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EXPERIMENTAL ARTICLES

Cytochromes *c* of the Anaerobic Methacrylate Reducer *Geobacter sulfurreducens* AM-1

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Abstract—Cytochromes *c* were found in the cells of the bacterium *Geobacter sulfurreducens* AM-1 grown on acetate and methacrylate. The periplasmic extract of *G. sulfurreducens* AM-1 contained about 88% of the total content of cytochromes *c* of intact cells. The analysis of cytochromes *c* from the native cells of *G. sulfurreducens* AM-1, from the periplasmic extract and from the cells treated by an alkaline solution showed the presence of nine proteins containing heme *c*. The molecular masses of cytochromes *c* from *G. sulfurreducens* AM-1 were 12.5, 15.5, 25.7, 29.5, 34.7, 41.7, 50.1, 63.1, and 67.6 kDa; localization of each cytochrome *c* was determined. Three heme-containing proteins (15.5 kDa, 25.7 kDa, and 29.5 kDa with the most intensive staining) were present mainly in the periplasm of the bacterium. The other two (50.1 and 67.6 kDa) were supposedly localized in the cell membrane. Cytochromes *c* with the molecular masses of 12.5, 15.5, and 67.6 kDa are considered as possible components of the methacrylate redox system of *G. sulfurreducens* AM-1.

Key words: anaerobic respiration, *Geobacter sulfurreducens* AM-1, periplasm, methacrylate reductase, cytochromes *c*, peroxidase activity of heme *c*.

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The interest in acetate-oxidizing iron reducers of the genus *Geobacter* (the δ-group of proteobacteria) is caused by their significance for bioremediation of radioactive metals [1, 2]. They reduce Fe(III) to Fe(II) and participate in humus decomposition, i.e. play an essential role in the global cycles of metals and carbon. Cytochromes *c* function in both of the above processes. An intensive 10 year study of the type strain *G. sulfurreducens* PCA resulted in decoding of the genome of this bacterium [3]. An interesting fact is that this genome contains an unusually large quantity of the genes encoding electron transport proteins. The genes of 111 cytochromes *c* have been found among them.

It is already known that at least eight cytochromes *c* of the type strain *G. sulfurreducens* PCA are expressed during its growth in the presence of insoluble Fe(III) oxides as electron acceptors [4–10]. The number of hemes of these cytochromes varies from two to six, and they are localized both in the membrane and in the periplasm. It is supposed that three cytochromes *c* are directly involved in the transfer of reducing equivalents to the terminal electron acceptor, Fe(III) [5, 6]. Five other cytochromes *c* participate in the regulation of expression of direct electron carriers, e.g., on the post-transcriptional level [7]. A number of strain PCA cytochromes *c* have been studied in detail [11–15]. Their three-dimensional structures [11–13] and thermodynamic characteristics [14, 15] have been determined.

The pure culture of another *G. sulfurreducens* strain designated as AM-1 was obtained by Russian researchers during the study of the pathways of methacrylate wastes decomposition [16]. The fact of reduction of methacrylate (2-methylpropenoate), a compound of anthropogenic origin, was of interest to researchers [17]. Strain AM-1 is the only known organism capable of complete oxidation of an organic compound (acetate) coupled to reduction of an organic substance (methacrylate). Methacrylate is a terminal acceptor of the reductase chain of the bacterium: it accepts reduction equivalents from the cytoplasmic TCA cycle through a menaquinone, with 8 isoprenoid residues in the side chain, found in the membrane [18]. Transformation of methacrylate into isobutyrate was shown to occur in the periplasm of strain AM-1. This process is catalyzed by a flavin-containing methacrylate reductase with the molecular mass of 50 kDa [19, 20]. The activity of methacrylate reductase depends on the periplasmic tetraheme cytochrome *c* with the molecular mass of 30 kDa, which is a physiological electron donor for the enzyme. However, no "transfer member" (carrier) from menaquinone to cytochrome *c* (30 kDa) was identified. The involvement of additional cytochrome(s) *c* in this process was suggested.

Cytochromes *c* are an extensive class of proteins possessing covalently bound heme(s) of type *c* [21].

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They are involved not only in electron transport during respiration and photosynthesis but also in the biosynthesis of the substances required for metabolism and detoxification. Cytochromes *c* have a typical absorption spectrum. In contrast to cytochromes *b* possessing a noncovalently bound heme and submerged in the cytoplasmic membrane, the cytochromes of group *c* are localized in the periplasm or in the cytoplasmic membrane protruding into the periplasm. Depending on the hydrophobicity of the amino terminus that holds ("anchors") hydrophilic proteins in the membrane, cytochromes *c* are submerged in the membrane to a greater or lesser extent.

