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EXPERIMENTAL ARTICLES

Lithoautotrophic Growth of the Freshwater Colorless Sulfur Bacterium *Beggiatoa "leptomitiformis"* D-402

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Abstract—The freshwater colorless sulfur bacterium *Beggiatoa "leptomitiformis"* D-402 was shown to be capable of lithoautotrophic growth in a batch culture under microoxic conditions at O_2 concentrations in the medium of no higher than 0.5 mg/l. The cell yield was maximum at a dissolved oxygen concentration of 0.15 mg/l. A high activity level of key enzymes of the Calvin cycle and of enzymes involved in dissimilatory oxidation of thiosulfate was recorded in the cells. The high rate of CO_2 assimilation (112–139 nmol/(min mg protein)) and the cell yield (12 mg dry cells/mmol thiosulfate oxidized), 91–92% of which was accounted for by CO_2 carbon, were close to those typical of autotrophic bacteria. Thiosulfate was oxidized almost completely to sulfate, and the fraction of intracellular sulfur in the final products did not exceed 0.2–1.7% of the thiosulfate sulfur. The cell membrane fraction contained cytochromes (*b* + *o*) and two cytochromes *c* with M_r of 23 and 26 kDa; the soluble fraction contained cytochrome *c* with M_r of 12 kDa.

Key words: freshwater filamentous sulfur bacteria, *Beggiatoa*, lithoautotrophy, ribulose-1,5-bisphosphate carboxylase, phosphoribulokinase, sulfite oxidoreductase, thiosulfate oxidoreductase, cytochromes.

The genus *Beggiatoa* comprises filamentous, gliding, colorless sulfur bacteria, which are poorly studied with respect to their taxonomy, metabolism, and phylogenetic relations. Representatives of this genus are widespread in freshwater and marine ecosystems. They develop on the surface of bottom sediments, forming sulfur mats at sites receiving hydrogen sulfide. Data on the metabolism of representatives of this genus are scarce due to difficulties related to obtaining and the maintenance of pure cultures. For two marine species, the capacity for lithoautotrophic growth at the expense of sulfur compounds was demonstrated [1, 2]. Numerous attempts to find lithoautotrophy or mixotrophy in freshwater strains, which were started in the early works of Pringsheim [3], have been unsuccessful [4–6]. Recently, we have shown the capacity for mixotrophic and strictly lithoautotrophic growth at the expense of sulfur compounds in the *Beggiatoa* strain D-402, isolated from a freshwater brook [7].

The aim of the present work was to check the ability of the freshwater strain *Beggiatoa "leptomitiformis"* D-402 to grow lithoautotrophically at the expense of thiosulfate oxidation. To do this, it was necessary to elaborate on a technique for the batch cultivation of these bacteria and to find the conditions favoring their autotrophic growth.

MATERIALS AND METHODS

The bacterium and conditions of its cultivation. This work used the *Beggiatoa "leptomitiformis"* D-402 strain isolated from a brook contaminated with domestic wastewater. The strain is maintained at the collection of the Laboratory of Ecology and Geochemical Activities of Microorganisms at the Institute of Microbiology, Russian Academy of Sciences. The composition of the medium used for cultivation was as follows (g/l distilled water): $NaNO_3$, 0.620; NaH_2PO_4, 0.125; $CaCl_2 \cdot 2H_2O$, 0.030; Na_2SO_4, 0.500; KCl, 0.125; $MgCl_2 \cdot 6H_2O$, 0.05 (pH 7.0). Before inoculation, the medium was supplemented with sterile solutions of trace elements and vitamins [8], and also with 100–500 mg/l of $NaHCO_3$ and a 10% solution of $Na_2S_2O_3 \cdot 5H_2O$ to a final concentration of 1–5 g/l. The solutions of $NaHCO_3$ and thiosulfate were sterilized by ultrafiltration. The conditions for lithoheterotrophic and mixotrophic cultivation of strain D-402 were described earlier [7].

