

## Characterization of the Maleylacetate Reductase MacA of *Rhodococcus opacus* 1CP and Evidence for the Presence of an Isofunctional Enzyme

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**Maleylacetate reductases (EC 1.3.1.32) have been shown to contribute not only to the bacterial catabolism of some usual aromatic compounds like quinol or resorcinol but also to the degradation of aromatic compounds carrying unusual substituents, such as halogen atoms or nitro groups. Genes coding for maleylacetate reductases so far have been analyzed mainly in chloroaromatic compound-utilizing proteobacteria, in which they were found to belong to specialized gene clusters for the turnover of chlorocatechols or 5-chlorohydroxyquinol. We have now cloned the gene *macA*, which codes for one of apparently (at least) two maleylacetate reductases in the gram-positive, chlorophenol-degrading strain *Rhodococcus opacus* 1CP. Sequencing of *macA* showed the gene product to be relatively distantly related to its proteobacterial counterparts (ca. 42 to 44% identical positions). Nevertheless, like the known enzymes from proteobacteria, the cloned *Rhodococcus* maleylacetate reductase was able to convert 2-chloromaleylacetate, an intermediate in the degradation of dichloroaromatic compounds, relatively fast and with reductive dehalogenation to maleylacetate. Among the genes ca. 3 kb up- and downstream of *macA*, none was found to code for an intradiol dioxygenase, a cycloisomerase, or a dienelactone hydrolase. Instead, the only gene which is likely to be cotranscribed with *macA* encodes a protein of the short-chain dehydrogenase/reductase family. Thus, the *R. opacus* maleylacetate reductase gene *macA* clearly is not part of a specialized chlorocatechol gene cluster.**

Maleylacetate reductases (EC 1.3.1.32) have long been known to be involved in the degradation of chloroaromatic compounds via chlorocatechols as intermediates (10, 31). By reduction of a carbon-carbon double bond they form 3-oxoadipate, a metabolite also of catechol catabolism, and thus compensate for the different oxidation states of chlorinated and nonchlorinated compounds. 2-Chloromaleylacetate, which is formed during turnover of several dichlorocatechols, is initially reductively dechlorinated and then reduced to 3-oxoadipate in a second reaction (22, 47).

Corresponding to the biochemical function in chlorocatechol degradation, the following maleylacetate reductase genes have been shown to be associated with dioxygenase, cycloisomerase, and dienelactone hydrolase genes as components of specialized chlorocatechol catabolic operons: *tfdF* and *tfdF<sub>II</sub>* on pJP4 from the 2,4-dichlorophenoxyacetate-utilizing strain *Ralstonia eutropha* (*Alcaligenes eutrophus*) JMP134 (29, 33, 37, 44), *tcbF* on pP51 from the 1,2,4-trichlorobenzene-degrading strain *Pseudomonas* sp. strain P51 (45), and *clcE* from the 3-chlorobenzoate catabolizing strains *Pseudomonas* sp. strain B13 and *Pseudomonas putida* AC866(pAC27) (15, 20, 21). Catechol degradation, in contrast, does not require a maleylacetate reductase activity, and corresponding genes do not belong

to the known catechol operons. Thus, while at least two of the chlorocatechol catabolic enzymes, i.e., the dioxygenases and cycloisomerases, appear to have been recruited from catechol catabolism, maleylacetate reductase genes must have had a different origin and original function (34).

The postulated original function of the maleylacetate reductases is still under discussion. In bacteria, these enzymes have been shown to play a role, for example, in quinol, resorcinol, and 2,4-dihydroxybenzoate degradation (6, 25, 41). Other aromatic growth substrates involving the action of maleylacetate reductase are more exotic, since they carry a fluorine substituent (35), a sulfo group (14), a nitro group (18, 40), or several chlorine substituents (8, 26, 48). Maleylacetate reductase genes have been shown to be part of a specialized gene cluster for 2,4,5-trichlorophenoxyacetate degradation (8, 9) and of a gene cluster for hydroxyquinol conversion which contributes to 4-nitrophenol turnover (4).