The goal of this work was to study the cytochromes *c* of *G. sulfurreducens* AM-1 grown on acetate and methacrylate, namely to determine their molecular masses and localizations.

MATERIALS AND METHODS

The object of investigation was a pure culture of the bacterium *Geobacter sulfurreducens* AM-1 from the culture collection of the Laboratory of Anaerobic Metabolism of Microorganisms, IBPM RAS (Pushchino).

Cultivation of the bacterium. Bacterial cells were grown in 10-l bottles at 37°C on a mineral medium prepared according to the principles of anaerobic cultivation [19, 20] and containing the following (g/l): NaCl, 0.9; $MgCl_2$, 0.2; $CaCl_2$, 0.1; NH_4Cl, 1.0; K_2HPO_4, 0.029; KH_2PO_4, 0.015; pH 7.0. The following solution of trace elements was added in the amount of 10 ml per 1 l of the medium (g/l): nitrilotriacetic acid, 12.8; $FeSO_4 \cdot 7H_2O$, 0.5; $MnCl_2 \cdot 4H_2O$, 0.1; $CoCl_2 \cdot 6H_2O$, 0.17; $CaCl_2 \cdot 2H_2O$, 0.1; $ZnCl_2$, 0.01; $CuCl_2$, 0.01; H_3BO_3, 0.01; $Na_2MoO_4 \cdot 2H_2O$, 0.01; NaCl, 1; Na_2SeO_3, 0.017; $NiCl_2$, 0.5; Na_2WO_4, 0.1. The vitamin solution added as 5 ml per 1 l of the medium contained the following (mg/l): biotin, 2.0; folic acid, 2.0; pyridoxine, 10.0; thiamine-HCl, 5.0; riboflavin, 5.0; nicotinic acid, 5.0; pantothenic acid, 5.0; cyanocobalamin, 0.1; *p*-aminobenzoic acid, 5.0; lipoic acid, 5.0. The medium contained 17 mM acetate and 50 mM methacrylate, as well as cysteine and sulfide (50 mg/l of each) as reducers.

Preparation of cell suspensions. The culture grown to the concentration of 0.25 g (wet biomass) in 1 l of the cultivation medium was aerobically centrifuged at 10000 *g* for 30 min at 4°C and the precipitate was washed with 100 mM Tris–HCl buffer (pH 7.8) followed by centrifugation at 2500 *g* for 15 min. The resultant cell precipitate was resuspended in the same buffer.

Preparation of the fraction of periplasmic proteins. The fraction of periplasmic proteins was obtained by the method described previously [22]. The cells were resuspended in the solution (at a ratio of 1 g of wet biomass per 10 ml of the solution) containing 50 mM Tris and 50 mM EDTA, with pH adjusted to 9.0 with Na_2CO_3. Periplasmic proteins were extracted into the solution after incubation of the cell suspension for 20 min at 37°C under continuous stirring on a magnetic stirrer. The treated cells were precipitated by centrifugation at 10000 *g* for 30 min at 4°C.

Protein was assayed by spectrophotometry at 595 nm according to Bradford [23]. Bovine serum albumin (BSA) was used as a standard.

The samples of intact cells and cells after extraction of periplasmic proteins were diluted by concentrated NaOH to the final alkali concentration of 0.2 N, boiled in a water bath for 30 min, and centrifuged at 10000 *g*. The supernatant was used for the protein assay.

The content of cytochromes *c* in the samples was determined from the differential spectra (dithionite-reduced minus oxidized) recorded by an UV-160 double-beam spectrophotometer (Shimadzu, Japan). The concentration of cytochromes *c* was calculated from the difference of absorption at 553 nm and 540 nm ($E_{553–540}$ = 80 $mM^{-1}cm^{-1}$) [20].

Electrophoresis under denaturing conditions. Proteins were separated by their molecular masses, using PAGE electrophoresis under denaturing conditions [24]. The proteins were separated in 15% gel on plates (10 × 7 cm) using a Mini Protean III apparatus for electrophoresis (Bio-Rad, United Kingdom).

