ISSN 0006-2979, Biochemistry (Moscow), 2009, Vol. 74, No. 7, pp. 793-799. © Pleiades Publishing, Ltd., 2009.
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Features of Structural Organization and Expression Regulation of Malate Dehydrogenase Isoforms from *Rhodobacter sphaeroides* Strain 2R

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Received July 16, 2008
Revision received September 4, 2008

Abstract—Two isoforms of malate dehydrogenase (MDH), dimeric and tetrameric, have been found in the purple non-sulfur bacterium *Rhodobacter sphaeroides* strain 2R, devoid of the glyoxylate shunt, which assimilate acetate via the citramalate cycle. Inhibitory analysis showed that the 74-kDa protein is involved in tricarboxylic acid cycle, while the 148-kDa MDH takes part in the citramalate pathway. A single gene encoding synthesis of the isologous subunits of the MDH isoforms was found during molecular-biological investigations. The appearance in the studied bacterium of the tetrameric MDH isoform during growth in the presence of acetate is probably due to the increased level of *mdh* gene expression, revealed by the real-time PCR, the product of which in cooperation with the citramalate cycle enzymes plays an important role in acetate assimilation.

DOI: 10.1134/S000629790907013X

Key words: malate dehydrogenase, purification, isoforms, expression regulation

Most phototrophic bacteria of the Rhodospirillaceae family, able to grow on acetate as the sole source of carbon, assimilate it via the tricarboxylic acid (TCA) cycle. A number of C_4-acids of the TCA cycle are used for biosynthetic purposes; they are compensated in many bacteria by the glyoxylate cycle that involves during each turnover two acetyl-CoA molecules, and a single succinate molecule is formed which is used in anabolism [1, 2]. Some species (*Rhodospirillum rubrum*, *Rhodobacter sphaeroides*) are able to assimilate acetate without the glyoxylate cycle via the recently discovered functionally analogous citramalate pathway [3, 4]. The main intermediate in the pathway is citramalate. It is transformed after a series of successive reactions to glyoxylate, condensed in the malate synthase reaction with an acetyl-CoA molecule and formation of malate, and incorporated in the TCA. This fills in the loss of the cycle intermediate substrates spent for biosynthetic needs.

Malate dehydrogenase (MDH, EC 1.1.1.37) is a multifunctional enzyme providing for all the above mentioned metabolic pathways. The MDH multifunctionality in eukaryotic cells is associated with the broad isoenzyme spectrum caused by genetic heterogeneity. It is known that six plant and two animal genes are responsible for biosynthesis of MDH isoenzymes [5, 6], while it was shown for some bacteria that the participation of MDH in the TCA and glyoxylate cycles involves isoforms emerging after structural rearrangements of the protein molecule. In this case, the emerging MDH dimeric and tetrameric forms contain identical subunits encoded by the same gene [7-9]. The mechanism of dimer transformation to tetramer is unknown, although a correlation was revealed between the main type of nutrition and quaternary structure in *Rhodopseudomonas palustris* and *Beggiatoa leptomitiformis*. It is interesting that *R. sphaeroides* strain 2R transforms acetate via the citramalate pathway, which also includes MDH, rather than by the glyoxylate shunt. The supposed genetic mechanism of protein polymorphism is unknown because the molecular biology of this strain has not been studied.

Abbreviations: MDH, malate dehydrogenase; TCA cycle, tricarboxylic acid cycle.

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However, according to the literature the genome of three other sequenced strains of *R. sphaeroides* contains the unique *mdh* gene present on chromosome I and encoding synthesis of the corresponding protein molecule [10, 11].

The goal of this work was to study the features of MDH structural organization and expression regulation in the bacterium *R. sphaeroides* strain 2R metabolizing acetate in the citramalate cycle.

