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EXPERIMENTAL ARTICLES

Regulation of Metabolic and Electron Transport Pathways in the Freshwater Bacterium *Beggiatoa leptomitiformis* D-402

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Abstract—The biomass yield of freshwater filamentous sulfur bacteria of the genus *Beggiatoa*, when grown lithoheterotrophically or mixotrophically, has been shown to increase 2 to 2.5 times under microaerobic conditions (0.12 mg/l oxygen) as compared to aerobic conditions (9 mg/l oxygen). The activity of the glyoxylate cycle key enzymes have been found to increase two to three times under microaerobic conditions (at an O_2 concentration of 2 mg/l), and the activities of the sulfur metabolism enzymes increased three to five times (at an O_2 concentration of 0.1–0.5 mg/l). It has also been found that, under microaerobic conditions, thiosulfate was almost completely oxidized to sulfate by the bacteria, without accumulation of intermediate metabolites. At the same time, a 2- to 15-fold decrease in the activities of the tricarboxylic acid cycle enzymes involved in the reduction of NAD and FAD was observed. Reorganization of the respiratory chain after changes in aeration and type of nutrition was also observed. It has been found that, in cells grown heterotrophically, the terminal part of the respiratory chain contained an aa_3-type oxidase, whereas, during mixotrophic, lithoheterotrophic, and autotrophic growth, aa_3-type oxidase synthesis was inhibited, and the synthesis of a cbb_3-type oxidase, which is induced under microaerobic conditions, was activated. The gene of the catalytic subunit CcoN of the cbb_3-type oxidase was sequenced and proved to be highly homologous to the corresponding genes of other proteobacteria.

Key words: freshwater *Beggiatoa*, mixotrophy, lithoautotrophy, microaerophily, sulfur metabolism, cytochromes aa_3 and cbb_3.

The habitats of filamentous colorless sulfur bacteria of the genus *Beggiatoa* are restricted to the junction of aerobic and anaerobic layers on the surface of H_2S-rich sulfur depositions on bottom sediments. As a consequence of their adaptation to growth in a zone where oppositely directed gradients of H_2S and oxygen develop, *Beggiatoa* cells form near-bottom mats, whose thickness varies from tenths of a millimeter to 10 cm, and, sometimes, even to 60 cm [1].

The results of the latest research on fossil layers in the Santa Barbara basin indicate that the *Beggiatoa* mats are of ancient phylogenetic origin [2]. Deep-water sulfur bacteria identical to the present-day *Beggiatoa* inhabited anoxic sulfur depositions on bottom sediments as early as in the late Archean and Proterozoic eras. At that time, the concentration of oxygen in the atmosphere was low, and the bottom of deep water bodies was a place where the damaging effects of ultraviolet radiation could be evaded. According to the symbiotic hypothesis of the origin of eukaryotes, deep-water *Beggiatoa* mats could have played an important role in the formation and maintenance of associated communities of aerobic and anaerobic protists, metazoans, and prokaryotes. Present-day *Beggiatoa* mats have been the source of isolation of a large number of new taxa. However, the study of associated organisms is complicated by the fact that it may not be possible to support their viability under laboratory conditions outside their community. The development of methods for maintenance of *Beggiatoa* in pure cultures may be an important step in the reconstruction of natural ecosystems and investigation of their component organisms.

There have been numerous attempts to maintain *Beggiatoa* in pure cultures. The possibility of cultivating *Beggiatoa* under both aerobic and anaerobic conditions has been demonstrated [3]; however, the cultivation conditions were usually heterotrophic. In natural environments, *Beggiatoa* most probably develop at the expense of inorganic sulfur compounds. It was not until recently that the ability of freshwater *Beggiatoa* to produce lithoautotrophic growth was demonstrated by the example of strain D-402. Lithoautotrophic growth

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occurred only under microaerobic conditions at O_2 concentrations that did not exceed 4–15 μM [4]. It should be noted that the natural habitats of *Beggiatoa* are microaerobic. It remains unknown, however, whether microaerobic conditions are crucial for the realization of other types of metabolism by *Beggiatoa* cells.