Three protein samples under study (native cells, periplasmic extract, and the cell fraction remaining after the obtaining of periplasmic extract) were applied to the two sides of the gel: from the left and from the right. After electrophoresis, the gel plate was divided into two equal parts. One part of the gel was treated with Coomassie brilliant blue G-250 to reveal protein bands and the other part, with 3,3',5,5'-tetramethylbenzidine in methanol to reveal cytochromes *c*. After the staining procedures, both parts of the gel plate were documented and superposed. In the case when the color of cytochromes *c* rapidly became dim, the stained bands corresponding to the positions of cytochromes *c* were slightly cut with a scalpel and then the cut gels were stained for protein.

Staining based on detection of the peroxidase activity of heme *c* was performed to reveal cytochromes *c* in polyacrylamide gels after SDS electrophoresis [25]. All the procedures were carried out in the dark. The gels, without preliminary staining with Coomassie brilliant blue G-250, were fixed twice during 10 min in 50 ml of 0.25 M Na acetate solution (pH 5.0). Then they were treated with 30 ml of freshly prepared 6.3 mM solution of 3,3',5,5'-tetramethylbenzidine in methanol. After 20 min, 2 to 15 ml of 30 mM H_2O_2 solution was added. Bright-blue bands corresponding to positions of the proteins containing heme *c* appeared after 10 to 60 min. For the removal of precipitated 3,3',5,5'-tetramethylbenzidine, purification of the gel background, and enhancement of staining intensity, the

Table 1. Distribution of cytochromes *c* in cell fractions of *G. sulfurreducens* AM-1

Sample	Protein, mg/ml	Cytochromes *c*		
		Specific content, μM per 1 mg protein	Total content, μM	Yield, %
Intact cells	14.06	1.42	229.6	100
"Washed cells"	51.03	1.04	31.8	13.9
"Periplasm"	4.53	4.7	200.9	87.5

Note: "Washed cells" are the cells obtained after extraction of periplasmic proteins. "Periplasm" is the periplasmic extract obtained by the treatment of cells by EDTA alkaline solution.

Table 2. Determination of the molecular masses and localizations of cytochromes *c* of *G. sulfurreducens* AM-1

R_f	Molecular mass, kDa	Intact cells	"Washed cells"	"Periplasm"
0.66	12.5	+		+
0.56	15.5	+		
0.34	29.5	+	+	+
0.27	34.7	+	+	+
0.12	50.1		+	
0.03	67.7	+	+	

gels were placed into isopropanol : 0.25 M Na acetate (3 : 7) solution (pH 5.0).

Documentation of the gels stained for heme *c* and protein was performed using a scanner and the Genus ColorPage-Vivid 1200XE software package.

Molecular masses of the detected cytochromes *c* after SDS electrophoresis were determined using the diagram plotted on the basis of electrophoretic mobility values R_f (abscissa axis) and $\log M$ (ordinate axis) of the marker proteins. Molecular masses of the marker proteins used in the experiments were 14.4, 18.4, 25.0, 35.0, 45.0, 66.2, and 166.0 kDa (Fermentas, Lithuania).

RESULTS

Obtaining the fraction of periplasmic proteins. The periplasmic extract of *G. sulfurreducens* AM-1 was obtained by the treatment of intact cells by an alkaline solution with Tris and EDTA (pH 9.0). In this case, the periplasmic proteins were released due to chelation of bivalent metal ions bound to lipopolysaccharides by the chelating agent, followed by the loss of structural integrity of the outer membrane [26, 27]. This procedure for determination of the localization of cytochromes *c* was chosen because it had already been repeatedly used previously [19, 20]. Periplasmic extract was obtained, among other purposes, for enzyme purification [20]. Hence, it was reasonable to define the distribution pattern of cytochromes *c* at the first step of obtaining the pure enzyme preparation.

The protein concentration and the content of cytochromes *c* were determined in native cells, the cells treated by an alkaline solution with EDTA, and the preparation of periplasmic proteins. The data on the content of cytochromes *c* obtained in one of the experiments are given in Table 1.

The concentration of cytochromes *c* was calculated from differential (dithionite-reduced minus oxidized) spectra (Fig. 1). For this purpose, the oxidized and dithionite-reduced absorption spectra were recorded in all three available samples. The spectra of oxidized samples had the maximum absorption at 409 nm. The spectra of dithionite-reduced samples had the maximum absorption at 552, 523, and 419 nm, typical of heme *c*.

As follows from Table 1, the cytochromes were distributed as follows: about 88% of cytochromes *c* (apparently the more hydrophilic ones) was revealed in the periplasmic extract and about 14% (more hydrophobic ones) was retained in the cell fraction after treatment with the alkaline solution containing Tris and EDTA. The latter proteins were most likely membrane cytochromes. The data on the quantity of cytochromes *c* in the periplasm of *G. sulfurreducens* AM-1 conform to the previous studies, where the content of cytochromes *c* in the periplasmic extract was 91% [19].