MATERIALS AND METHODS

The purple non-sulfur phototrophic bacterium *R. sphaeroides* strain 2R from the collection of the Department of Microbiology of Moscow State University was the object of investigation. The bacterium was grown in Pfenning's nutrient medium [12] of following composition (g/liter): KH_2PO_4, 0.33; $MgCl_2 \cdot 6H_2O$, 0.33; $NH_4Cl \cdot 4H_2O$, 0.33; Na_2SO_4, 0.33; KCl, 0.33; $CaCl_2 \cdot 2H_2O$, 0.1; Difco yeast extract, 0.1; sodium succinate, 1.0; sodium acetate, 1.0; distilled water to total volume of 1 liter, pH 7.6. Vitamins and microelements were added after sterilization. Cells were pelleted by centrifugation for 15 min at 8000*g*, washed with 0.05 M Tris-HCl, pH 7.5, and sonicated using a UZDN-2T disintegrator for 2 min at 500 W and frequency 22 kHz in an ice bath. Cell fragments were pelleted by centrifugation for 5 min at 4000*g* and 4°C.

MDH activity was determined spectrophotometrically with a LOMO SF-46 spectrophotometer (Russia) at 340 nm by changes in NADH content [13]. In the case of the oxaloacetate reduction rate determination, the reaction medium contained 50 mM Tris-HCl, pH 8.0, 1.5 mM oxaloacetate, and 0.15 mM NADH. The enzyme amount that converted 1 μmol NADH in the back reaction in 1 min at 25°C was taken as the unit of MDH activity.

Succinate dehydrogenase activity (EC 1.3.99.1) was studied using the phenazine methosulfate method [14]. Activity of fumarate hydratase (EC 4.2.1.2) was determined by a method based on estimation of the difference in the malate and fumarate absorption at 240 nm [15]. Activity of isocitrate dehydrogenase (EC 1.1.1.42) was determined by the rate of $NADP^+$ reduction [16]. Activity of isocitrate lyase (EC 4.1.3.1) was determined with phenylhydrazine according to Dixon and Kornberg [17]. Malate synthase (EC 4.1.3.2) was measured spectrophotometrically by glyoxylate-dependent CoA formation from acetyl-CoA with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) [17]. Protein amount was determined according to Lowry et al. [18].

For MDH purification a six-stage scheme was used that includes fractionation by ammonium sulfate (45-80% saturation), gel filtration through a column (1.5 × 20 cm) of Sephadex G-25 (Pharmacia, Sweden), ion-exchange chromatography on a column (1.5 × 12 cm) of DEAE-Toyopearl (ToyoSoda, Japan) (elution was carried out in a linear KCl concentration gradient (0-300 mM)), and gel chromatography through a Sephadex G-200 column (2 × 40 cm).

Gel chromatography through Sephadex G-200 column (2 × 40 cm) was used for determination of quaternary structure and molecular mass of native MDH [19]. Its elution volume (V_e) and void volume of the chromatographic column (V_o) was determined using blue dextran (Serva, Germany). Molecular mass of the studied enzyme was determined using the formula obtained from the calibration plot:

$$\log M_r = 6.698 - 0.987(V_e/V_o).$$

Electrophoresis of native MDH was carried out in 9% polyacrylamide gel according to Davis' modification [20]. The gel was specifically developed by the tetrazolium method [21] and the protein in the gel was universally stained using the silver nitrate technique [22]. SDS-PAGE was carried out according to Laemmli in 12.5% polyacrylamide gel [23]. Each specimen contained 3-5 μg protein. Standard marker proteins (Sigma, USA) were used for calibration curve plotting (kDa): phosphorylase *b*, 94.5; BSA, 66.2; ovalbumin, 43.0; carboanhydrase, 31.0; trypsin inhibitor, 21.5; lysozyme, 14.4.

