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The Fermenting Bacterium *Malonomonas rubra* is Phylogenetically Related to Sulfur-Reducing Bacteria and Contains a *c*-Type Cytochrome similar to those of Sulfur and Sulfate Reducers

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Summary

Malonomonas rubra is a microaerotolerant fermenting bacterium which can maintain its energy metabolism for growth by decarboxylation of malonate to acetate. 16S rRNA sequence analysis revealed that *M. rubra* is closely related to the cluster of mesophilic sulfur-reducing bacteria within the delta subclass of the Proteobacteria, with the fermenting bacterium *Pelobacter acidigallici* and the sulfur reducers *Desulfuromusa kysingii, D. bakii* and *D. succinoxidans* as closest relatives. The cells contain high amounts (up to 12% of the total cell protein content) of a *c*-type cytochrome which is present mainly (>60%) in the cytoplasm and to minor parts in the periplasm (>20%) and associated with the membrane fraction (>10%), independent of the growth substrate. This cytochrome is a tetraheme cytochrome of 13,700 Da molecular mass with a midpoint redox potential of –0.210 V. *M. rubra* does not reduce sulfur or ferric iron compounds. Since this cytochrome appears not to be involved in the energy metabolism it is concluded that it is a remnant of sulfur-reducing ancestors of this bacterium, without a conceivable physiological function in its present energy metabolism.

Key words: cytochrome *c* – sulfur-reducers – sulfat-reducers – decarboxylation – malonate

Introduction

Malonomonas rubra was isolated as a fermenting bacterium able to grow by decarboxylation of malonate to acetate (DEHNING and SCHINK, 1989). It can also ferment malate or fumarate to succinate and CO_2. The red pigmentation of the cells was conspicuous from the beginning, staining colonies in deep agar culture intensely red. Spectral analysis revealed that this pigmentation was due to a high content of cytochrome *c* in the cells, however, the metabolism of this bacterium gave no clue to a possible function of this cytochrome in electron transfer reactions.

The present study aimed at a characterization and elucidation of possible functions of this cytochrome in the energy metabolism of *M. rubra*. Moreover, comparative 16S rRNA sequence analysis was performed in order to obtain possible hints for phylogenetic relationships to bacteria with other metabolic capacities.

Materials and Methods

Strains source, media, and growth conditions: *Malonomonas rubra* strain GraMal1, DSM 5091, was from our own culture collection, and was checked for purity at regular intervals by microscopy after growth in mineral medium with malonate as sole substrate, or in complex medium (Difco AC medium; diluted 1:10). *M. rubra* was grown in bicarbonate/ CO_2-buffered, sulfide-reduced mineral medium adapted for marine bacteria (WIDDEL and PFENNIG, 1981), containing 7-vitamin solution (WIDDEL and PFENNIG, 1981), selenite-tungstate solution (TSCHECH and PFENNIG, 1984) and the trace element solution SL10 (WIDDEL et al., 1983). The final pH of the medium was adjusted to 7.2–7.4. The growth temperature was 28 °C. Substrates were added from sterile, neutralized stock solutions. Mass cultures were grown in 5 l carboys with 20 mM malonate as substrate. Cells were harvested by centrifugation at 10,000 × g for 25 min.

Phylogenetic analysis: *In vitro* amplification and direct sequencing of 16S rRNA encoding DNA fragments was done as described earlier (SPRINGER et al., 1992). The sequence data

have been deposited at the EBI database under accession number 17712. The new 16S rRNA sequence was fitted into an alignment of about 10,000 homologous full and partial primary structures available in public databases (LUDWIG, 1995) using the respective automated tools of the ARB software package (LUDWIG and STRUNK, 1996). Distance matrix, maximum parsimony and maximum likelihood methods were applied for tree reconstruction as implemented in the ARB software package. Different data sets varying with respect to included outgroup reference organisms (sequences) as well as alignment positions were analyzed.