The aim of this work was to study the effect of different O_2 concentrations on the metabolism of *Beggiatoa* and on the components of its electron transport chain.

MATERIALS AND METHODS

Organisms and cultivation conditions. Freshwater filamentous sulfur bacterium *Beggiatoa leptomitiformis* strain D-402 (=DSMZ 14946), taken from the collection of the Laboratory of the Ecology and Geochemical Activity of Microorganisms, Institute of Microbiology, Russian Academy of Sciences, was used in this work. The composition of the media and conditions of autotrophic and mixotrophic cultivation were as described in [4]; the conditions of microaerobic cultivation and methods of obtaining cell suspensions and enzyme preparations were as described in [5].

Determination of enzymatic activity. The activity of tricarboxylic acid cycle and glyoxylate cycle enzymes were determined on a SF-26 spectrophotometer using commonly applied spectrophotometric methods [6]. The activity of sulfur metabolism enzymes was measured in supernatants of cell homogenates according to previously described methods [5, 7]. Specific activity was expressed in micromoles of substrate (or reaction product) formed (consumed) per minute per milligram of protein.

Chemical and spectral analyses. Determination of hydrogen sulfide, thiosulfate, sulfates, tetrathionate, elementary sulfur, and protein; spectral analyses of bacterial membranes; and electrophoretic separation of cytochromes *c* were performed according to the procedure described in [8].

DNA extraction was conducted using the Marmur method [9].

The search for genes and amplification of DNA fragments were carried out on a CycloTemp amplifier (NPF STM-Ts, Russia). The reaction mixture contained 10–20 ng of the DNA under study, 2 pM of each primer, and 2 U of *Tag* DNA-polymerase (DiaTag, Helikon, Russia). The temperature regime was as follows: 94°C for 3 min and then 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s. The following degenerated primers were used for the fragment of the *ccoN* gene:

Fw: 5'-CA(C,T)AC(C,T,G,A)TC(G,C,T)GC(G,C)GT(G,C)AT(C,T)TTCGC(C,T)TTC-3';
Rv: 5'-CGACATCATCGG(C,A,G)CC(C,T)TCGAA-3'.

Sequencing of the amplified DNA fragments was performed on an automatic four-capillary DNA sequencer ABI Prism 3100-Avant Genetic Analyzer using an ABI PRISM® BigDye™ Terminator v 3.1 reagent kit at the Genome Center at the Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, with the support of the Russian Foundation for Basic Research. The nucleotide sequence of the fragment of the *ccoN* gene of *Beggiatoa leptomitiformis* D-402 was deposited with GenBank (accession AJ891039).

RESULTS AND DISCUSSION

Influence of the concentration of oxygen in the cultivation medium on the metabolism type and enzymatic activity. The ability of freshwater strain *Beggiatoa leptomitiformis* D-402 to utilize reduced sulfur compounds as sources of energy when grown lithoautotrophically and mixotrophically has already been demonstrated [4]. In the absence of organic compounds in the growth medium, strain D-402 is capable of lithoautotrophic growth under strictly microaerobic conditions, with its cell yield (*Y*) exceeding the that of marine autotrophic strains of *Beggiatoa*. It has also been shown that *B. leptomitiformis* D-402 is incapable of autotrophic growth on mineral medium with thiosulfate under aerobic conditions or at O_2 concentrations in the medium corresponding to $p_{O_2} = 0.01$ or higher (1–9 mg/l). Autotrophic growth is registered only under microaerobic conditions at O_2 concentrations lower than 0.5 mg/l [4]. During autotrophic growth, thiosulfate was primarily oxidized to sulfate, and elemental sulfur was accumulated in minor quantities. The $S^0 : SO_4^{2-}$ ratio averaged 1 : 50. For lithoheterotrophic growth, the $S^0 : SO_4^{2-}$ ratio averaged 1 : 3 (Table 1). The specific rate of thiosulfate oxidation on the first day of cultivation was 6–7 times higher for lithoautotrophic growth than that for mixotrophic growth and amounted to 0.11–0.18 μmol/(min mg protein) and 0.02–0.025 μmol/(min mg protein), respectively (Table 1). The effects of O_2 concentrations on the biomass yield during mixotrophic growth on a medium with thiosulfate and succinate are presented in Fig. 1. The biomass yield was inversely related to the initial concentration of O_2 in the medium: maximal yield (46.5–59.0 mg/l) was registered at 0.12–0.21 mg O_2/l.