The technique of batch cultivation at various concentrations of O_2. The bacteria were cultivated in 1.2 l shake flasks sealed with butyl rubber and closed with screw caps. The flasks contained 125 ml of liquid medium. Gas phases with varying oxygen concentrations were obtained by completely filling the flasks with freshly boiled sterile medium and then forcing out

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Table 1. Effect of oxygen concentration in mineral medium on the biomass yield of *Beggiatoa* "*leptomitiformis*" D-402 (mg protein/l)

Culture age, h	O_2 concentration in the medium, mg/l				
	2.0	1.0	0.5	0.2	0.15
	O_2 concentration in the gas phase, %				
	1.42	0.72	0.36	0.14	0.09
24	0	1.0	1.5	1.3	7.5
48	0	1.0	3.0	8.3	11.3
72	0	1.0	4.5	10.5	15.3
96	0	1.0	3.3	1.5	3.3
120		1.0	1.5		1.5

a certain volume of the medium, first with argon and then with air (Table 1). Argon and air were sterilized by passing them through 0.2-μm-pore-size Millipore filters. After one day, the oxygen content in the gas phase was determined on a LKhM-80 gas chromatograph. The content of dissolved oxygen in the medium was determined by the Perfil'ev's modification [9] of the Winkler method, without adding thiosulfate to the medium. This technique allows the content of oxygen to be determined in a small sample volume; samples and reagents are taken with a 5- or 10-ml vacuumed syringe washed with freshly boiled water. After the introduction of thiosulfate to a final concentration of 1–5 g/l, inoculum (1 ml per 125 ml of the medium) was introduced with a syringe. All analyses were performed after three or four culture transfers in each variant of the medium. The composition of the gas phase was monitored by periodically measuring the O_2 content by gas chromatography. At a gas phase–liquid phase ratio of 1 : 10, the content of O_2 in the gas phase changed insignificantly during the growth of bacteria in all cultivation variants. In cases where the O_2 concentration in the gas phase decreased by more than 25–30% (in variants with the initial O_2 concentration of 0.21 and 0.15 mg O_2 per 1 l of gas), the gas phase was periodically (one or two times per day) supplemented with a certain volume of air to restore the initial O_2 concentration. During lithoheterotrophic and mixotrophic growth, the bacteria were cultivated in shake flasks under cotton plugs providing free access of air [7].

The intensity of $NaHCO_3$ assimilation was determined with ^{14}C radiocarbon by a conventional procedure [10] using 5 mM $NaH^{14}CO_3$ with a specific activity of 1×10^7 cpm/mmol $NaH^{14}CO_3$. The rate of autotrophic fixation of CO_2 was judged from the incorporation of the radiocarbon of $NaH^{14}CO_3$ into cellular protein. The bacteria were incubated for 24 h in a medium with $NaH^{14}CO_3$ (2×10^6 cpm/mmol) at an oxygen concentration in the medium of 0.3 mg/l. After that, a sample aliquot was filtered through a 0.2-μm-pore-size membrane filter, and the radioactivity of cells was measured on a Mark-II scintillation counter. The content of protein in the cells was assumed to be 43–44% of the cell carbon [11].

Obtaining of cell suspensions and membrane preparations. Cell suspensions were obtained by centrifugation (5000*g*, 4°C, 30 min) of bacterial cultures and washing the cells twice with 0.1 M Tris–HCl buffer (pH 7.5). Cell homogenate was obtained by disrupting bacterial cells in a UZDN-2T ultrasonic disintegrator (500 W, 22 kHz, 2 min, on an ice bath). The homogenate was centrifuged (9000*g*, 4°C, 30 min), and the supernatant was collected.

Determination of key enzymes of the Calvin cycle. The activity of ribulose-1,5-bisphosphate carboxylase (RubisCo, EC 1.1.39) was determined in the supernatant using labeled bicarbonate ($NaH^{14}CO_3$) with a specific activity of 10^6 cpm/mmol. The activity of the enzyme was judged from the rate of ^{14}C incorporation into the acid-stable product of the reaction [12]. Nonspecific fixation of CO_2 was determined in the control lacking ribulose-1,5-bisphosphate. Since the active center of RubisCo is sensitive to oxygen, bacterial cells and supernatant were kept in an argon atmosphere to avoid enzyme inactivation.