Localization experiments: Cells were harvested at the end of exponential growth by 25 min centrifugation at 10,000 × g, washed one and resuspended in 50 mM Tris-HCl, pH 7.0. 20% (w/v) sucrose, 2 mM EDTA and 1 mg lysozyme per ml (21,500 U × mg protein^{-1}) were added, and the suspension was incubated for at least 1 hour at 28 °C. Spheroplast formation was followed microscopically. Spheroplasts were removed by 30 min centrifugation at 5,000 × *g*, and the supernatant was cleared of cell debris afterwards by 30 min centrifugation at 45,000 × *g* to give the periplasmic fraction. Cytoplasm and membrane fractions were obtained from spheroplasts by centrifugation at 120,000 × *g* after sonication. Malate dehydrogenase activity was measured as a tracer of the cytoplasmic fraction in the periplasmic fraction and culture supernatant measuring NADH oxidation with oxaloacetate (STAMS et al., 1984). Pyridine hemochromes were extracted after published procedures (WESTON and KNOWLES, 1973).

Purification of cytochrome *c*: Purification started with the periplasmic fraction which was applied to a 0.6 × 5 cm cation exchanger column (Mono S, prepacked, Pharmacia, Sweden) preequilibrated with 25 mM sodium phosphate buffer, pH 6.3, as eluent. In a linear gradient up to 1 M NaCl, the cytochrome eluted at 270–300 mM NaCl concentration. The fraction containing cytochrome *c* (detected by the absorption spectrum of its reduced form) was loaded on a gel filtration column (1.25 × 30 cm, Superose 12 prepacked, Pharmacia, Sweden) run with 0.15 M ammonium acetate buffer, pH 6.3. The fraction containing the cytochrome was concentrated by lyophilization since the cytochrome tended to attach to ultrafiltration membranes. The concentrate in a 1.7 M ammonium acetate concentration was loaded on a hydrophobic interaction column (1.25 × 10.5 cm, Phenyl Superose prepacked, Pharmacia, Sweden). The cytochrome eluted with 1.7 M ammonium acetate, whereas the remaining contaminating proteins were retained and eluted at lower ionic strength. The cytochrome fraction was lyophilized, redissolved in two steps with ten volumes of distilled water each, and lyophilized again.

Characterization of cytochrome *c*: For SDS gel electrophoresis the method of LAEMMLI (1970) was applied with 12% or 14% polyacrylamide for resolving gels and 4% for stacking gels. Samples were diluted in sample buffer containing 60 mM Tris/HCl, 2% (w/v) SDS, 10% (w/v) glycerol, 0.025% (w/v) bromophenol blue, and no mercaptoethanol. Electrophoresis was carried out ion a dual slab cell (Mini-Protean II, Bio-Rad, USA) with Tris/Glycin/SDS-buffer (25 mM, 250 mM, 0.1% w/v, respectively), starting at 30 mA until samples entered the resolving gel, and separating at 40 mA. Heme staining in SDS gels was performed as described earlier (THOMAS et al., 1976) with modifications by GOODHEW et al. (1986).

The molecular mass of the purified cytochrome *c* was estimated by gel filtration chromatography on a Hi-Load Superdex 75 preparation-grade column (Pharmacia BioSystems, Freiburg) equilibrated with 50 mM potassium phosphate buffer, pH 7.2, containing 0.5 M NaCl. The void volume was determined with dextran blue (1 mg per ml), and the column was calibrated with the low-molecular-weight calibration kit (Pharmacia, Freiburg).

The midpoint redox potential was determined by titration with 100 µM benzyl viologen (E° = −360 mV), flavin adenin dinucleotide (E° = −219 mV), flavin mononucleotide (E° = −190 mV) and 100 µM indigodisulfonate (E° = −125 mV) as redox indicators, dithionite as reductant, and air as oxidant. Absorptions of dyes and cytochrome were recorded with a double-beam UV/VIS spectrophotometer (Uvikon 860, Kontron, Switzerland).

For determination of total iron content, cytochrome solutions were dissolved in 0.1 M NaOH, and the iron concentration was determined with an atomic absorption spectrophotometer (303B, Perkin Elmer, Norwalk, CT, USA) at 284.3 nm.