Analyses of the thiosulfate oxidation products revealed that thiosulfate was primarily oxidized to elemental sulfur and sulfates and that tetrathionate was accumulated in minor quantities. The ratio of oxidation products depended on the extent of aeration (Table 1). Under aerobic conditions, the $S^0 : SO_4^{2-}$ ratio was 1 : 2.6

Table 1. Influence of the concentration of dissolved oxygen in the growth medium on the intensity of thiosulfate oxidation and composition of the end products during autotrophic and mixotrophic growth of *B. leptomitiformis* D-402

Time, h	Oxidized, mg S/l	Accumulated, mg S/l			Protein, mg/l	*Y**
	$S_2O_3^{2-}$	$S_4O_6^{2-}$	S^0	SO_4^{2-}		
Autotrophic growth under microaerobic conditions (0.12 mg O_2/l)						
24	87	2.0	0.5	89	6.5	12.8
48	124	0.3	3.2	126	7.5	10.0
72	138	0.0	4.0	141	9.0	10.0
Lithoheterotrophic growth under microaerobic conditions (0.12 mg O_2/l)						
24	46	1.8	4.8	44	28.0	–
48	131	0.0	25.4	108	46.5	–
72	204	0.0	38.4	156	55.0	–
Lithoheterotrophic growth under aerobic conditions (9 mg O_2/l)						
24	23	0.0	4.5	17	10.0	–
48	87	0.0	19.8	52	18.0	–
72	116.78	4.0	28.1	81	24.0	–
Lithoautotrophic growth under microaerobic conditions (0.3 mg O_2/l)						
24	30	–	–	–	0.76	3.4

* Molar growth yield, mg dry cells/mmol thiosulfate.

after 48 h of incubation, whereas, at 0.12 mg O_2/l, it was 1 : 3.8. Tetrathionate was not accumulated under microaerobic conditions, sulfates being the major end products, which means an increase in the energy output of oxidative reactions.

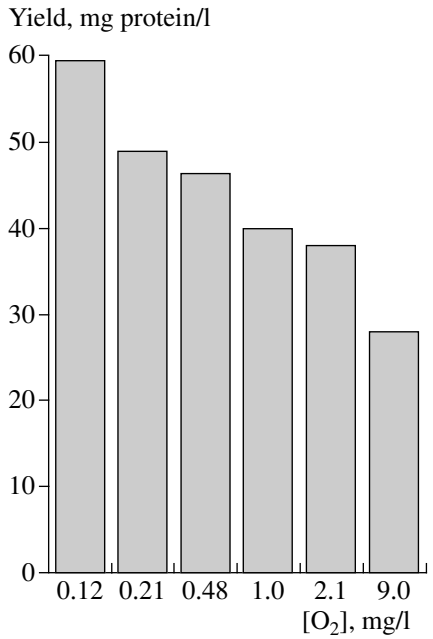

Fig. 1. Influence of the concentration of dissolved oxygen in the medium on the yield of *B. leptomitiformis* grown mixotrophically.