The chlorocatechol pathway of the chlorophenol-utilizing strain *Rhodococcus opacus* (*erythropolis*) 1CP obviously evolved functionally convergent to the corresponding pathway in the proteobacteria mentioned above (13, 39). Thus, it is not surprising that the chlorocatechol gene cluster of strain 1CP is organized differently from the corresponding proteobacterial operons; in fact, its characterization showed that it does not comprise a maleylacetate reductase gene (13). Thus, the nature of the gene cluster(s) encoding a maleylacetate reductase in *R. opacus* remained to be elucidated. Such gene clusters could complement otherwise incomplete pathways, and they might also have provided the source from which the maleylacetate reductase gene was recruited during evolution of dedicated pathways, such as the proteobacterial chlorocatechol catabolic route.

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TABLE 1. Plasmids used in this study

Plasmid	Relevant characteristics <sup>a</sup>	Source (reference)
pBluescript II SK(+) pET11a*	Phagemid derived from pUC19, P <sub>lac</sub> , lacZ, Ap <sup>r</sup> , fl(+) origin, ColE1 origin Derivative of pET11a with additional cloning sites ( <i>Kpn</i> I, <i>Xho</i> I, <i>Sal</i> I), P <sub>T7lac</sub>	Stratagene (1) J. Altenbuchner, Stuttgart, Germany (42)
pMARRE0	Ap <sup>r</sup> ; 0.982-kb PCR product of primers NH2-3 and HOM in the <i>Eco</i> RV site of pBluescript II SK(+)	This study
pMARRE1	Ap <sup>r</sup> ; pBluescript II SK(+) containing a 5,657-bp <i>Kpn</i> I fragment of <i>R. opacus</i> 1CP DNA	This study
pMARRE2	Ap <sup>r</sup> ; pBluescript II SK(+) containing a ca. 7-kb <i>Xho</i> I fragment of <i>R. opacus</i> 1CP DNA	This study
pMARRE3	Ap <sup>r</sup> ; pBluescript II SK(+) containing a 4,628-bp <i>Pst</i> I fragment of <i>R. opacus</i> 1CP DNA	This study
pMARRE5	Ap <sup>r</sup> ; 2,079 bp from pMARRE1 on <i>Eco</i> RI- <i>Hind</i> III fragment in pBluescript II SK(+)	This study
pMARRE10	Ap <sup>r</sup> ; PCR-amplified <i>macA</i> in <i>Nde</i> I and <i>Bam</i> HI site of pET11a*	This study

<sup>a</sup> Abbreviation: Ap<sup>r</sup>, ampicillin resistance.

(Some of the results presented here have previously been reported in a preliminary communication [38].)

#### MATERIALS AND METHODS

**Bacterial strains, plasmids, and culture conditions.** *R. opacus* 1CP, previously assigned to the species *R. erythropolis*, has been reported to utilize 4-chlorophenol and 2,4-dichlorophenol as sole sources of carbon and energy (17, 43). Cells for enzyme purification were grown with 4-chlorophenol as described previously (39). *Escherichia coli* DH5 $\alpha$  (32) was obtained from GIBCO BRL, and *E. coli* BL21(DE3)(pLysS) (32, 42) was obtained from Novagen. *E. coli* strains were grown aerobically in baffled Erlenmeyer flasks with constant shaking (170 rpm) at 37°C in 2 $\times$  YT medium (32), if appropriate, with ampicillin at a final concentration of 100  $\mu$ g/ml. The plasmids used in this study are listed in Table 1.

**DNA manipulations, cloning procedure, and sequence analysis.** Genomic DNA from *R. opacus* 1CP was prepared as described by Eulberg et al. (11). Plasmid DNA from *E. coli* was isolated with a Pharmacia Flexiprep kit or by a modified boiling method (30). Other general genetic methods were performed by standard procedures (3, 32), usually as described previously (11).