Analytical methods: Cytochrome *c* was quantified taking redox difference absorption spectra of dithionite-reduced minus air- or hydrogen peroxide-oxidized preparations. The specific absorption coefficient was determined with the purified cytochrome, and applied for quantification in cell subfractions. Spectra were recorded with double beam UV/VIS spectrophotometers (Uvikon 860 or 930, Kontron, Switzerland) at room temperature. Glass and quartz cuvettes used had 1 ml total volume, 1 cm light path, and were sealed with rubber stoppers and gassed with nitrogen for anoxic measurements. Peak heights were measured relative to a baseline drawn between the troughs at 530–535 and 565–570 nm. Protein was quantified according to BRADFORD (1976).

Chemicals: All chemicals were of analytical or reagent grade quality and were obtained from Biomol (Ilvesheim, Germany), Boehringer (Mannheim, Germany), Eastman Kodak (Rochester, NY, USA), Fluka (Neu-Ulm, Germany), Merck (Darmstadt, Germany), Pharmacia (Freiburg, Germany), Serva (Heidelberg, Germany), and Sigma (Deisenhofen, Germany). Gases were purchased from Messer-Griesheim (Darmstadt, Germany), and Sauerstoffwerke Friedrichshafen (Friedrichshafen, Germany).

Results

16S rRNA sequence analysis

16S rRNA encoding DNA from *Malonomonas rubra* was amplified *in vitro* and directly sequenced. The sequence was deposited at the EBI data library under accession number 17712. A comparative database analysis revealed highest sequence similarity (95.6%; Table 1) with *Pelobacter acidigallici* (SCHINK and PFENNIG, 1982). These two organisms together with *Desulfuromusa* species represent a monophyletic cluster sharing 94.6% and more 16S rRNA similarity (Table 1). This cluster is part of one of the major phylogenetic groups of the delta subclass of the *Proteobacteria* comprising the genera *Desulfuromonas*, *Desulfuromusa*, *Geobacter*, *Malonomonas*, and *Pelobacter* (Figure 1).

Cytochrome contents of cells and cell subfractions

Cells of *M. rubra* contained cytochromes which stained colonies in deep-agar cultures orange-red (DEHNING and SCHINK, 1989). Redox-difference spectra of cell-free extracts exhibited absorption bands at 551, 552, and 419 nm wavelength as typical of a *c*-type cytochrome. After growth with malonate, 60–65% of this cytochrome was found in the cytoplasm, 20–22% in the periplasmic space, and 10–15% associated with the

Table 1. Overall 16S rRNA sequence similarities of *Malonomonas rubra*, its closest relatives and the major phylogenetic groups of the delta subclass of the *Proteobacteria*. Mean values are given for phylogenetic groups (*Desulfuromonas, Geobacter, Desulfobacter, Desulfobulbus, Syntrophobacter,* Myxobacteria and *Desulfovibrio*). Abbreviations: Da, *Desulfobacter*; DI, *Desulfobulvus*; Do, *Desulfuromonas*; Du, *Desulfuromusa*; Dv, *Desulfovibrio*; G., *Geobacter*; M., *Malonomonas*; My, Myxobacteria; P., *Pelobacter*; Sb, *Syntrophobacter*; Su, *Syntrophus*; [1], the group comprises *Desulfuromonas acetexigens, "Desulfuromonas palmitatis", Pelobacter acetylenicus, P. carbinolicus* and *P. venetianus*; [2], the group comprises *Geobacter metallireducens, G. sulfureducens, "G. hydrogenophilus", Pelobacter propionicus* and *"P. chapelleii"*; [3], the group comprises the genera *Desulfobacter, Desulfobacterium, "Desulfobacula", "Desulfobotulus", Desulfococcus, Desulfonema* and *Desulfosarcina*; [4], the group comprises the genera *Desulfobulbus, Desulfofustis, "Desulforhopalus"* and *Desulfocapsa*; [5], the group comprises the genera *Syntrophobacter, "Desulfoacinom"* and *"Desulforhabdus"*; [6], the group comprises the genera *Angiococcus, Archangium, Cystobacter, Melitangium, Myxococcus* and *Stigmatella*; [7], the group comprises the genera *Desulfovibrio, Desulfohalobium* and *Desulfomicrobium"*.