Together with an increase in the biomass yield, a decrease in aeration led to changes in the specific activity of the carbon and sulfur metabolism enzymes (Tables 2, 3). In a previous study, we established that, during chemolithoheterotrophic growth, a key role in the dissimilatory oxidation of thiosulfate is played by sulfite oxidoreductase and, to a lesser extent, thiosulfate oxidoreductase [4]. The specific activity of oxidoreductases of sulfur compounds are high and comparable to those in marine lithotrophic strains of *Beggiatoa* [10]. It was shown that, for strain D-402, a decrease in O_2 concentration from 9 to 0.1 mg/l resulted in a threefold increase in specific sulfite oxidoreductase activity, whereas specific thiosulfate oxidoreductase activity increased by 2.4 times. At the same time, there was a decrease in the specific activity of the tricarboxylic acid cycle enzymes participating in the reduction of NAD and FAD, i.e., in the activity of isocitrate dehydrogenase, 2-oxoglutarate dehydrogenase, succinate dehydrogenase, and malate dehydrogenase. However, growth under microaerobic conditions was accompanied by an increase in the activity of the key enzymes of the glyoxylate cycle, i.e., isocitrate lyase and malate synthase, which provide intermediates for constructive metabolism (Table 3). A significant increase in aconitate hydratase activity under microaerobic conditions partly results from the antioxidative effect of thiosulfate, as has been shown for the microaerophilic sulfur bacterium *Spirillum winogradskii* [11]. Thiosulfate reduces the inhibitory effects of reactive oxygen species on hydratases. Hydratases contain a $[4Fe\text{-}4S]^{2+}$

cluster, which was earlier shown [12] to be a sensor of the superoxide anion.

Thus, our results indicate that a decrease in the O_2 concentration in the cultivation medium is accompanied by changes in the *B. leptomitiformis* D-402 energy metabolism: the role of inorganic compounds increases, whereas that of organic compounds decreases.

Cytochrome components of the electron transport chain in the membranes of cells with different types of metabolism under various aeration regimes. According to the difference spectra obtained during successive reduction of cytochromes with ascorbate, *N,N,N′,N′*-tetramethyl-*n*-phenylenediamine (TMPD), and dithionite, the membranes of *B. leptomitiformis* cells grown lithoheterotrophically, organotrophically, and autotrophically, contain cytochromes *c* and *b* with peaks in at the α absorption bands at 551–558 nm. The reduction of all the cytochromes is shown in Fig. 2a. After organotrophic growth in the absence of thiosulfate, the membrane spectra had a peak at 605 nm, corresponding to the maximum of the cytochrome *c* α band (Fig. 2a, *2*). The CO difference spectra revealed a peak at 590 nm and trough at 608 nm characteristic of cytochrome a_3 (Fig. 2b, *2*). Cytochrome a_3 was detected only in the organotrophically grown cultures, which indicates the presence of an aa_3-type oxidase. The factors inducing this oxidase in *B. leptomitiformis* have not yet been described.

The data presented in Fig. 3 (*2B*) show that the cytochrome *b* share of the total of all the cytochromes (light-gray columns) was constant for all the nutrition types and amounted to 56–57% whereas the CO-binding cytochrome *b* share (dark-gray columns) increased by 2–3 times during autotrophic and lithoheterotrophic growth as compared to organotrophic growth. The CO spectrum of the membranes in the 542–572 nm region (Fig. 2b), despite the seeming similarity to the *Escherichia coli* cytochrome *o* spectrum, exhibited characteristics typical of CO-binding cytochromes *b* (with peaks at 541 and 572 nm and a trough at 557 nm) [13]. The ability of cytochromes *b* to bind CO indicated that the respiratory chain included a cbb_3-type oxidase, which was repressed under conditions favoring synthesis of the aa_3-type oxidase (Fig. 3, *2B*). According to the electrophoresis data, the qualitative composition of cytochromes *c* was not influenced significantly by the growth conditions. In all cases, soluble cytochrome *c* and two membrane cytochromes *c* with molecular weights of 12, 23, and 26 kDa, respectively, were detected. The general scheme of the electron transport chain in *B. leptomitiformis* is presented below.