Experiments on protein molecule association and dissociation were carried out for 15 min at 25°C in 50 mM phosphate buffer. In experiments on the effect of different pH values on oligomeric state of the protein molecule, MDH was incubated in phosphate buffer (pH 6.0-9.0). To study the metabolite effects on the enzyme aggregation level, MDH was incubated at pH 7.5 in the presence of 5 mM $MgCl_2$, 0.5 mM NAD^+, 5 mM malate, 0.3 mM NADH, 2 mM oxaloacetate, and 3 mM acetyl-CoA. Cross-linking with glutaraldehyde was studied according to Jacob et al. [24] with some modifications. After cross-linking of the protein with 4 mM glutaraldehyde (Reanal, Hungary) for 20 min at 25°C in 50 mM phosphate buffer, sodium tetraborate dissolved in 0.2% NaOH was added to the mixture (2 : 1). The excess of sodium tetraborate was removed with sodium pyruvate, and the reaction medium was kept for 60 min at 4°C. Then an equivalent amount of Laemmli buffer was added to reaction mixture, and SDS-PAGE was carried out [25].

For inhibitory analysis, the aconitate hydratase inhibitor fluoroacetate (3 mM) and the propionyl-CoA carboxylase (one of key enzymes of citramalate cycle) inhibitor itaconate (5 mM) were added to the medium for bacterial growth [26].

Genomic DNA was isolated from 100 μl bacterial culture using the guanidine–thiocyanate–phenol–chloroform technique using cetyltrimethylammonium bromide (CTAB) [27]. The DNA obtained was dissolved in 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA and used for PCR analysis. For total RNA isolation, the sample was

extracted by phenol–chloroform with LiCl as precipitant [28].

Electrophoresis in 1% agarose gel was used for visualization and qualitative analysis of isolated nucleic acid. Gels were stained with 0.1% alcohol solution of ethidium bromide.

The reverse transcription of mRNA was carried out using M-MULV reverse transcriptase (SibEnzyme, Russia) for synthesis of the first cDNA strand according to the manufacturer's instruction.

Primers were selected by base sequences of MDH from different organisms using the Primer 3 program [29].

The polymerase chain reaction was used to identify the malate dehydrogenase gene [30]. The sample was amplified in a Biometra thermocycler (Biometra, Germany). The product size was determined by comparison with the known length DNA markers (SibEnzyme). The following primer sequences for were used for the *mdh* gene: forward 5′-ggaatgacacgrgatggacctg-3′ and reverse 5′-tgaaaacttckgctgtgaatg-3′ (T_m = 60°C).

Quantitative real-time PCR was carried out on a Bio-Rad DNA Engine Thermal Cycler Chromo 4 (BioRad, USA) using SYBR Green I for staining. The cDNA obtained on 100 ng total cellular RNA was used for the reaction. The elongation factor gene *ef-1ά* was used as normalizer [31], and base sequences used for the *mdh* gene identification were used as primers. The following amplification parameters were used: preliminary denaturing for 5 min at 95°C; cycle – 95°C, 30 sec; 60°C, 30 sec, and 72°C, 30 sec (detection); final elongation at 72°C for 10 min. Total RNA without reverse transcription was used as negative control.

Relative expression of the studied genes was determined using the $2^{-\Delta\Delta Ct}$-technique [32] with Opticon Monitor™ Software (BioRad).

RESULTS AND DISCUSSION

Determination of activity of some TCA and citramalate cycle enzymes in *R. sphaeroides* variants has shown the dependence of their functioning on the type of nutrition (Table 1). The activity of the TCA cycle enzymes was found independently of nutrition type and carbon source, while functioning of malate synthase was detected only in the case of acetate utilization by the bacteria. In this case, complete absence of isocitrate lyase activity in all experimental variants against the background of high malate synthase activity showed that acetate assimilation in *R. sphaeroides* takes place during the citramalate cycle, which was confirmed by results of investigation of other citramalate pathway enzymes in this strain [3].

Since upon chemoorganoheterotrophic growth on acetate the citramalate cycle was also active in bacterial cells along with the TCA cycle and MDH had maximal specific activity, specific enzyme staining was used after native electrophoresis in polyacrylamide gel. Two enzyme isoforms with relative electrophoretic mobility 0.42 and 0.73 were found (Fig. 1a).