Phosphoribulokinase (EC 2.7.1.19) was determined from the formation rate of alkali-hydrolyzable phosphorus from ribulose-1,5-bisphosphate [12]. The determination was carried out in an argon atmosphere. The incubation medium contained (mM) Tris–HCl (pH 7.8), 100; $MgCl_2$, 5; DTT, 5; ribose-5-phosphate Na, 10; and $ATPNa_2$, 5. The enzyme preparation (supernatant) was added in an amount of 0.05 ml per 0.5 ml of the incubation medium. The reaction was terminated by the addition of trichloroacetic acid. Alkali-hydrolyzable phosphorus was determined in the presence of a 3% solution of $(NH_4)_2MoO_4$ and a 1% solution of ascorbic acid. Pyridoxal phosphate was used as the enzyme inhibitor.

Determination of carboanhydrase (EC 4.2.1.1). The activity of this enzyme was determined from the rate of the change in the indicator coloration during the enzymatic reaction [12] and expressed in the Wilbur and Andersen units [12].

Thiosulfate–ferricyanide oxidoreductase (EC 1.8.2.2) and sulfite–cytochrome *c* oxidoreductase (EC 1.8.3.1) [13] were determined in the supernatant by spectrophotometrically measuring the reduction rates of ferricyanide ($\lambda = 420$ nm) or cytochrome *c* ($\lambda = 550$ nm) in the presence of oxidizable substrate.

Other biochemical methods. Sulfur-containing compounds, cytochromes *c*, and protein were determined as described earlier [14]. Prior to protein determination, sulfur was extracted with 50% ethanol for 2 h, after which cells were washed with 0.05 M Tris–HCl and hydrolyzed in 1 N NaOH for 10 min at 90°C.

The membrane fraction was obtained from cells treated with egg lysozyme (2 h, 37°C) and then mechanically disrupted by the cell suspension pass-

Table 2. Dynamics of thiosulfate oxidation, product accumulation, and molar biomass yield in *Beggiatoa "leptomitiformis"* D-402 autotrophic cultures grown under different conditions

Duration of cultivation, h	Oxidized, mg/l			Accumulated, mg/l			Cell protein, mg/l	$Y_{S_2O_3^{2-}}$
	$S/S_2O_3^{2-}$	S/SO_3^{2-}	$S/S_4O_6^{2-}$	$S/S_4O_6^{2-}$	S^0	S/SO_4^{2-}		
At 0.15 mg dissolved O_2 per 1 l medium								
24	102.1	0	0	3.07	0.4	104	6.1	7.5
	72.9	0	0	0	0.5	74	7.05	12.2
48	145.9	0	0	0.6	2.6	148	7.3	
	102.1	0	3.5	0	3.8	104	8.0	
72	160.5	0	1.8	0	3.6	163	8.9	
	116.7	0	0	0	4.5	119	9.0	
At 0.5 mg dissolved O_2 per 1 l medium								
24	30						0.76	3.4
	25						0.54	2.7

Note: The table shows data of two replicate experiments. Protein was assumed to make up 50% of the weight of dry cells. $Y_{S_2O_3^{2-}}$ is the molar biomass yield (mg dry cells/mmol thiosulfate oxidized).

Table 3. Specific activities of the Calvin cycle key enzymes (nmol/(min mg protein)) and carboanhydrase (arbitrary units) in cells of *Beggiatoa* "*leptomitiformis*" D-402 grown lithoautotrophically under microaerobic conditions (0.3 mg O_2/l)

Enzyme	Measurement conditions		
	without inhibitor	+ pyridoxal phosphate (2.8×10^{-5} M)	+ PCMB (10^{-5} M)
RubisCo	73.0 ± 5	–	5.0 ± 5
Phosphoribulokinase	2138.0 ± 50	503 ± 50	–
Carboanhydrase*	23.8 ± 2	–	–

* The activity of carboanhydrase in cells grown lithoheterotrophically under microaerobic conditions was 5.6 arbitrary units.