Protein electrophoresis under denaturing conditions. Proteins were separated by molecular masses using denaturing electrophoresis in PAAG (15%). In this case, the proteins are separated by molecular

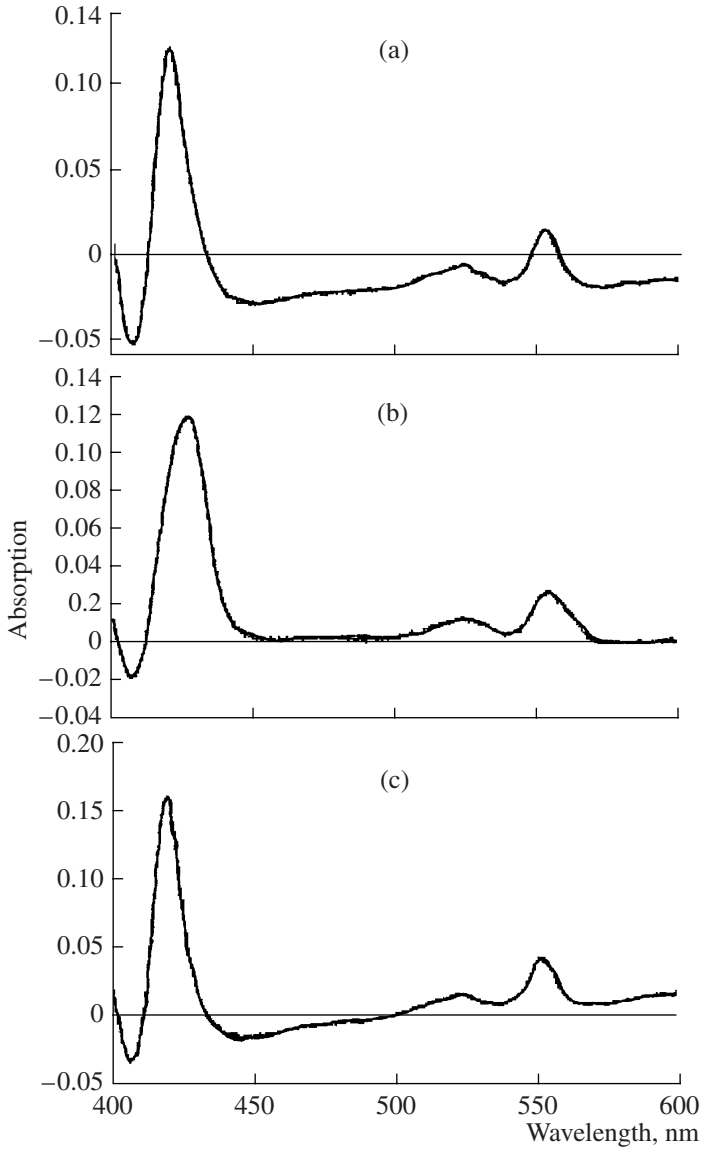

Fig. 1. Differential spectra of intact cells (a), cells after extraction of periplasmic proteins (b), and periplasmic extract of *G. sulfurreducens* AM-1 (c).

masses only. The molecular masses of cytochromes *c* determined by electrophoresis as is shown in Fig. 2, are presented in Table 2.

Cytochromes *c* of intact cells of *G. sulfurreducens* AM-1. The analysis of several electrophoregrams of intact cell proteins revealed the bands corresponding to 4–5 cytochromes *c*. As a rule, the most intensive and persistent staining (as compared with "washed cells" and "periplasm") was observed in the fraction of intact cells, which was probably due to the presence of the total pool of cytochromes in these samples. The results of the analysis of several electrophoregrams are presented in Table 3.

Insufficient reproducibility of the electrophoresis results is caused by the variations of protein separation and staining conditions. Besides, one should take into consideration the instability of the group *c* cytochromes under aerobic conditions [21] used in the study and the instability of staining for heme *c*.

As follows from Table 3, the cytochrome *c*, which is stained most intensively and revealed at each electrophoresis, has a molecular mass of 29.5–30.0 kDa. Most probably, the same cytochrome *c* serves as a direct donor of reducing equivalents for methacrylate reductase. Cytochrome *c* with the molecular mass of 30 kDa has already been obtained in a pure form [20]. The samples of intact cells were often shown to contain cytochromes *c* with molecular masses of 12.5, 15.5, 26, and 68–69 kDa.

