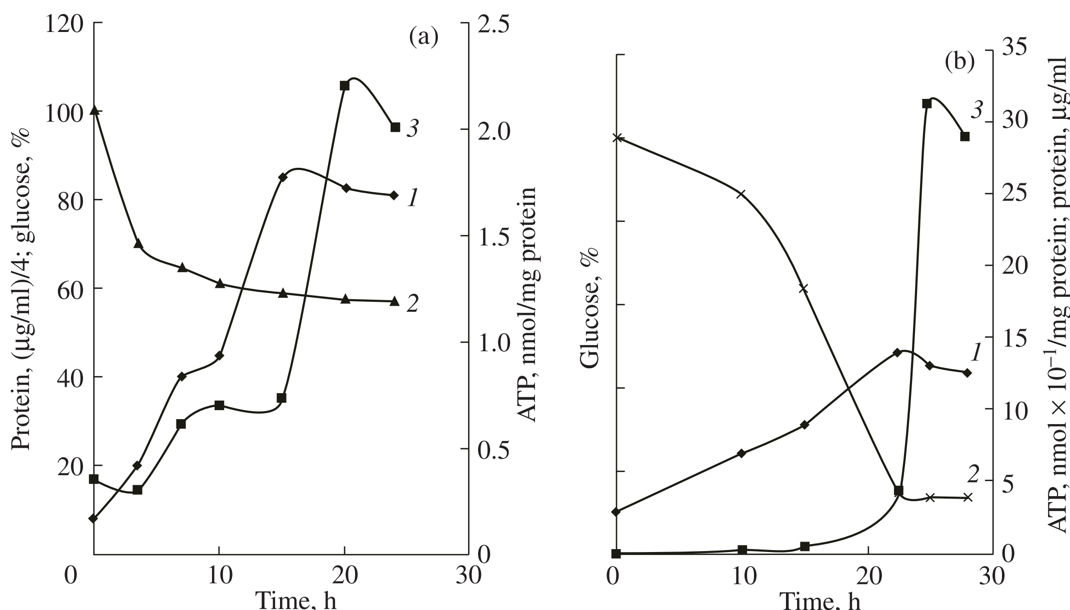

Fig. 2. Organotrophic growth of strains (a) *S. thermotolerans* Kr1 and (b) *A. tolerans* K1 on the medium with glucose and yeast extract: (*1*) protein; (*2*) glucose utilization, % of the initial content; (*3*) changes in the ATP concentration.

phase curve of this strain growth suggests that there may be a transition from the preferential oxidation of glucose to that of yeast extract. The total glucose utilization by the *Sulfobacillus* cells was 43% of the added amount after 20-h cultivation (Fig. 2a, curve *2*); the peak rate of its oxidation (20 mg/h) was detected in the first hours of growth. Under the same conditions, the growth rate of *A. tolerans* K1 was lower (0.13 h^{-1}), and the amount of the biomass produced by the culture was smaller (Fig. 2b, curve *1*). Strain K1 utilized glucose at a lower rate (12.8 mg/h) than the *Sulfobacillus* strain; however, it utilized it almost completely in 25 h (Fig. 2b, curve *2*). In both cultures grown under chemoorganoheterotrophic conditions, the proportion of cells with spores was 5–10%.

The ATP concentrations in strains Kr1 and K1 grown under organotrophic conditions were 0.3–2.2 and 0.1–3.1 nmol/mg protein, respectively; the differences were insignificant. In both cultures, the maximum ATP concentrations were detected in stationary phase cells. This can be attributed to the equilibrium between the ATP synthesis and utilization during active growth and to the imbalance between these processes upon the onset of the stationary phase (Figs. 2a and 2b; curves *3*).

The specific rate of chemoorganoheterotrophic growth of the bacterium *A. cycloheptanicus* 4006, used for comparison with the studied mixotrophs, was high (μ_{max} = 0.2 h^{-1}), as well as the biomass yield (Fig. 3). The glucose concentration in the BAC medium was 50 times higher than in the growth media of oligotrophic mixotrophic bacteria, and 82% of its amount was utilized by *A. cycloheptanicus* 4006 in 25 h (Fig. 3, curve *2*) at a maximum rate of 1110 mg/h. Spore formation was not detected by the end of the cultivation, which could be due to its suppression by the high glucose concentrations. The dynamics of the ATP content in the cells at various growth phases was oscillatory. The maximum ATP level (12.3 nmol/mg protein) was detected at the end of the lag phase; cells at the stage of exponential growth exhibited low ATP concentrations (Fig. 3, curve *3*). The second increase in the ATP content (3.0 nmol/mg protein) was observed in the culture upon the onset of retarded growth. During this period, *A. cycloheptanicus* 4006 oxidized glucose and, probably, some components of yeast extract. Thus, during linear growth of the obligately heterotrophic bacterium, a constant ATP concentration was observed.