Table 4. Activity of specific oxidases of sulfur compounds in cells of *Beggiatoa* "*leptomitiformis*" D-402 grown lithoautotrophically and mixotrophically

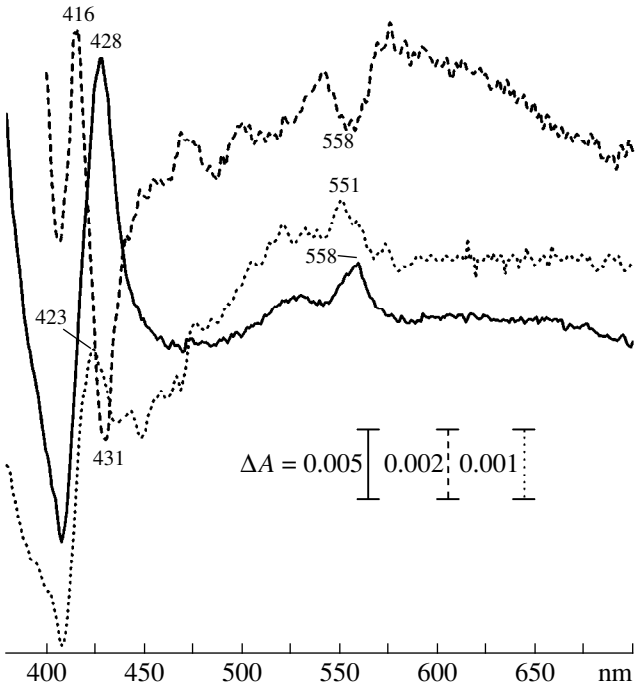
O_2, mg/l medium	Sulfite–cytochrome *c* oxidoreductase, nmol/(min mg protein)	Thiosulfate–ferricyanide oxidoreductase, nmol/(min mg protein)
	Lithoheterotrophic growth*	
9.0	300	31
	Mixotrophic growth**	
9.0	90	60
	Lithoautotrophic growth	
0.1	1500	260

Note: The table presents data of representative experiments.

* Cultivation was performed in medium with acetate and thiosulfate in the presence of 50 μM rotenone (NADH dehydrogenase inhibitor).

** Cultivation was performed in medium with acetate and thiosulfate without rotenone.

ing through the French press five times. Membrane fragments were sedimented by centrifugation (200 000*g*, 4°C, 3 h). Difference absorption spectra of membrane suspensions (dithionite-reduced versus air-oxidized and dithionite-reduced and treated with CO versus dithionite-reduced) were recorded on a Hitachi U-3400 spectrophotometer at room temperature.



Difference absorption spectra of *Beggiatoa "leptomitiformis"* D-402 membrane particles. Solid line, dithionite-reduced minus air-oxidized; broken line, dithionite-reduced and treated with CO minus dithionite-reduced; dotted line, ascorbate-reduced minus air-oxidized.

RESULTS

Conditions of lithoautotrophic growth. Our attempts to cultivate *Beggiatoa "leptomitiformis"* D-402 in a thiosulfate-containing mineral medium with free access of air or at an oxygen concentration of higher than 1% in the gas phase (1–9 mg/l of medium) did not produce positive results. Autotrophic growth in the mineral medium with thiosulfate occurred only in microaerobic conditions at an O_2 concentration in the medium not higher than 0.5 mg/l (Table 1). The biomass yield during autotrophic growth increased (reaching 15.3 mg protein/l) with a decrease in the dissolved oxygen content from 0.5 to 0.1 mg/l. Under microaerobic conditions, the growth pattern of the filamentous sulfur bacteria changed: they grew as short, highly motile filaments and were evenly distributed in the medium.