Organisms	Overall 16S rRNA sequence similarity (%)												
	Pa	Dub	Duk	Dus	Doa	Do	G	Da	DI	Sb	Su	My	Dv
M. rubra	95.6	95.0	94.6	94.8	92.2	91.9	87.6	84.0	83.8	84.0	85.1	81.8	82.1
P. acidigallici		94.6	94.8	94.8	92.2	92.2	88.7	83.9	84.2	84.3	85.3	82.4	82.6
Du. bakii			98.8	98.3	90.7	91.8	87.3	83.5	83.5	83.5	84.1	81.7	82.2
Du. kysingii				98.3	90.7	92.0	87.4	83.5	83.5	83.7	84.3	81.8	82.4
Du. succinoxidans					90.9	91.6	87.1	83.2	83.5	83.9	84.1	81.6	82.1
Do. acetoxidans						89.9	88.9	84.2	84.5	84.4	87.1	83.4	82.9
Desulfuromonas[1]							90.1	83.6	84.0	84.6	86.3	83.2	82.5
Geobacter[2]								83.7	84.1	84.3	84.1	83.9	82.1
Desulfobacter[3]									83.0	82.4	85.2	80.3	81.2
Desulfobulbus[4]										83.8	86.0	81.3	81.8
Syntrophobacter[5]											84.4	81.9	81.3
Syntrophus												83.0	82.1
Myxobacteria[6]													79.8
Desulfovibrio[7]													

membrane fraction. Malic dehydrogenase as a tracer of cytoplasmic proteins was found to 99.5% in the cytoplasm, 0.4% in the periplasm, and less than 0.1% in the membrane fraction, indicating that the above distribution was not altered significantly by the fractionation procedure. The membrane-associated cytochrome *c* could not be solubilized with 0.2 M KCl but was resolved from the membranes nearly quantitatively (>90%) with 1% Triton X-100.

Purification of the cytochrome

The cytochrome was most easily accessible at high concentration in the periplasmic fraction, and enrichment and isolation started from this fraction, therefore. Part of the contaminating proteins was bound to an anion exchanger DE 52 in 50 mM Tris-HCl buffer, pH 7.0, which did no bind the cytochrome. A Mono S cation exchanger equilibrated with 25 mM sodium phosphate, pH 7.0, bound 83% of the cytochrome which was eluted subsequently with a linear NaCl gradient at 0.27–0.30 M concentration. Gel filtration on Sepharose 12 and hydrophobic interaction chromatography yielded a homogeneous cytochrome preparation which exhibited only one protein band after SDS polyacrylamide gel electrophoresis and silver staining. This band was also stained with high sensitivity by specific heme staining. The enrichment was 5.9-fold, with a yield of 11.6%.

Enrichment of the cytoplasmic cytochrome by separation on S-Sepharose and Superdex 75 led to a cytochrome preparation with spectroscopic properties nearly identical to those of the periplasmic cytochrome preparation. The membrane fraction contained, beyond the *c*-type cytochrome, also a small fraction of a *b*-type cytochrome which could be identified by pyridine hemochrome extraction (absorption bands 556, 540 and 410 nm) but was not characterized further.

Characterization of the periplasmic cytochrome *c*

The molecular mass of the purified cytochrome *c* was determined by gel filtration chromatography to be 13.7 ± 0.3 kD, in comparison with standard proteins of 1.4–26.6 kD molecular mass.

Absorption spectra of the purified cytochrome *c* in it oxidized and reduced form are shown in Fig. 2. The reduced cytochrome had absorption maxima at 551, 522, and 418 nm, the oxidized form a maximum at 407 nm. The specific absorption of the α band of the reduced form of the cytochrome at 551 nm wavelength was 32.6 $mM^{-1} \times cm^{-1}$, calculated on the basis of the determined molecular mass. With the specific absorption coefficient of the purified cytochrome, it could be estimated that up to 12% of the total protein content of *M. rubra* cells was cytochrome protein.

Atomic absorption spectroscopy of the periplasmic cytochrome *c* revealed four heme per molecule, as compared to commercially available monoheme cytochrome *c* from horse heart (Sigma biochemicals).