Autotrophic growth. Let us consider the growth of mixotrophic bacteria under autotrophic conditions. During growth in the medium with ferrous iron as the sole energy source (Fig. 4a), strain *S. thermotolerans* Kr1 grew (after a brief lag phase) at a specific rate of 0.12 h^{-1} (Fig. 4a, curve *1*) and oxidized iron during the whole growth period (curve *2*). The peak rate of iron oxidation was 120 mg/h. By the end of cultivation, the cell length and width decreased; some of the cells produced forespores. The maximum ATP concentration in cells, 0.3 nmol/mg protein, was detected before the onset of the exponential growth phase. On completion of this growth phase (in 10 h), growth slowed down (μ = 0.08 h^{-1}); the rate of iron oxidation was 100 mg/h. The ATP concentration did not exceed 0.1 nmol/mg protein.

Strain *A. tolerans* K1 grew under autotrophic conditions (Fig. 4b, curve *1*) at a specific rate of 0.09 h^{-1}; the

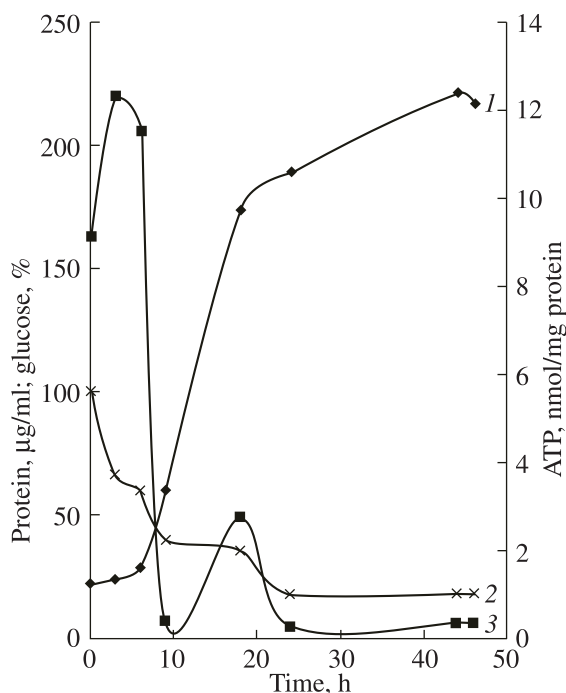

Fig. 3. Organotrophic growth of the bacterium *A. cycloheptanicus*: (*1*) protein; (*2*) glucose utilization, % of the initial content; (*3*) changes in the ATP concentration.

rate of iron oxidation was as low as 20 mg/h (curve *2*). By the end of cultivation, a majority of cells were represented by refractory, swollen, and sporulating cells. After 25 h of cultivation, the amount of spores constituted 25% of the total population. Two small increases in the ATP concentration (to values less than 0.1 nmol/mg protein) were observed (curve *3*), likely due to the utilization of substrates, iron, and reduced sulfur (data on the reduced sulfur utilization are not presented).

The autotrophic bacterium *At. ferrooxidans* TFBk, used for comparison, grew on the medium with ferrous iron (Fig. 5, curve *1*) at a higher rate (0.19 h^{-1}) than the studied cultures of mixotrophic bacteria. Iron oxidation occurred during the whole growth period (curve *2*). The maximum rate of the energy substrate oxidation was 364–400 mg/h, which exceeded the rate of its oxidation by strains Kr1 and K1. Some oscillatory fluctuations of the ATP content in the cells of strain TFBk were recorded, with maximum concentrations detected at the start and end of growth (6.5 and 17.0 nmol/mg protein, respectively, curve *3*).