High-purity MDH preparations from *R. sphaeroides* strain 2R were obtained for analysis of the enzyme quaternary structure. In the course of six-stage purification, two MDH isoforms were isolated with specific activity levels 14.70 ± 0.44 (purification extent 40.1) and 11.00 ± 0.33 U/mg protein (purification extent 30.6) (Table 2). The values of specific activity for *R. sphaeroides* are close to those for MDH from other members of this bacterial group. For example, the level of specific activity of dimeric MDH from another purple phototrophic microorganism, *R. palustris*, was 6.97 U/mg protein [7].

Electrophoretic analysis of purified preparations showed that in polyacrylamide gel after universal staining

Table 1. Specific activity (nmol/min per mg protein) of TCA cycle enzymes and malate synthase of *R. sphaeroides* cells grown under different conditions (n = 8, $p \le 0.05$)

Enzyme	Cultivation conditions			
	photoorganoheterotrophic		chemoorganoheterotrophic	
	acetate	succinate	acetate	succinate
Malate dehydrogenase	179 ± 5.37	126 ± 3.78	437 ± 13.11	374 ± 11.22
Isocitrate dehydrogenase	2.9 ± 0.09	2.38 ± 0.07	35.6 ± 1.07	26.2 ± 0.79
Succinate dehydrogenase	12.6 ± 0.08	6.2 ± 0.19	60.9 ± 1.82	36.8 ± 1.1
Fumarate hydratase	27.9 ± 0.84	17.6 ± 0.53	27.2 ± 0.82	10.4 ± 0.31
Malate synthase	18.3 ± 0.55	0	23.6 ± 0.71	0
Isocitrate lyase	0	0	0	0

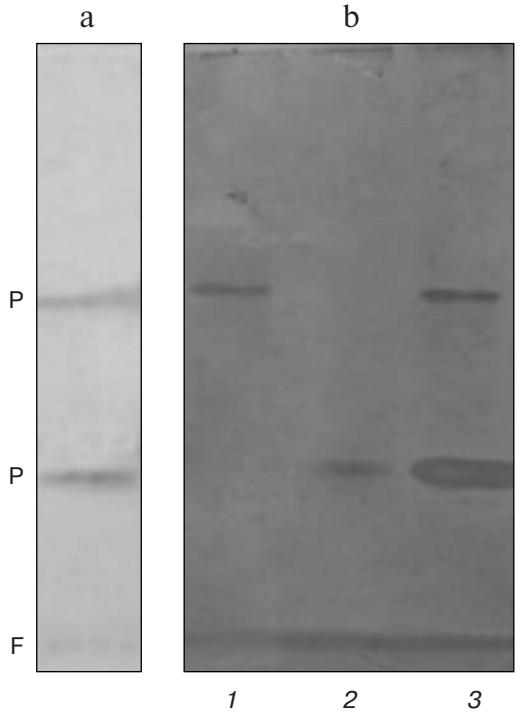

Fig. 1. Electrophoregrams of MDH from *R. sphaeroides* strain 2R grown under chemoorganoheterotrophic conditions in the presence of acetate: a) specific enzyme development; b) development by silver nitrate; *1*, *2*) after gel chromatography on Sephadex G-200; *3*) after ion-exchange chromatography on DEAE-Toyopearl. P, protein band; F, bromophenol blue front.

for proteins and after specific development, only one protein band corresponding to each MDH isoform (with relative electrophoretic mobilities 0.42 and 0.73) was found upon the chemoorganoheterotrophic growth of the phototroph on acetate (Fig. 1).

Molecular mass values of isolated MDH isoforms determined by gel filtration through Sephadex G-200 were 75 ± 2.22 and 148 ± 5.18 kDa upon bacterial growth in the presence of acetate. The molecular mass of a single subunit determined by SDS-PAGE was 37 ± 0.74 kDa for both enzyme isoforms (Fig. 2). This means that MDH obtained from *R. sphaeroides* under conditions of chemoorganoheterotrophic growth on acetate was represented by a mixture of two isologous forms, dimeric and tetrameric. Numerous investigations of different authors have shown that both dimeric and tetrameric MDH forms involved in different metabolic processes appear in bacterial cells. For example, it is known that MDH of *B. leptomitiformis* is a dimer under conditions of organotrophic growth, whereas under conditions of lithotrophic growth tetrameric enzyme form also is active in the bacterial cells [2].