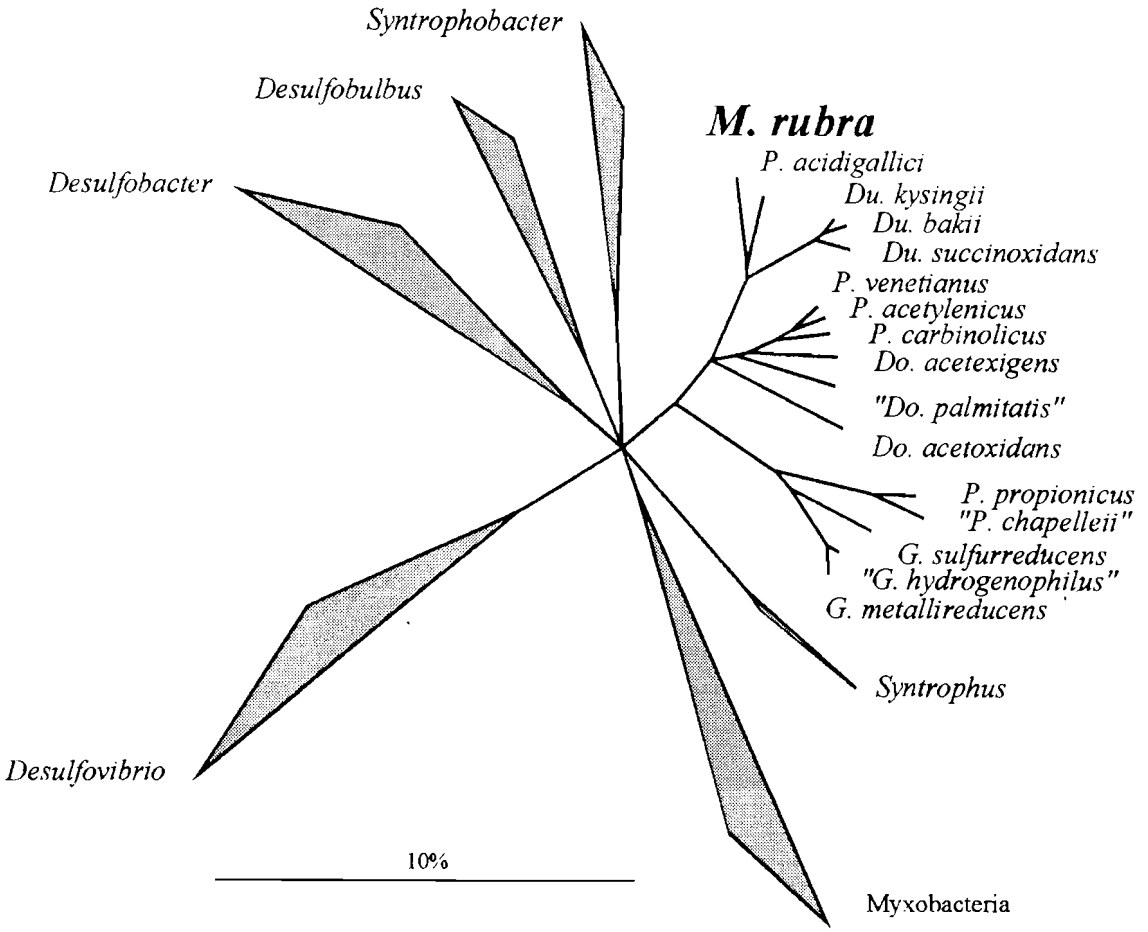

Fig. 1. 16S rRNA based tree reflecting the relationships of *Malonomonas rubra*, its closet relatives and the major phylogenetic groups of the delta-subclass of the *Proteobacteria*. The topology of the tree is based on the results of a distance matrix analysis. Only sequence positions which share identical residues among 50% of all available (at least almost) complete 16S rRNA sequences from the delta subclass proteobacteria were included for tree construction. Multifurcations indicate that a common relative branching order was not supported by the results obtained performing different treeing methods. Phylogenetic groups are indicated by triangles. The bar indicates 10% estimated sequence divergence. Abbreviations are as specified in the legend to Table 1.