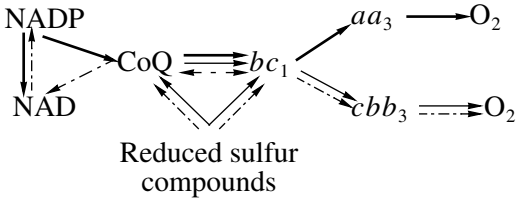

Table 2. Influence of the concentration of dissolved oxygen in the growth medium on the activity of sulfur metabolism enzymes in *B. leptomitiformis* D-402 grown mixotrophically and lithoautotrophically

O_2, mg/l	Sulfite oxidoreductase activity, nmol/(min mg of protein)	Thiosulfate oxidoreductase activity, nmol/(min mg protein)
Mixotrophic growth		
9.0	300	31
2.1	450	64
0.48	600	140
0.12	920	75
Lithoautotrophic growth		
0.12	1530	260

Table 3. Influence of the concentration of dissolved oxygen in the growth medium on the activity of carbon metabolism enzymes in homogenates of *B. leptomitiformis* D-402 cells grown mixotrophically*

Enzymes	Activity, nmol/(min mg protein)	
	2.1 mg O_2/l**	9 mg O_2/l
Aconitate hydratase	30.0	15.0
Isocitrate dehydrogenase	1.4	24.0
Succinate dehydrogenase	6.0	36.0
Fumarate hydratase	54.0	97.0
Malate dehydrogenase	120.0	270.0
2-oxoglutarate dehydrogenase	ND	12.0
Citrate synthase	11.0	8.5
Isocitrate lyase	11.0	5.0
Malate synthase	18.8	6.5

* Growth in a medium with 2 g/l thiosulfate and 0.5 g/l succinate.
** These determinations used cells whose prehistory included four successive culture transfers under microaerobic conditions (2 mg O_2/l).

The arrows indicate electron transfer under various growth conditions: the thick, thin, and dashed arrows refer to organotrophic, lithoheterotrophic, and lithoautotrophic growth, respectively.

The genes encoding the cbb_3-type oxidase were detected by PCR with oligonucleotide primers targeting conservative sites of the gene *ccoN* which encodes the major enzyme subunit. Sequencing of the PCR products showed their high homology to the CcoN subunits of the cbb_3-type oxidases of other proteobacteria. Comparison of the deduced amino acid sequence corresponding to the gene fragment from *B. leptomitiformis* with the respective fragment from *Rhodobacter sphaeroides*, a typical holder of a cbb_3-type oxidase [14], revealed 71% homology (Fig. 4).