DISCUSSION

According to the published data, the ATP content in microbial cells is low and makes up 0.1–10 mg per one gram of absolutely dry biomass (ADB), or approximately 0.2–20 μmol/g ADB, or 0.3–30 nmol/mg protein [7]. Some authors have demonstrated that the ATP

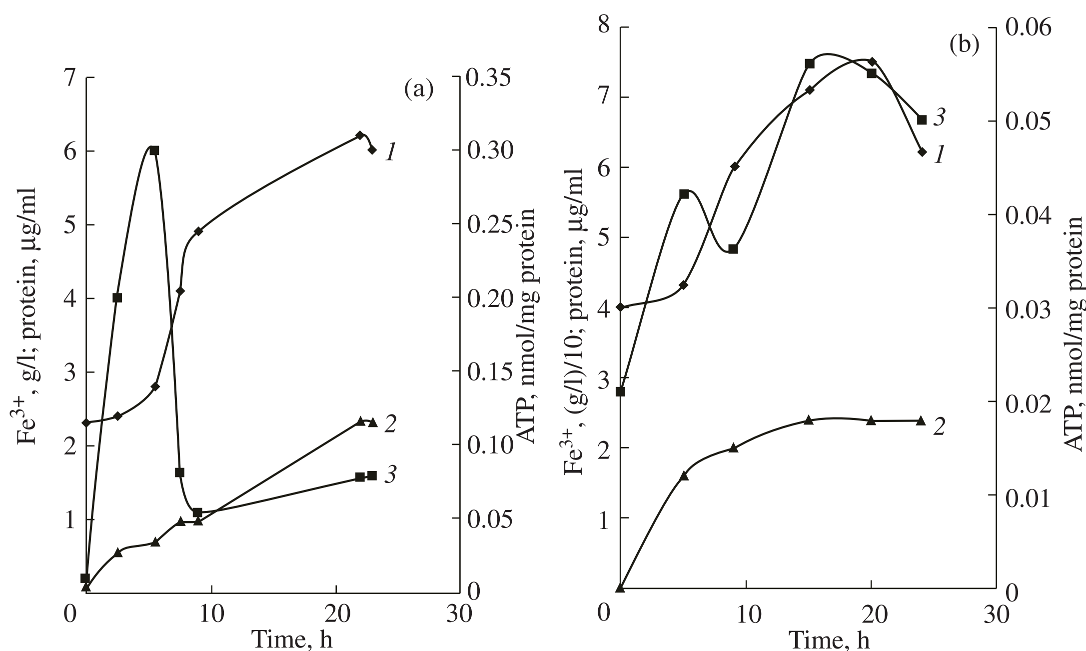

Fig. 4. Lithotrophic growth of strains (a) *S. thermotolerans* Kr1 and (b) *A. tolerans* K1: (*1*) protein; (*2*) Fe^{2+} oxidation; (*3*) changes in the ATP concentration.

content in heterotrophic bacteria is, as a rule, higher than that in autotrophic microorganisms; however, the opposite results have also been reported. For example, the ATP content in *Alicyclobacillus acidocaldarius* cells was 7 nmol/mg protein [14]; in *Bdellovibrio bacteriovorus* cells, it was 30 nmol/mg protein [15]. The maximum ATP content in cell supressions of heterotrophic bacteria *Hyphomicrobium* sp. and *Catenococcus thiocyclus* grown on a medium with acetate and thiosulfate reached 7–8 nmol/mg protein [16], whereas the ATP content in *Escherichia coli* cells was 4.2–8.2 nmol/mg ADB [17]. Washed cells of the heterotrophic sulfur bacterium *Spirillum winogradskyi* showed high rates of ATP production, 5.6 nmol/(min mg protein) [18]. In cell supressions of the alkaliphilic sulfate-reducing bacteria *Natroniella acetigena* and *Desulfonatronum lacustre*, the ATP content was 0.5–1.5 nmol/mg protein [19].

In the cells of the autotrophic bacterium *Nitrobacter winogradskyi*, the maximum ATP concentration was 0.9–2.5 nmol/mg protein [20]; in the cells of *Thiobacillus ferrooxidans*, it was 5 nmol/mg protein [21].