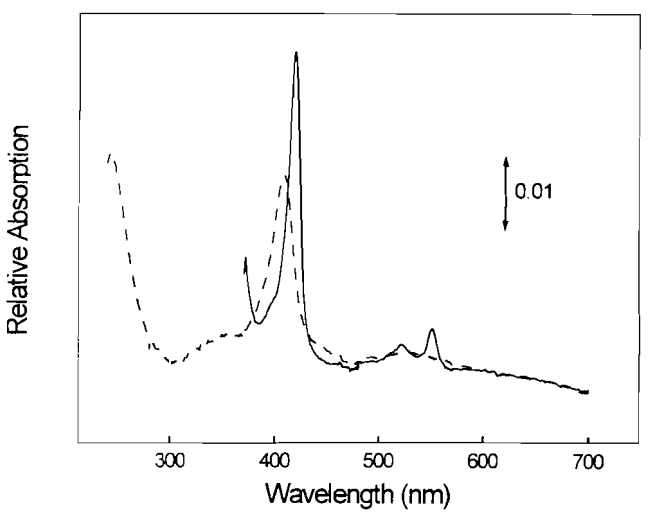

Fig. 2. Dithionite reduced (solid line) and H_2O_2 oxidized (dashed line) spectra of cytochrome *c* from the periplasm of *Malonomonas rubra*.

Electron donor and acceptors

The periplasmic cytochrome *c* was reduced by dithionite, Ti(III) NTA, H_2S, or dithioerythritol, but not by cysteine or ascorbate. It was oxidized quickly by O_2. A redox titration with indigodisulfonic acid, flavin mononucleotide, flavin adenin dinucleotide, and benzyl viologen resolved three redoxpotentials of the cytochrome. The identified redox potentials of individual heme groups were −0.120 V, −0.220 V and −0.370 V (each with a standard error of ±0.020 V).

Physiological experiments

The cytochrome *c* in intact cells was reduced upon addition of malonate or acetate, and oxidized by oxygen. Under anoxic or microoxic conditions (≤5% air saturation), malonate was fermented to acetate without substantial increase in cell density. The cytochrome content of intact cells was identical after growth with malonate

or fumarate; it did not change either upon exposure to air oxygen. Elemental sulfur, polysulfide, or ferric iron hydroxide [ferrihydrite, $Fe(OH)_3$] was not reduced with malonate or fumarate as electron donor. No oxygen-dependent oxidation o facetate could be observed, not even in dense cell suspensions.

Discussion

Phylogenetic position of *Malonomonas rubra*

The status of *Malonomonas rubra* as a species representing its own genus is supported by substantial 16S rRNA sequence difference (4.4%) to its so far closest relative *Pelobacter acidigallici* (Table 1, Figure 1). This is the type species of the genus *Pelobacter* (SCHINK and PFENNIG, 1982). These two genera, together with *Desulfuromonas* and *Geobacter,* are members of one of the major phylogenetic groups of the delta subclass of the *Proteobacteria.* The group is monophyletic and significantly separated from he other delta proteobacteria as supported by the results of various analyses using differing datasets and applying alternative treeing methods, as shown in the tree of Figure 1. However, within this group only *Desulfuromusa* and *Geobacter* are monophyletic genera, whereas *Desulfuromonas* and especially *Pelobacter* are phylogenetically heterogenous. Given that *Pelobacter acidigallici* is the type species of its genus and *Malonomonas rubra* its closest relative, a taxonomic reevaluation of the genus *Pelobacter* may appear necessary in the future.