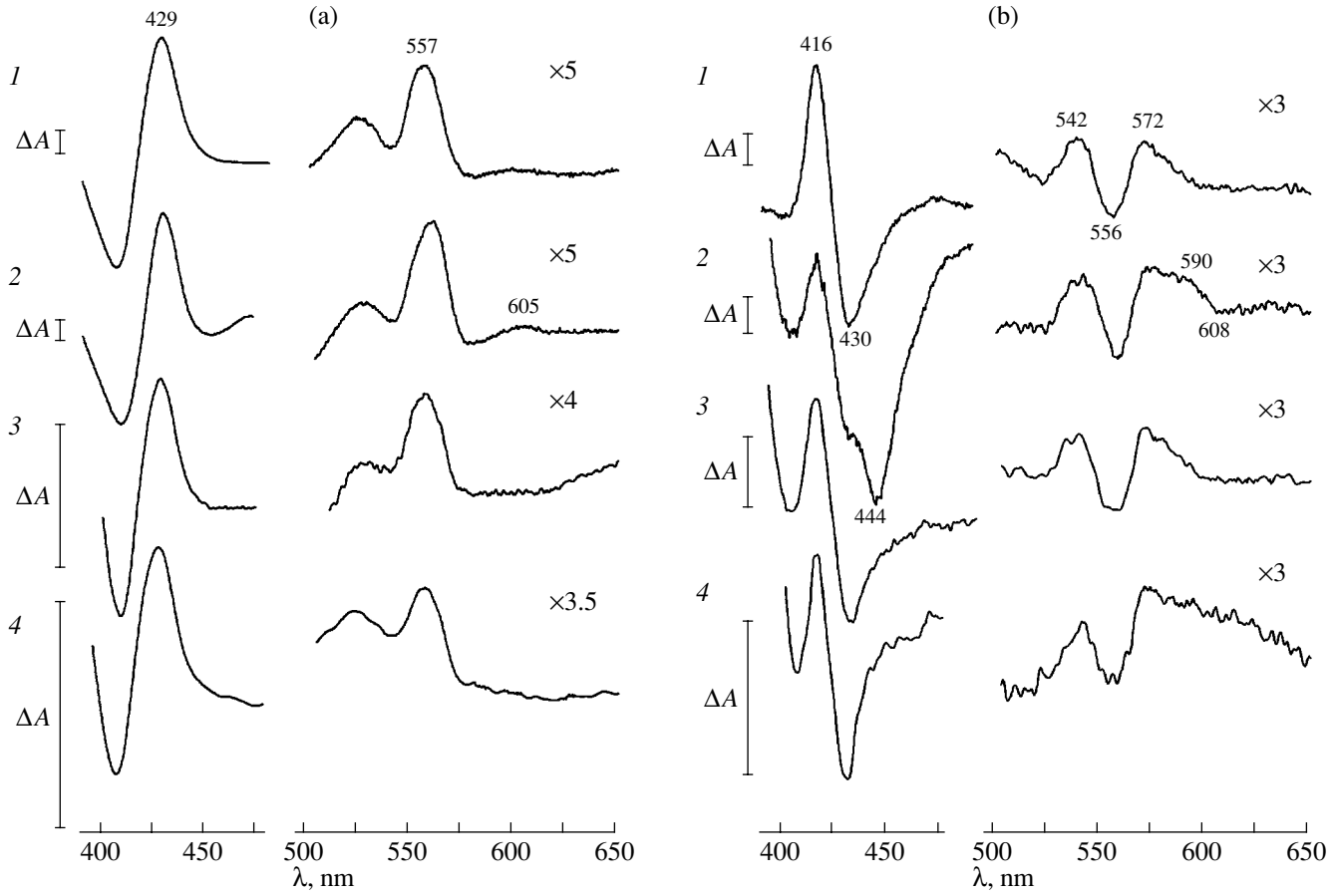

Fig. 2. Difference spectra of membrane particles derived from *B. leptomitiformis* D-402 cells after (*1*) aerobic lithoheterotrophic, (*2*) aerobic organotrophic, (*3*) microaerobic lithoheterotrophic, and (*4*) microaerobic lithoautotrophic growth. (a) Dithionite-reduced minus air-oxidized particles; (b) dithionite-reduced CO-treated particles minus dithionite-reduced particles. ΔA corresponds to (a) 0.01 and (b) 0.002.