Cytochromes *c* of *G. sulfurreducens* AM-1 "washed cells". The analysis of heme *c*-containing proteins in the samples of cells pretreated by the alka-

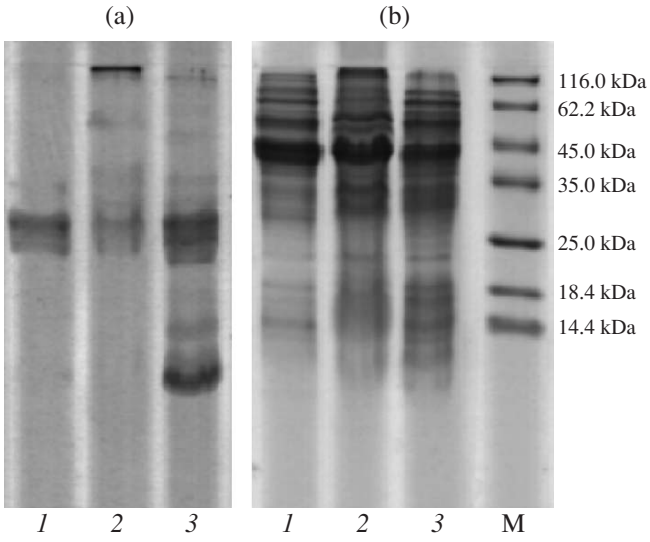

Fig. 2. Electrophoregram of cytochromes *c* (a) and proteins (b) from *G. sulfurreducens* AM-1 in 15% SDS PAGE: periplasmic extract (*1*); cells after extraction of periplasmic proteins (*2*); intact cells (*3*); M, marker proteins of molecular mass.

line solution with EDTA ("washed cells") is presented in Table 4. In no case were cytochromes *c* with the molecular masses of 15.5 and 26 kDa revealed on electrophoregrams. At the same time, the samples contained cytochromes *c* with higher molecular masses not detected in the intact cells, e.g., cytochromes of 50.1 and 72.4 kDa. Cytochromes *c* of 29.5, 41.7, and 67.6 kDa were stained most intensively and revealed most often. The cytochrome *c* of 63.1 kDa was found twice.

After extraction of the periplasmic proteins, the fraction of membrane components in the cells increases relative to their initial amount in the native cells. Thus, it was logical to suggest that the cytochromes *c* with molecular masses above 34 kDa, which have not been found in the intact cell samples (50.1 and 72.4 kDa), are the membrane cytochromes not soluble in the periplasm. The presence of the 29.5-kDa cytochrome *c* in the samples of "washed cells" is probably explained by its incomplete extraction from the cells (according to the data in Table 1, "washed cells" contain 14% of the total pool of cytochromes *c*). Besides, it is possible that cytochrome *c* with the molecular mass of 29.5 kDa has a high intensity of staining and can be revealed easier than other cytochromes as it contains not one but four hemes *c* [20].

Table 3. Analysis of electrophoregrams of cytochromes *c* of intact cells of *G. sulfurreducens* AM-1

Molecular mass, kDa	Experiment					
	I	II	III	IV	V	VI
12.5	+	+				
15.5	+				+	+
25.7				+	+	
29.5	+	+	+		+	+
34.7	+					
41.7		+				
63.1		+				
67.6	+				+	
77.6				+		

Cytochromes *c* of the periplasmic extract of *G. sulfurreducens* AM-1 cells. The periplasmic extract was characterized by the presence of six cytochromes *c* with molecular masses of 12.5 to 42 kDa. A bright band corresponding to the 30-kDa cytochrome *c* was found in all experiments. Besides, the periplasmic extract contained cytochromes *c* of 12.5 and 15.5 kDa. The results of the "periplasm" studies are presented in Table 5. The cytochromes of 50.1, 67.6, and 72.4 kDa were not revealed in any of the cases.

DISCUSSION

Cytochromes c of G. sulfurreducens AM-1

As one can see from the Summary Table 6 presenting the data of the six electrophoregrams and the three preceding tables (Tables 3–5), the cells of *G. sulfurreducens* AM-1 were found to possess nine heme *c*-containing proteins of 12.5, 15.5, 25.7, 29.5, 34.7, 41.7, 50.1, 63.1, and 67.6 kDa. The cytochrome *c* with the molecular mass of 29.5–30.0 kDa is stained most intensively and was revealed most frequently. The previous studies have shown that it is a tetraheme periplasmic cytochrome *c*, the direct donor of reducing equivalents for methacrylate reductase [20]. Two of the nine cytochromes (15.5 and 25.7 kDa) were found in the periplasmic extract (and were absent in "washed cells"). Two other cytochromes (50.1 and 67.6 kDa) most likely were not extracted from the membrane, as they were revealed only in the samples of "washed cells". The brightest staining of bands in the gels after electrophoresis was typical of 29.5-, 12.5-, and 15.5-kDa cytochromes *c*.