Comparison of the periplasmic *c*-type cytochrome with other known cytochromes

The redox difference spectrum of the cytochrome *c* purified from the periplasmic fraction of *M. rubra* is typical of the class III (multiheme) cytochromes found in sulfate- and sulfur-reducing bacteria: there was to 695 nm absorption in the oxidized form and a shoulder on the short wavelength side of the Soret band (PETTIGREW and MOORE, 1987). Compared to known tri- and tetra-heme class III cytochromes (PETTIGREW and MOORE, 1987) from sulfate- and sulfur-reducing bacteria, the cytochrome *c* described here does not differ substantially by its molecular mass and its midpoint redox potential. Cytochromes of *Desulfovibrio vulgaris* strain Miyazaki (SOKOL et al., 1980), *D. vulgaris* strain Hildenborough (DERVARTANIAN et al., 1978; BRUSCHI et al., 1984), *D. salexigens* strain Benghazi (DRUCKER et al., 1970), *D. gigas* (XAVIER et al., 1979), *D. desulfuricans* strain Norway (BRUSCHI et al., 1977; BRUSCHI, 1981), and *Shewanella putrefaciens* (MORRIS et al., 1994) have molecular masses of 13 kD or higher, and standard redox potentials below –0.2 V. *Desulfobulbus elongatus* has a cytochrome of a standard redox potential of –0.165 V and a molecular mass of 13.7 kD (SAMAIN et al., 1986). All cytochromes mentioned have nearly identical absorption maxima (±2 nm wavelength) both in their reduced and oxidized forms. The cytochrome *c* from *Desulfuromonas acetoxidans* has standard redox potentials of the three hemes at –0.102, –0.177, and –0.177 V (according to FIECHTNER and KASSNER, 1979) or –0.140, –0.210 and 0.240 V (according to BRUSCHI et al., 1984). The specific absorption coefficient at the α absorption band (at 551 nm wavelength) of the reduced *M. rubra* cytochrome was 32.6 $mM^{-1} \times cm^{-1}$, similar to that of the three-heme cytochrome *c* of *D. acetoxidans* (30.8 $mM^{-1} \times cm^{-1}$; PROBST et al., 1977).

Possible physiological function of the periplasmic cytochrome *c*

The cytochrome described here was oxidized in intact cells by oxygen, and reduced by their growth substrates malonate and fumarate, but also by their fermentation product acetate. There is no indication of an involvement of this *c*-type cytochrome in reduction of fumarate to succinate (DEHNING and SCHINK, 1989), rather, the *b*-type cytochrome present in the cytoplasmic membrane is more prone to a function in such an electron transport. The observed reduction of the cytochrome in intact cells by substrates such as malonate or acetate is probably enabled by oxidation of acetyl CoA through the tricarboxylic acid cycle. *M. rubra* is known to contain all reactions of this cycle which operates either in acetyl CoA oxidation for cell carbon synthesis from acetyl residues during growth with malonate or in a non-cyclic manner during growth with fumarate (DEHNING and SCHINK, 1989). Electrons for reduction of the cytochrome with its comparably low midpoint redox potential (–0.210 V) are most likely to be provided in the isocitrate dehydrogenase (–0.350 V, NAD-dependent) and the 2-oxoglutarate ferredoxin oxidoreductase (–0.370 V; benzylviologen-dependent) reaction. Nonetheless, since no external electron acceptors are reduced in acetate oxidation, this cytochrome appears not to fulfil a visible function in growth during fermantation of malate or fumarate.

The biochemistry of malonate decarboxylation has been elucidated. Malonate binds to the enzyme protein to form an S-malonyl enzyme complex, in exchange for a bound acetyl residue, and is decarboxylated in the S-malonyl form by carboxyl transfer to a biotin carrier (HILBI et al., 1992). Thus, the *c*-type cytochrome appears not to be involved in the decarboxylation reaction either; it had been hypothesized earlier that it could act as a radical generator if the free malonate molecule was decarboxylated by a radicalic mechanism.

The obvious phylogenetic relationship of *M. rubra* to sulfur-reducing bacteria suggested that the cytochrome present, similar to those found in sulfur reducers, might act in electron transfer to sulfur as external electron acceptor. Also ferric iron-reducing bacteria are phyletically related to the sulfur reducers on the basis of 16S rRNA sequence similarities (CACCAVO et al., 1994), and also these bacteria contain cytochromes, either associated with the cytoplasmic membrane and the periplasmic space (MYERS and MYERS, 1992) or in the periplasm and even outside the cells (SEELIGER et al., 1998). We therefore checked again thoroughly for possible reduction of

sulfur or ferric iron by *M. rubra* but could not find any indication of such activities.

We conclude that the *c*-type cytochrome of *M. rubra* does not fulfill an essential physiological function in the energy metabolism of this bacterium but may be a remnant from sulfur- or ferric iron-reducting ancestors to which it appears to be related on the basis of 16S rRNA sequence similarity data.

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