Intensity of carbon dioxide fixation. During the cultivation of the bacteria with radioactive $NaH^{14}CO_3$ in mineral medium at an O_2 concentration of 0.5 mg/l, the yield of cell protein over the first 24 h of incubation (exponential growth phase) was 1.0 and 1.4 mg/l in two experiments (0.54 and 0.76 mg of protein carbon per 1 l). The accumulation of cell carbon, measured radioisotopically, amounted to 1.13 and 1.62 mg/l (or, according to our calculations, 0.49 and 0.7 mg protein carbon per 1 l). Thus, the fraction of protein carbon originating from carbon dioxide fixation comprised 91–92% (a value close to those characteristic of chemolithotrophic bacteria studied so far). The calculated rate of labeled bicarbonate fixation ranged from 112 to 139 nmol/(min mg protein) in replicate experiments. During growth in mineral medium, 96–99.5% of thiosulfate was oxidized to sulfate. Elemental sulfur accumulated intracellulary in minor amounts (0.4–3.7% of thiosulfate sulfur) (Table 2).

The values of the molar biomass yield, calculated from data on biomass yields and thiosulfate oxidation rates in the exponential growth phase during the first day of cultivation, significantly depended on the cultivation conditions. If the concentration of dissolved oxygen was 0.1 mg/l, $Y_{S_2O_3^{2-}}$ equaled to 7.5–12.2 mg dry biomass per 1 mmol thiosulfate oxidized. When the concentration of dissolved oxygen was 0.5 mg/l, the molar biomass yield was about three times lower, varying in replicate experiments from 2.7 to 3.4 mg dry biomass per 1 mmol thiosulfate oxidized.

The activity of key enzymes of the Calvin cycle and carboanhydrase. High activities of the key enzymes of the Calvin cycle, namely RubisCo (73 nmol/(min mg protein)) and phosphoribulokinase (2138 nmol/(min mg protein)), were recorded in cells grown lithoautotrophically in microaerobic conditions (Table 3). The addition to the reaction mixture of specific inhibitors, namely *p*-chloromercuribenzoate (PCMB) or pyridoxal phosphate, resulted in an abrupt decrease in the enzymatic activities: RubisCo was inhibited by 93%, and phosphoribulokinase was inhibited by 76%. In autotrophically grown cells, the activity of carboanhydrase, involved in the transformation of a bicarbonate ion into CO_2, which directly reacts with RubisCo, proved to be more than four times higher than in cells grown lithoheterotrophically.

The activity of sulfur metabolism enzymes. Earlier, we found that during the mixotrophic and chemolithoheterotrophic growth of *Beggiatoa "leptomitiformis"* D-402, the main role in thiosulfate oxidation (linked to the functioning of the electron transport chain) is played by sulfite oxidoreductase and, to a lesser extent, by thiosulfate oxidoreductase [7]. During lithoautotrophic growth, both of the above-mentioned enzymes exhibited high activities: 1500 and 260 nmol/(min mg protein), respectively. As can be seen from Table 4, the enzymatic activities in autotrophically grown cells were 5 and 8 times higher than in chemolithotrophically grown cells, and 17 and 4 times higher than in mixotrophically grown cells.

Cytochrome content. The difference absorption spectra of membrane particles exhibited bands characteristic of cytochromes *c* (absorption maximum in the alpha band at 551 nm) and cytochromes *b* (maximum absorption in the alpha band at 558 nm) (figure). The content of these cytochromes was 0.22 and 0.17 nmol/mg membrane protein. A more detailed study of cytochromes *c* by electrophoresis in polyacrylamide gel with SDS showed the presence of at least three cytochromes *c* in the cells: a soluble cytochrome *c* with a molecular mass of 12 kDa and two membrane-

bound cytochromes *c* with molecular masses of 23 and 26 kDa. Difference CO-spectra revealed only cytochromes of the *b* + *o* type.

DISCUSSION

Investigation of the habitats of colorless sulfur bacteria with the use of selective microelectrodes showed that the development of *Beggiatoa* spp. in marine bottom sediments is confined to a narrow zone of O_2 and H_2S gradients with micromolar concentrations of O_2 and H_2S [15]. Successful isolation of autotrophic marine species of *Beggiatoa* proved possible with the use of gradient semisolid mineral media [1]. Numerous *Beggiatoa* isolates from freshwater habitats, which are usually more oxidized than marine habitats, were obtained by a number of researchers under aerobic cultivation conditions; however, attempts of autotrophic cultivation proved unsuccessful. We made an attempt to find conditions favorable for lithoautotrophic growth of our freshwater isolate, paying attention primarily to the oxygen concentration in the medium. Comparison of the values of the biomass yield and the molar biomass yield allowed us to establish the oxygen concentration range within which lithoautotrophic growth is possible. This concentration range of dissolved oxygen proved to be 3–16 mM (0.1–0.5 mg O_2 /l); the growth maximum was observed at the lowest concentration tested.