Gel filtration through Sephadex G-200 revealed dependence of quaternary structure of MDH from purple bacteria *R. sphaeroides* on the nature of its growth substrate. Thus, only dimeric enzyme form was involved in the case of bacterial growth in the presence of succinate, i.e. when only the TCA cycle was active, whereas in the case of acetate utilization, which is oxidized to glyoxylate in the recently discovered citramalate cycle, an additional tetrameric MDH form was induced in *R. sphaeroides* cells independently of the energy source used by the bacteria.

Probably the equilibrium between enzyme isoforms can be the molecular basis of efficient enzyme activity regulation *in vivo*. Experiments on the association–dissociation of the homogeneous dimeric preparation with cross-linking by bifunctional reagent for detection of factors influencing the MDH oligomeric state revealed the pH dependence of the process (Fig. 3). Increasing the pH from 7.0 to 8.5 caused association of dimers with formation of tetramers, while lowering of pH to 6.0 was accompanied by dissociation of enzyme dimers with formation of MDH monomers. The effect of cofactors, coenzymes, and different metabolites like Mg^{2+}, NAD^+, NADH, oxaloacetate, and acetyl-CoA was also studied. Addition of magnesium ions to homogeneous enzyme stimulated protein aggregation and formation of enzyme tetramers (Fig. 3). A similar effect was described earlier for malic enzymes of plant origin for which the increasing level of free magnesium and medium alkalization in illuminated chloroplast stroma is able to regulate the malic enzyme activity mainly by influencing its quaternary structure

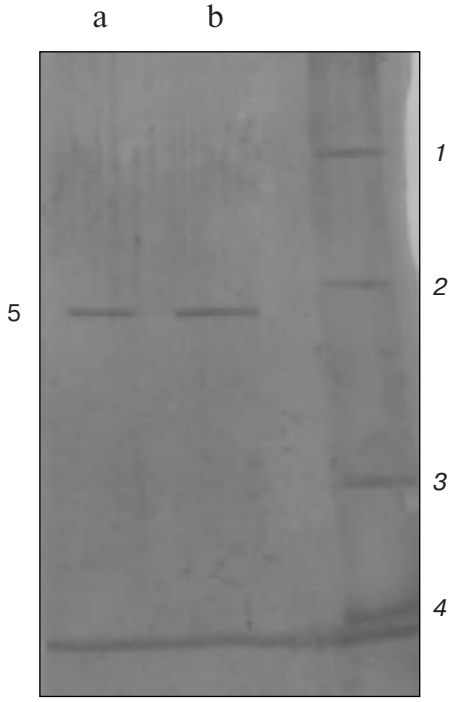

Fig. 2. Results of molecular mass determination of the *R. sphaeroides* strain 2R MDH subunit by SDS-PAGE: a) MDH dimer; b) MDH tetramer; *1*) phosphorylase *b*; *2*) ovalbumin; *3*) trypsin inhibitor; *4*) lysozyme; *5*) MDH under study.

Table 2. Purification of MDH from *R. sphaeroides* strain 2R grown under chemoorganoheterotrophic conditions in the presence of acetate ($n = 8$, $p \le 0.05$)