Involvement of Cytochromes c in Methacrylate Reduction

The cytochrome *c*-methacrylate oxidoreductase (50 kDa) are known to be similar to flavoproteins from different groups of proteobacteria [18, 20, 28]. The highest amino acid homology was observed for two flavoproteins, AAN56611 (95% similarity) and AAN54479 (90% similarity), from *Shewanella oneidensis* and for the periplasmic protein FccA (76% similarity) from *Wolinella succinogenes.* It was assumed that the physiological role of the FccA protein

Table 4. Analysis of electrophoregrams of cytochromes *c* of "washed cells" of *G. sulfurreducens* AM-1

Molecular mass, kDa	Experiment					
	I	II	III	IV	V	VI
12.5		+			+	
29.5	+	+	+			+
34.7	+					
41.7		+		+		+
50.1				+	+	+
63.1				+		+
67.6	+		+			+
72.4		+				
77.6				+		

Table 5. Analysis of electrophoregrams of the periplasmic extract of *G. sulfurreducens* AM-1

Molecular mass, kDa	Experiment					
	I	II	III	IV	V	VI
12.5	+	+				
15.5			+		+	
25.7				+	+	
29.5	+	+	+	+		+
34.7	+			+		
41.7		+		+	+	
63.1		+				
77.6		+				

from *W. succinogenes* is related to methacrylate and acrylate reduction [29]. The operon encoding the flavin-containing protein FccA (52 kDa) contains two more open reading frames, FccB and FccC, which encode tetraheme cytochromes *c* [30]. FccC (24 kDa) is a membrane cytochrome. FccB (14 kDa) is a periplasmic cytochrome directly interacting with protein FccA in the oxideoreductase complex.

In the scheme of the transfer of reducing equivalents from the TCA cycle to the terminal acceptor methacrylate in the strain *G. sulfurreducens* AM-1, the question about the electron acceptor from menaquinone is still open [20]. It is only known that the direct donor of reducing equivalents for the flavoprotein (50 kDa) with methacrylate reductase activity is cytochrome *c* (30 kDa), which is also present in the periplasm of *G. sulfurreducens* AM-1.

The data on the cytochromes *c* of *G. sulfurreducens* AM-1 suggest a number of assumptions concerning the additional components of the methacrylate reductase system. For instance, the missing link in the transfer of electrons and protons may be cytochrome *c* with the molecular mass of 67.6 kDa since it is a membrane cytochrome. With the same probability, one could assume participation in this process of cytochromes *c* with molecular masses of 12.5 kDa and 15.5 kDa, because they show practically the same bright staining for the presence of heme *c* as cytochrome *c* (30 kDa). Probably more than one cytochrome *c* is missing. In any case, the presented results give a primary notion of the quantity, localization, and molecular masses (as classification element) of cytochromes *c* of the bacterium *G. sulfurreducens* AM-1 grown on acetate and methacrylate.

Table 6. The most frequently revealed cytochromes *c* of the bacterium *G. sulfurreducens* AM-1

Molecular mass, kDa	Intact cells	"Washed cells"	"Periplasm"
12.5	++	++	++
15.5	+++		++
25.7	++		++
29.5	+++++	++++	+++++
34.7	+	+	++
41.7	+	+++	+++
50.1		+++	
63.1	+	++	+
67.6	++	+++	

Further studies are required for the exact definition of the role of cytochromes *c* in reduction of synthetic methacrylate. In particular, they may be based on the comparison of amino acid sequences of the cytochromes *c* from *G. sulfurreducens* AM-1 with amino acid sequences of other known cytochromes. The data on the quantity of synthesized cytochromes *c*, localization of each of them, and their involvement in the methacrylate redox system will substantially extend our knowledge of the carriers of respiratory chains of *G. sulfurreducens* AM-1 and of the mechanisms of reduction of double bonds by anaerobes. Besides, the results of such studies will promote the application of component proteins of the methacrylate reductase complex in nature conservation and ecologically safe cleanup activities.

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