The highest specific amount of total cytochromes corresponded to lithoheterotrophic growth under aerobic conditions, whereas the lowest was recorded under lithoautotrophic conditions. As can be seen from Fig. 3 (*1B* and *1C*), the increase in the cytochrome content during aerobic lithoheterotrophic growth (columns *1*) resulted mainly from the activation of synthesis of cytochromes *b* and *c*. Their amounts increased by 1.5 and 2 times, respectively. Evidently, using thiosulfate as an energy source, in addition to organic substrates, requires completion of the electron transport chain with cytochromes *b* and *c*. An assumption can be made that this branch of electron transfer is inducible, and its content is regulated by the presence of sulfur sources of energy in the cultivation medium. As Fig. 3 (*1B* and *1C*) shows, the levels of cytochromes *b* and *c* decreased under microaerobic conditions (columns *3*) as compared to aerobic conditions (columns *1*). If the assumption made above is correct, the transfer to microaerobic conditions evidently results in elimination of those cytochromes *b* and *c* that are not involved in microaerobic respiration. It is noteworthy that the share of cytochromes *b* (Fig. 3, panels *2A* and *2B*) was constant in the presence of thiosulfate under all the growth conditions studied (columns *1, 3*, and *4*), and, consequently, so was the ratio of cytochromes *b* and *c*. Thus, the shape of the difference spectra (Fig. 2a, *1, 3*, and *4*) remained constant and did not reflect changes in the respiratory chain. A significantly different cytochrome composition was observed on the media without thiosulfate (Fig. 3, panels *A–C*, columns *2*). In the absence of thiosulfate, cytochrome aa_3 is appeared in the *B. leptomitiformis* cell membranes. It was not detected under other growth conditions. We have shown for the first time that a cbb_3-type oxidase functions in *B. leptomitiformis* cells in addition to an aa_3-type oxidase. It is known that the affinity of the latter to oxygen is an order of magnitude higher, and it is usually induced by low concentrations of oxygen. According to our data, synthesis of the cbb_3-type oxidase in *B. leptomitiformis* is enhanced under microaerobic conditions. The share of this oxidase amounted to 23% of the total cytochrome content, as compared to 8 and 15% during aerobic organotrophic and mixotrophic growth, respectively (Fig. 3 *2B*, dark-gray columns). As low oxygen concentrations favor the maintenance of a low redox potential, it is