Purification stage	Total volume, ml	Total activity, U	Protein, mg	Specific activity, U/mg	Purification extent	Yield, %
Homogenate	7.0	46.8 ± 1.4	130.0 ± 3.9	0.246 ± 0.007	1	100
Supernatant	6.8	37.5 ± 1.1	89.4 ± 2.7	0.420 ± 0.013	1.2	80.2
Fractionation by $(NH_4)_2SO_4$, 45-80%	2.0	26.96 ± 0.80	20.74 ± 0.62	1.30 ± 0.04	3.6	57.6
Gel filtration through Sephadex G-25	3.0	19.8 ± 0.6	12.6 ± 0.4	1.570 ± 0.047	4.36	53.8
Ion-exchange chromatography on DEAE-Toyopearl	3.0	3.07 ± 0.09	0.270 ± 0.008	9.86 ± 0.29	27.3	5.6
Gel chromatography on Sephadex G-200						
dimer	3.0	0.810 ± 0.002	0.055 ± 0.002	14.70 ± 0.44	40.1	2.2
tetramer	3.0	0.770 ± 0.023	0.070 ± 0.003	11.00 ± 0.33	30.6	1.65

[25, 33, 34]. Other metabolites had no effect on the oligomeric state of MDH from *R. sphaeroides*.

The results show again that MDH might exist *in vivo* in different oligomeric states. In the case of oligomerization, MDH is converted into conformational states exhibiting different catalytic activities, and change in pH is important in this process. Besides, dynamic equilibrium might depend both on intracellular enzyme and its substrate concentrations and on the presence of some metabolites, temperature, and ionic strength of the solution [25]. For example, dissociation of enzyme tetramer to dimers and monomers was registered for maize and wheat malic enzymes on lowering temperature from 42 to 0°C. Addition of glycerol caused enzyme aggregation, while increase in ionic strength (ammonium sulfate, NaCl) had the opposite effect on protein structure.

Specific inhibitors of the TCA and citramalate cycles made it possible to determine the functional role of the MDH dimers and tetramers. Upon addition to growth medium of 5 mM itaconate that caused 84.5% inhibition of propionyl-CoA carboxylase [26], only the 74 kDa MDH dimer was found in the cells exhibiting chemoheterotrophic growth in the presence of acetate. Growing bacteria in the presence of fluoroacetate resulted in inhibition of aconitate hydratase, one of the TCA cycle enzymes not involved in the citramalate cycle. In this variant, the MDH tetramer of 148 kDa was detected.

There were no data in the literature on the molecular biology of this strain of *R. sphaeroides*. Therefore, molecular investigations were necessary to clarify genetic determination of the found numerous molecular forms of MDH. PCR analysis carried out with isolated genomic DNA and specific primers revealed the presence of a single gene encoding the malate dehydrogenase protein molecule. Results of amplification show that the MDH peptide component is determined in the genome of *R. sphaeroides* by a single gene (Fig. 4). The amplicon size well correlates with theoretical data obtained during selection of primers using the Primer 3 program [29].

The relative level of *mdh* gene expression under various conditions of *R. sphaeroides* growth was studied using real-time quantitative PCR. The *mdh* gene transcription level during chemoheterotrophic growth in the presence of acetate exceeded that upon growth in the presence of succinate by about 30% (Fig. 5). It is known that in a eukaryote intensification of *mdh* gene expression by 62%

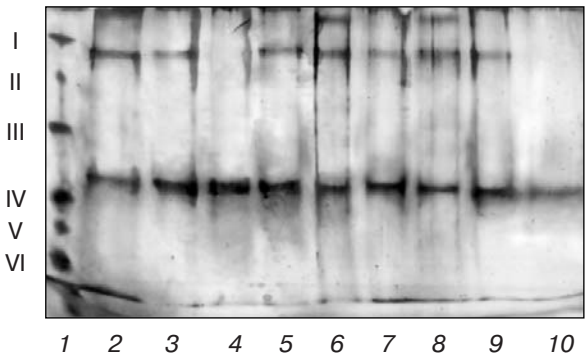

Fig. 3. SDS-PAGE of dimeric MDH cross-linked by glutaraldehyde in the presence of different metabolites. *1*) Protein markers: I, phosphorylase *b*; II, BSA; III, ovalbumin; IV, carboanhydrase; V, trypsin inhibitor; VI, lysozyme; *2*) 0.5 mM NAD^+; *3*) 0.3 mM NADH; *4*) pH 6.0; *5*) pH 7.0; *6*) pH 8.5; *7*) 5 mM malate; *8*) 5 mM $MgCl_2$; *9*) control without added metabolites; *10*) control without glutaraldehyde treatment.