Primer NH2-3, 5'-(A/C)G(G/C)GT(G/C)GT(G/C)TTCGG(G/C)GC(G/C)GG-3', was custom synthesized corresponding to the amino acid sequence RVVF GAG, which is conserved close to the N termini of TfdF and of the previously purified maleylacetate reductase (TfdF<sub>11</sub>) from *R. eutropha* JMP134 (37). Primer HOM, 5'-GG(A/T)CG(G/C)GG(A/G)TT(G/C)GG(A/G)TA(C/T)TG-3', was designed to fit to a conserved region of the maleylacetate reductase genes *tfdF* and *tcbF* (positions 976 to 995 and 973 to 992, respectively). PCR mixtures contained, in a total volume of 50  $\mu$ l, 50 pmol of each primer, 0.1  $\mu$ g of genomic template DNA, 100  $\mu$ M deoxynucleoside triphosphates, 1.5 mM MgCl<sub>2</sub>, 0.3 U of Eurogentec Goldstar polymerase, and the corresponding buffer. PCR was performed with 30 s of denaturing (94°C), 30 s of annealing (45°C), and 60 s of polymerization (72°C) for 33 cycles, with an additional 3 min of denaturing prior to the first cycle (polymerase addition after 2 min) (7) and 15 min of polymerization after the last cycle.

Correctly sized amplification products (ca. 980 bp) were ligated into the T-tailed *Eco*RV site of pBluescript II SK(+), yielding pMARRE0. After digoxigenin labeling, the cloned PCR product served to identify hybridizing bands on a Southern blot (Amersham Hybond N<sup>+</sup> nylon membrane) with *Xho*I-, *Pst*I-, and *Kpn*I-digested genomic DNA from *R. opacus*. DNA from corresponding regions on a second gel was eluted and ligated into pBluescript II SK(+). The labeled insert of pMARRE0 was used again to identify by colony hybridization those *E. coli* DH5 $\alpha$  clones which contained the recombinant plasmids pMARRE1, -2, and -3.

The nucleotide sequences of both strands of the inserts of pMARRE1 and -3 were determined and analyzed as described previously (11). In addition to FASTA, the BLAST programs (2, 16) were used under default conditions to screen databases.

**Overproduction of the maleylacetate reductase MacA in *E. coli*.** To detect the polypeptide encoded by the predicted maleylacetate reductase gene, the T7lac expression system of Studier et al. (42) was used. For overexpression of maleylacetate reductase, the *macA* gene was PCR amplified from pMARRE5 by using primer PND (5'-GATCATATGGCTGAGCGCTCCTCCGAG-3'), with an *Nde*I site (underlined) and the start of *macA* (in boldface), as well as primer PBA (5'-GTGGATCCCTATTCGGGGCGGGGATTG-3') with a *Bam*HI site (underlined) and 19 bases (in boldface) corresponding to the end of *macA*. The PCR mixture was as described above except that different primers and template (pMARRE5) and 0.5 U of polymerase were used. The PCR program differed from the one given above in that (i) annealing conditions were 45 s at 50°C and (ii) only 22 cycles were performed. The resulting PCR product was digested with *Nde*I and *Bam*HI and inserted into pET11a\*, digested with the same enzymes, to yield plasmid pMARRE10, which contained the intact *macA* gene under the control of the T7lac promoter of pET11a\*.

*E. coli* BL21(DE3)(pLysS) (42) was used as the host strain for inducible overexpression of maleylacetate reductase. The strain containing the recombinant plasmid pMARRE10 was grown at 30°C in shaken 3-liter Erlenmeyer flasks

containing 1 liter of 2 $\times$  YT medium supplemented with 100  $\mu$ g of ampicillin per ml, 2.5 mM betaine, and 660 mM sorbitol. For induction of *macA*, 0.4 mM IPTG (isopropyl- $\beta$ -D-thiogalactopyranoside) was added to the cultures at an optical density at 600 nm of 1, and the cells were harvested after 7 h of incubation at 30°C. Cell pellets were washed with 50 mM BisTris [bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane]-HCl (pH 6.5)-1 mM dithiothreitol (DTT) and then stored at -20°C until required.

**Preparation of cell extracts.** Cells of *E. coli* BL21(DE3)(pLysS)(pMARRE10) were thawed on ice, resuspended in 50 mM BisTris-HCl (pH 6.5)-1 mM DTT, and disrupted by passage through an Aminco French pressure cell (37). Cells of *R. opacus* 1CP were resuspended in the same buffer and disintegrated by grinding with glass beads (11). In both cases, clear extracts were obtained by ultracentrifugation plus filtration (11).