The ATP consumed during biomass synthesis represents only a portion of the total ATP pool consumed. Some of the energy is used for the production of the transmembrane electrochemical potential, as well as for CO_2 fixation, mechanical movement, cell maintenance processes, etc.; some of it is released into the surroundings in the form of heat. There is no universal pattern of ATP pool formation and its utilization for growth. Under unfavorable conditions that inhibit biosynthetic reactions, bacteria are able to maintain the rate of energy metabolism, so as to rapidly restore the high growth rate under favorable conditions.

A comparison of the rates of ATP accumulation and utilization during organotrophic growth on glucose of both mixotrophic bacteria under study (Figs. 2a and 2b) showed that the growth of strain *A. tolerans* K1, despite high rates of ATP synthesis, was slower (according to the rate of protein production) than that of *S. thermotolerans* Kr1. Under heterotrophic conditions, the maximum concentration of ATP in the *Alicyclobacillus* cells was higher than that in *Sulfobacillus* cells. In strain K1, which has the complete tricarboxylic acid (TCA) cycle, as well as a higher activity of the enzymes of carbohydrate catabolism [11, 22], it was 3.1 nmol/mg protein, whereas in strain Kr1, it was 2.2 nmol/mg protein. In the alicyclobacillus (strain K1), this ATP level was probably due to the more complete glucose oxidation as compared to strain Kr1. For instance, during organotrophic growth, *A. tolerans* K1 utilized glucose at a rate which was 50% higher than that of the *Sulfobacillus* strain. This observation explains the fact that *A. tolerans* K1, which, unlike the *Sulfobacillus* strain, is able to utilize almost completely the glucose added to the medium, survives numerous subculturings on media that contain only organic substrates [5].

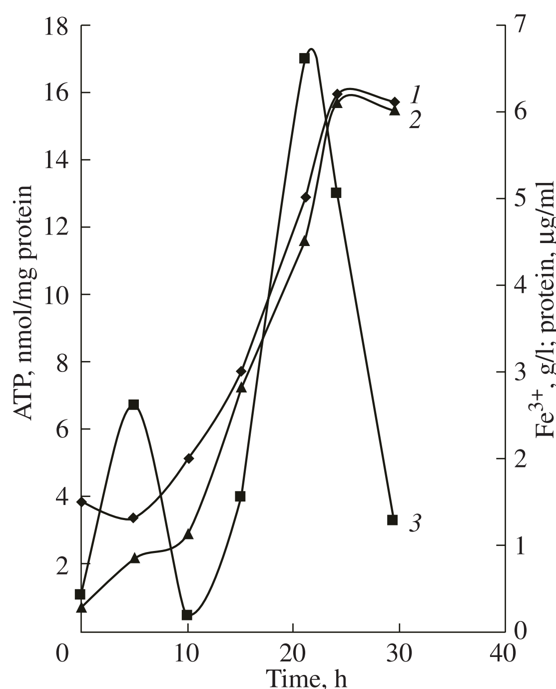

Fig. 5. Lithotrophic growth of the bacterium *At. ferrooxidans* TFBk: (*1*) protein; (*2*) Fe^{2+} oxidation; (*3*) changes in the ATP concentration.

The earlier established limited ability of strain K1 to utilize glucose (growth after three or four culture transfers) may be associated with the low rate of glucose uptake, because of certain defects in the transport system, as demonstrated for *S. acidophilus* ALV [23], as well as with low activity of the enzymes responsible for the glucose utilization as a carbon and energy source. Our previous studies of *A. tolerans* K1 showed a decrease in the activity of enzymes of sugar metabolism and of the TCA cycle when mixotrophic conditions gave way to heterotrophic ones; the glyoxylate shunt was not functioning as well because of the lack of the isocitrate lyase activity. Although functioning of the Entner–Doudoroff pathway instead of the oxidative pentose phosphate pathway [11] resulted in a somewhat higher energy yield, it could impair the production of nucleotide and nucleic acid precursors.