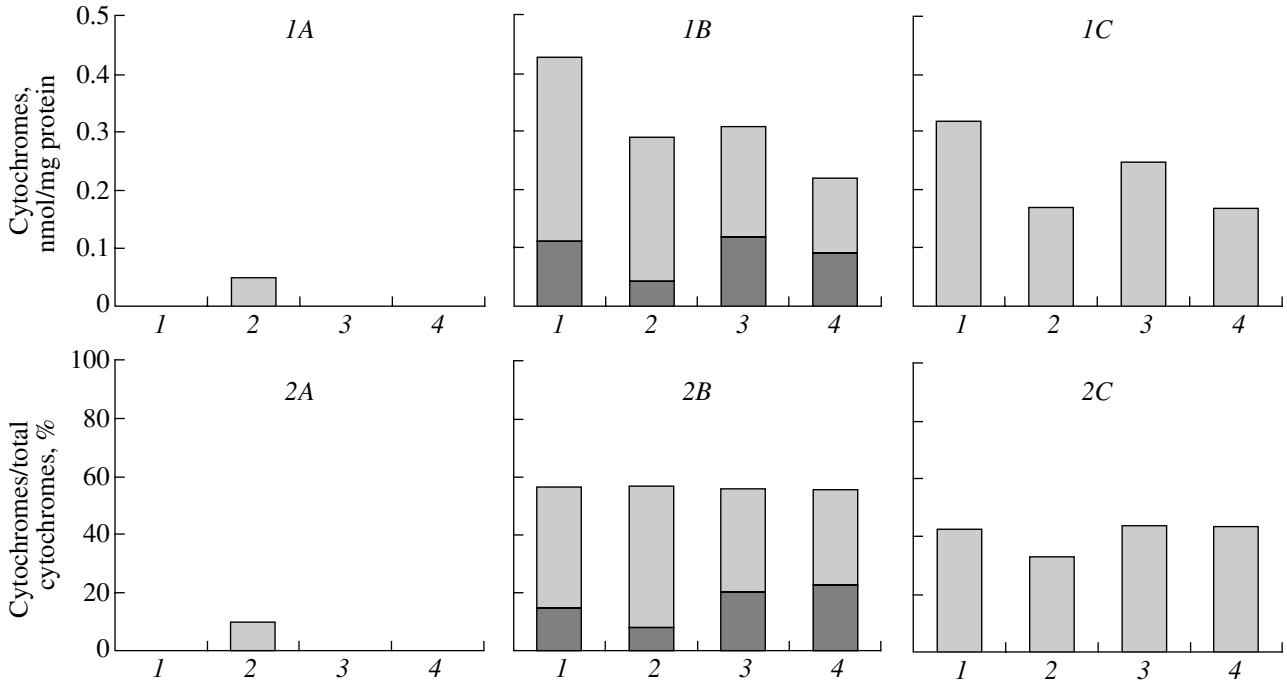

Fig. 3. Contents of cytochromes (*1A* and *2A*) *a*, (*1B* and *2B*) *b*, and (*1C* and *2C*) *c* in the cell membranes of *B. leptomitiformis* D-402 as dependent on the cultivation conditions (*1, 2, 3*, and *4*, see the caption to Fig. 2). Quantitative determination of cytochromes *c* in the presence of cytochromes *b* was performed by recording the difference spectra of membrane particles incubated with 2.5 mM potassium cyanide for 20 h and then reduced with 5 mM potassium ascorbate and 10 μM TMPD for 30 min versus air-oxidized membrane particles. The quantity of cytochromes *b* was evaluated by subtracting the difference spectra obtained for determination of cytochromes *c* as described above from the difference spectra of dithionite-reduced versus air-oxidized membrane particles.

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Beggiatoa                                                                MGYTSSKE
Rhodobacter sphaeroides     121 AVIFAFGGNA LIATSFYVVQ RTSAARLWGG NLGWFVFWGY NLFIVLVAQS YLLGATQSKE 180

Beggiatoa                        YAELEWPIDI LIAIVWVTYA IVFFGTMIKR KTKHIYVGNW FFGAFIIAVA ILHIVNNVEM
Rhodobacter sphaeroides     181 YAEPEWYLDL WLTIVWVCYL AAFLGTIIKR KEPHIYVANW FYLAFIVTVA MLHIFNNLSI 240

Beggiatoa                        PVSL--IKSY SAYAGATDAM IQWWYGHNAV GFFLTAGFLG MMYYFMPKQA ERPMYSYRLS
Rhodobacter sphaeroides     241 PVSFFGSKSV QVFSGVQDAM VQWWYGHNAV GFFLTAGFLG MMYYFVPKQA ERPVYSYKLS 300

Beggiatoa                        IVHFWALIIL YMWAGPHHLH YTALPDWAQS MGMVMSIILL APSWGGMING MMTLSGAWHK
Rhodobacter sphaeroides     301 IVHFWALIFL YIWAGPHHLH YTALPTWTST LGMVFSIMLW MPSWGGMING LMTLSGAWDK 360

Beggiatoa                        LRTDPIMRFM I
Rhodobacter sphaeroides     361 LRTDPIIRMM VVSIGFYGMS TFEGPMMSIK AVNSLSHYTD WTIGHVHSGA LGWNGMITFG 420
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Fig. 4. Comparison of the amino acid sequences of fragments of the CcoN catalytic subunits of the cbb_3-type cytochrome *c* oxidases of *B. leptomitiformis* D-402 (deduced from the nucleotide sequences determined in the present study) and *Rhodobacter sphaeroides* [14].

possible that the synthesis of this enzyme depends on the redox characteristics of the cultivation medium as well as on the O_2 concentration itself.

The presence of a cbb_3-type oxidase in the filamentous sulfur bacteria *Beggiatoa* is quite natural, as they are typical representatives of microaerophilic associations. According to various data, the cbb_3-type oxidase is characterized by a K_m coefficient of 8–20 nM; i.e., its affinity to oxygen is an order of magnitude higher than that of the aa_3-type oxidases. Despite the adaptation of the cbb_3-type oxidases to operation under microaerobic conditions, the efficiency of energy transformation by these enzymes is not less than that of the aa_3-type oxidases of mitochondria and most aerobic bacteria. According to the data from an analysis of 16S rRNA, the colorless sulfur bacteria *Beggiatoa* belong to the class *Gammaproteobacteria* [15]. A lot of data have been accumulated showing that cbb_3-type oxidases are widespread among representatives of various classes of proteobacteria [16], and it has been stated that this

enzyme is a characteristic component of the electron transport chain of representatives of the phylum *Proteobacteria* [17].

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