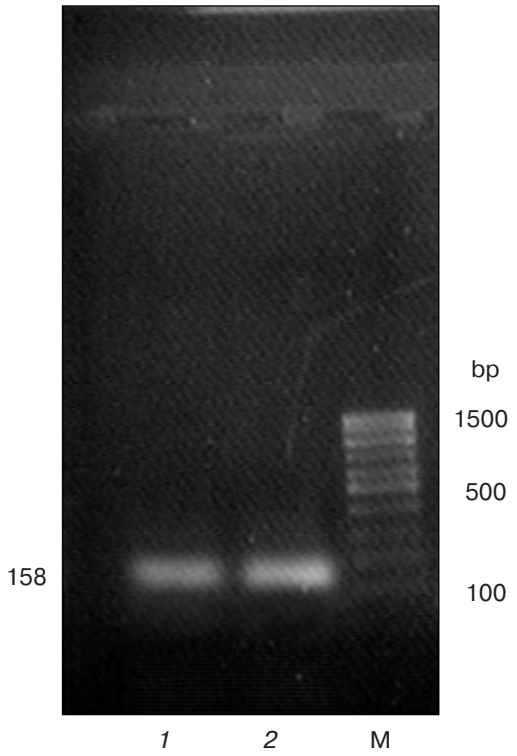

Fig. 4. Amplification with MDH-specific primers of *R. sphaeroides* strain 2R genomic DNA upon chemoorganoheterotrophic growth on succinate (*1*) and acetate (*2*). M, markers of DNA molecular mass.

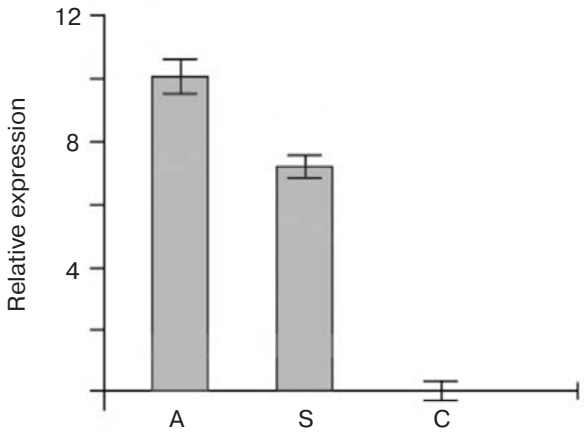

Fig. 5. Expression level of *mdh* gene of *R. sphaeroides* strain 2R on acetate (A) and succinate (S). The cDNA obtained with 100 ng of total RNA was used for the reaction. Relative gene expression is given as the sample ratio to control (*ef-1α*) using the $2^{-\Delta\Delta Ct}$ technique. C, control.

causes the increase in MDH activity by 32% [35]. In our case, the increase in the *mdh* gene transcription on acetate type of nutrition can be indicative of increased synthesis of the enzyme additional subunits necessary for formation of MDH tetramer functioning in the citramalate cycle. The increased level of *mdh* gene expression upon growth in the presence of acetate correlates with data of other authors who showed that metabolism of *Corynebacterium glutamicum*, *Escherichia coli*, and other bacteria grown on acetate as the sole source of carbon is characterized by several-fold increased expression levels of acetate assimilation genes as well as of genes of key TCA cycle enzymes (*sdhA*, *sdhB*, *fumH*, and *mdh*) [36, 37].

Thus, our investigation showed that two bands of MDH activity found on electrophoregram after specific development (Fig. 1a) are isoforms whose isologous peptide components are determined by a single gene in *R. sphaeroides*. The emergence in of tetrameric MDH isoform during growth on acetate is evidently due to increased level of *mdh* gene expression. Dimer dimerization might be the result of a change in the ionization of the protein molecule. The product of this process, MDH tetramer, in cooperation with the citramalate cycle enzymes plays an important role in acetate assimilation.

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