Importantly, the glucose transport into cells of the mixotrophic strains is dependent on the energy produced during the oxidation of inorganic compounds, since the addition of inorganic substrates simultaneously with organic ones increased the intensity of glucose utilization (Figs. 1a and 1b). These peculiarities of the operation of the carbon metabolism pathways in the *Sulfobacillus* and *Alicyclobacillus* strains result in lower yields of their organotrophic growth (see the table). The maximum ATP concentration in the studied strains during their chemoorganoheterotrophic growth on glucose (2.2–3.1 nmol/mg protein) was four to six times lower than that in the cells of the typical

Growth parameters and ATP concentrations in the cells of the studied cultures

Cultivation conditions	Maximum growth rate, h^{-1}	Maximum rate of iron oxidation, mg/h	Glucose utilization, %	Maximum protein content, mg/l	Maximum ATP concentration, nmol/mg protein
			S. thermotolerans Kr1		
Mixotrophic	0.26	250	63.0	32.1	3.8
Heterotrophic	0.17	–	43.0	21.0	2.2
Autotrophic	0.12	120		6.2	0.3
			A. tolerans K1		
Mixotrophic	0.17	30	100	38.7	0.6
Heterotrophic	0.13	–	87	14.0	3.1
Autotrophic	0.09	20		7.5	<0.1
			At. ferrooxidans TFBk		
Autotrophic	0.20	400		6.2	17.0
			A. cycloheptanicus 4006		
Heterotrophic	0.20		82	220.0	12.3

aerobic heterotrophic bacterium *A. cycloheptanicus* 4006 (Fig. 3). In the cells of strain 4006, the ATP concentration reached 12.3 nmol/mg protein. At the early growth stage, the ATP concentration in strain 4006 cells was one or two orders of magnitude higher than that in the cells of strains Kr1 and K1 grown on medium with organic substrates. This may account for the weaker organotrophic growth of *A. tolerans* K1 and *S. thermotolerans* Kr1 as compared to their mixotrophic growth.

During lithotrophic growth, the oxidation of Fe^{2+} by *S. thermotolerans* Kr1 and *A. tolerans* K1 occurred immediately after changing the optimal mixotrophic conditions (Figs. 4a and 4b). The maximum ATP concentration in the cells of *S. thermotolerans* Kr1 (0.3 nmol/mg protein) was approximately five times higher than that in the cells of *A. tolerans* K1 (less than 0.1 nmol/mg protein) (curves *3*). In the cells of the chemolithoautotroph *At. ferrooxidans* TFBk, used for comparison, the ATP content was 17.0 nmol/mg protein (Fig. 5), which was approximately 60 and 300 times higher than that in the cells of *S. thermotolerans* Kr1 and *A. tolerans* K1, respectively. Both in the beginning and at the end of growth, the ATP content in the cells of *At. ferrooxidans* TFBk (1.0–2.5 nmol/mg protein) was on average 6 and 30 times higher than that in *Sulfobacillus* and *Alicyclobacillus* strains, respectively. A much higher ATP content was also detected in other autotrophs mentioned above [20, 21]. Thus, the obtained results on ATP production by strains K1 and Kr1 confirmed that these bacteria are able to grow in autotrophic conditions using inorganic substrates as energy sources and electron donors and CO_2 as a carbon source. Under these conditions, the *Sulfobacillus* strain oxidized completely the ferrous iron added to the medium in 22 h. The *Alicyclobacillus* strain oxidized only 20% of the added iron (or about 10% of the iron oxidized by the *Sulfobacillus* strain) and switched to the utilization of reduced sulfur compounds (thiosulfate, which was present in the medium) (unpublished data). The rates of the autotrophic growth of both bacteria were low, 0.09 and 0.12 h^{-1}. The autotrophic CO_2 assimilation, which occurred due to the activity of ribulose bisphosphate carboxylase via the Calvin cycle, requires a great amount of energy. The switch from mixotrophic to autotrophic growth conditions had an adverse effect on the biomass yield and the culture morphology. Strain K1 retained only the glycolytic pathway of carbohydrate metabolism [11, 22], which can only provide for a low ATP level in cells. The results of our experiments suggest that the amount of energy released during the oxidation of inorganic compounds under autotrophic conditions is not large enough to provide for cell growth comparable to mixotrophic growth. The cells have to use ATP in such energy-consuming biosynthetic processes as carbon dioxide assimilation through the reductive pentose phosphate pathway, for the transmembrane transport, mechanical movement, membrane energization, and, probably, for the reverse electron transfer. It has been established that, under unfavorable conditions, the maintenance expenditures of ATP increase from 10 to 30% [7].