**Enzyme assays and estimation of protein concentrations.** Maleylacetate reductase activities were determined by monitoring NADH oxidation in the presence of maleylacetate as described by Schlömann et al. (35). Kinetic experiments (in the presence of 0.2 mM NADH as cosubstrate) were also performed with 2-chloromaleylacetate. Protein concentrations were determined by the method of Bradford (5), with bovine serum albumin as the standard. Maleylacetate and 2-chloromaleylacetate were prepared by alkaline hydrolysis from *cis*-dienelactone (*cis*-4-carboxymethylenebut-2-en-4-olide) or its 2-chlorosubstituted analog, which were kindly provided by S. Kaschabek and W. Reineke (Wuppertal, Germany).

**Purification of the maleylacetate reductase MacA from an overexpressing *E. coli* clone.** Purification was performed at room temperature by using a fast protein liquid chromatography system as well as chromatography media from Pharmacia and 50 mM BisTris (pH 6.5)-1 mM DTT as the basal buffer for all steps. The initial anion-exchange chromatography of the cell extract (volume, 37 ml; protein, 2,040 mg; total activity, 1,470 U) was performed in two parallel runs on a Q Sepharose High Performance HR16/10 column with a linear NaCl gradient of 0.1 to 0.8 M over 300 ml (elution at ca. 0.3 M NaCl). From the following Red Sepharose CL-6B column (bed volume, 85 ml), proteins were eluted by first washing with buffer and by then applying an NaCl step of 0 to 2 M NaCl over 1 ml, which yielded the maleylacetate reductase at the high salt concentration. After concentration of the preparation by ultrafiltration (Amicon PM 10 filters) and addition of NaCl to 3 M (final concentration), the preparation was subjected to two parallel runs of a phenyl-Superose HR 10/10 chromatography with a gradient of 3 to 0.4 M NaCl over 65 ml (elution at ca. 1.7 M NaCl) and to concentration and partial salt removal by ultrafiltration. During the subsequent Mono Q HR 5/5 chromatography (three parallel runs) with an NaCl gradient of 0 to 0.4 M over 92 ml, the maleylacetate reductase eluted at ca. 0.3 M NaCl. In the next step, the maleylacetate reductase pool was chromatographed in four parallel runs on a Blue Sepharose CL-6B HR 10/10 column with an NaCl gradient of 0 to 2 M NaCl over 35 ml (elution at ca. 1 M NaCl). A concentrated and partially desalted preparation was further purified by using a weak anion exchanger (Mono P HR 5/5; three parallel runs) with a gradient of 0 to 0.7 M NaCl over 50 ml (elution at ca. 0.3 M NaCl). The final purification step was another Mono Q HR 5/5 run with an NaCl gradient from 0.2 to 0.7 M over 60 ml. The maleylacetate reductase preparation was concentrated, and 50% (vol/vol) glycerol was added for stabilization.

**Partial purification of a maleylacetate reductase induced in 4-chlorophenol-grown cells of *R. opacus* 1CP.** The purification made use of some of the same chromatographic media and the same buffer solution (50 mM BisTris [pH 6.5]-1 mM DTT) as for the procedure outlined above. A cell extract (volume, 600 ml; protein, 1,650 mg; total activity, 900 U; specific activity, 0.55 U/mg) which had been obtained from 77 g (wet weight) of cells was initially applied to a Q Sepharose High Performance HR16/10 column. Elution with a gradient of 0 to 0.5 M NaCl over 200 ml resulted in two activity peaks. Corresponding pools were further purified in separate runs on a Blue Sepharose CL-6B column (XK 26/20; bed volume, 50 ml) by using a gradient of 0 to 1.5 M NaCl over 300 ml for elution. Active fractions originating from the first peak on Q Sepharose High Performance chromatography appeared to contain relatively pure protein (protein, 0.03 mg; total activity, 6.5 U; specific activity, 217 U/mg) and thus were concentrated and analyzed by electrophoresis.