The values of the parameters of *Beggiatoa "leptomitiformis"* D-402 lithoautotrophic growth (activities of the enzymes of the Calvin cycle and sulfur metabolism and the molar growth yield) were similar to those earlier established for lithoautotrophic thiobacilli [11, 16] and much higher than values earlier determined in gradient cultures of two marine strains of *Beggiatoa* [17, 18]: the activity of RubisCo was 2.4-fold higher, the activity of sulfite oxidase was 1.5- to 2-fold higher, and the molar biomass yield during thiosulfate oxidation was 1.5-fold higher. It should be noted that the $Y_{\max_{H_2S}}$ value observed during the chemostat cultivation of sulfur bacteria was shown to be two times higher than the $Y_{\max_{S_2O_3^{2-}}}$ value; i.e., the efficiency of H_2S oxidation is two times higher than the efficiency of thiosulfate oxidation. Therefore, it can be anticipated that the efficiency of sulfide-dependent lithoautotrophic growth of the freshwater strain would be much higher than that determined in marine strains.

The higher molar growth yield observed in the freshwater strain D-402 can be caused by different reasons: either by a higher activity of the enzymes involved in the oxidation of sulfur compounds or by more efficient energy conservation. In marine strains, energy conservation occurs via both oxidative phosphorylation and substrate-level phosphorylation, which is less efficient. Our study of the cytochrome composition showed that the higher molar growth yield of the freshwater strain may also be due to the peculiarities of the composition of its electron transport chain (ETC). In autorophically grown cells of strain D-402, we found cytochromes *b*, three types of cytochromes *c*, and CO-binding cytochromes (*b* + *o*). The latter may represent the oxidase of the bb_3 type, and this allows us to assume the existence of two sites of coupling of electron transport with ATP synthesis. In the cells of marine *Beggiatoa*, only two *c*-type cytochromes were found: a low-molecular cytochrome c_{553} and a multiheme complex with an M_r of 210 kDa [19]; this suggests the presence of only one coupling site in the ETC. As far as freshwater *Beggiatoa* isolates are concerned, data on the composition of their ETC during organotrophic growth are contradictory and probably reflect diversity of sulfur metabolism pathways. Cells of a *B. alba* strain grown with acetate and sulfide contained tree types of cytochrome c_{553} and a CO-binding flavocytochrome c_{554} [20]. Earlier studies of the same strain only reported the presence of cytochromes *c* [20]. However, the difference spectra presented in the paper cited give a clear indication of the presence of cytochromes *b* (a shoulder in the 553–562 nm region). Summarizing the data available on the cytochrome composition of freshwater isolates, we can state that, despite the opinion of some authors, the presence of cytochromes *b*, *c*, *a*, and flavocytochrome c_{554} has been definitely established in their heterotrophically grown cells. Our study of lithoautotrophically grown cells of strain D-402 revealed neither cytochrome *a* nor flavocytochromes. Further investigations of the cells of this strain grown under different cultivation conditions will show whether the aforementioned difference is due to species-specific peculiarities of the ETC composition or to differences in growth conditions.

Special attention should be paid to the regulatory role of oxygen with respect to the intensity of the lithoautotrophic growth of strain D-402. It follows from data presented in Tables 1 and 3 that the decrease in biomass yield and molar biomass yield with and increase in the concentration of dissolved oxygen in the medium (down to complete lack of growth) cannot be explained by the inhibition of oxidases of sulfur compounds alone. Their activities remained sufficiently high even under aerobic conditions during lithoheterotrophic growth and were close to the activities observed in marine strains. The main factor determining the decrease of biomass yield seems to be the repression of RubisCo.

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