Under optimal mixotrophic conditions, the growth rates of strains *S. thermotolerans* Kr1 and *A. tolerans* K1 were the highest, 0.17 and 0.26 h^{-1}, respectively (Figs. 1a and 1b; curves *1*). In the presence of ferrous iron (and thiosulfate in the case of strain K1), glucose,

and yeast extract, *S. thermotolerans* Kr1 showed the maximum biomass yield (32.1 mg protein/l) after 15-h cultivation, and strain *A. tolerans* K1 showed the maximum biomass yield (38.7 mg protein/l) after 34-h cultivation. A competition between the glucose and ferrous iron utilization was detected. In strain *S. thermotolerans* Kr1, Fe^{2+} inhibited glucose utilization. On the contrary, glucose inhibited iron utilization in strain *A. tolerans* K1. Each of these cultures utilized primarily inorganic or organic substrates. The high growth rate of the *Sulfobacillus* strain was mainly due to the high rate and completeness of iron oxidation, whereas the high growth rate of the *Alicyclobacillus* strain was due to the high rate and completeness of glucose utilization. The rate of iron oxidation by *S. thermotolerans* Kr1 was four to eight times higher than that in the case of *A. tolerans* K1 (140–250 and 30 mg/h, respectively). Besides, the cells of *S. thermotolerans* oxidized approximately three times more iron than *Alicyclobacillus* cells. On the contrary, strain K1 *A. tolerans* utilized glucose at a higher rate than strain *S. thermotolerans* Kr1. After 12-h cultivation, the *Alicyclobacillus* cells utilized glucose almost completely, whereas the *Sulfobacillus* cells utilized only 63% of it by the end of growth (in 25 h).

Under these conditions, in the presence of glucose and Fe^{2+}, all the enzymes of the carbohydrate metabolism, as well as the majority of the TCA cycle enzymes, were more active in *A. tolerans* K1 than in *S. thermotolerans* Kr1. The utilization of glucose via the three main pathways of sugar catabolism, the fructose-bisphosphate, oxidative pentose phosphate, and Entner–Doudoroff pathways [11], enable the *Alicyclobacillus* cells to obtain the energy it needed. In the cells of *S. thermotolerans* Kr1, the same three pathways of sugar catabolism operate under mixotrophic conditions. However, the activity of the key enzymes of the Entner–Doudoroff pathway and of the pentose-phosphate pathway of glucose utilization (6-phosphogluconate dehydrogenase, 6-phosphogluconate dehydratase, and 2-keto-3-deoxy-6-phosphogluconate aldolase) is low [24]. The impaired oxidation of organic acids via the TCA and glyoxylate cycles (disruption of the TCA cycle at the level of 2-oxoglutarate due to the absence of 2-oxoglutarate dehydrogenase and isocitrate lyase activities) indicates that the *Sulfobacillus* strain is capable of utilizing only limited quantities of glucose as an energy source and electron donor.

When comparing the maximum ATP content in the cells of both cultures grown under mixotrophic conditions, it was found that the ATP concentration in strain *S. thermotolerans* Kr1 was five times higher than that in strain *A. tolerans* K1 (3.8 and 0.6 nmol/mg protein, respectively) (Figs. 1a and 1b; curves *4*). This fact points to the strain Kr1 higher capacity for chemolithotrophy, whereas the ability of strain K1 to utilize glucose almost completely attests to its pronounced heterotrophy. It is possible that, due to the active iron oxidation, the *Sulfobacillus* strain is able to obtain more reducing equivalents. Strain *A. tolerans* K1 consumes a considerable portion of the energy obtained during the process of glucose utilization for operation of the transport processes and the Calvin cycle. That is why the maximum ATP concentration in the sulfobacillus during mixotrophic growth is higher than that in the alicyclobacillus. Hence, the coupling of the processes of production and consumption of energy under optimal growth conditions are clearly seen in the studied thermotolerant bacteria. There is a pronounced correlation between the beginning of active bacterial growth and the decrease in the ATP concentrations in the cells.

The data on the biomass yield, iron oxidation, glucose utilization, and ATP concentrations in the bacterial cells grown under various conditions are presented in the table. It may be concluded that the 60- to 300-fold differences in the ATP concentrations between mixotrophic and autotrophic cells and the 4- to 6-fold differences between the mixotrophic and heterotrophic cells can explain the cessation of the lithotrophic and organotrophic growth of the studied strains after several culture transfers.

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