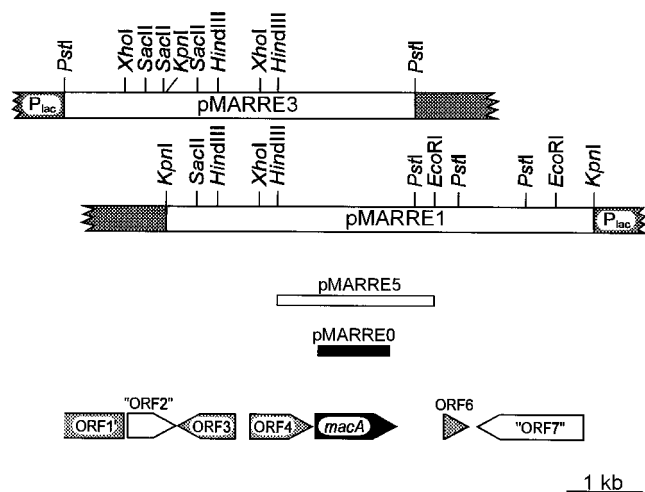


FIG. 1. Restriction and genetic map of the inserts of the original plasmids pMARRE1 and pMARRE3 carrying *R. opacus* 1CP DNA in the multiple cloning site of pBluescript II SK(+) (vector segments are indicated by shading). The insert of subclone pMARRE5 is shown, as is the PCR product (black) cloned to give pMARRE0, which was used as a digoxigenin-labeled probe for cloning. The positions and orientations of open reading frames are indicated by arrows below the restriction maps. The insert of pMARRE2 (not shown) extends from the *XhoI* site of pMARRE1 ca. 7 kb to the right.

**SDS-polyacrylamide gel electrophoresis and protein sequencing.** Discontinuous sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and staining of gels were performed basically as described previously (11, 37). If bands were to be analyzed by protein sequencing, they were cut from Western blots (for details, see reference 12) and subjected to automated sequencing (Applied Biosystems model 473A).

**Investigation of 2-chloromaleylacetate turnover.** The conversion of 2-chloromaleylacetate and the formation of a product from it were investigated in a reaction mixture (100  $\mu$ l, 37°C) containing 50 mM Tris-HCl (pH 7.5), 0.8 mM NADH, 0.8 mM 2-chloromaleylacetate, and 3.5 U of maleylacetate reductase (MacA) purified from *E. coli*. After 5 min, an additional 0.8 mM NADH was added. In samples taken from this mixture after 1, 3, 5, 7, and 10 min, the reaction was stopped by addition of equal volumes of solvent for high-performance liquid chromatography (HPLC) and subsequent centrifugation. The substrate consumption and product formation were analyzed by reversed-phase HPLC under conditions described previously (37). The NADH amounts used for the reduction of maleylacetate and 2-chloromaleylacetate, respectively, were determined by recording the absorption at 340 nm over 30 min of a reaction mixture (1 ml, 37°C) containing 50 mM Tris-HCl (pH 7.5), 0.2 mM NADH, 0.08 mM maleylacetate or 2-chloromaleylacetate, and 3.5 U of maleylacetate reductase (MacA) purified from *E. coli*.

**Nucleotide sequence accession number.** The sequence data reported in this article will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession no. AF030176.

## RESULTS AND DISCUSSION

**The cloned maleylacetate reductase MacA is distantly related to its proteobacterial counterparts but an efficient catalyst of 2-chloromaleylacetate conversion.** With primers NH2-3 and HOM, which were designed assuming a considerable sequence similarity between proteobacterial and rhodococcal maleylacetate reductases, it was possible to amplify, from genomic DNA of *R. opacus* 1CP as template, a fragment with the expected size of ca. 980 bp. Labeling of this fragment and its use as a probe allowed the isolation of three recombinant plasmids, pMARRE1, pMARRE2, and pMARRE3 (Table 1; Fig. 1). Sequence analysis of the inserts of pMARRE1 and -3 revealed four complete open reading frames, designated *macA*, ORF3, ORF4, and ORF6, as well as one incomplete open reading frame, designated ORF1' (Fig. 1). In addition, we detected two regions with homology to entries in the database which comprised parts of different open reading frames

and which were thus designated "ORF2" and "ORF7," respectively.

The 1,074-bp maleylacetate reductase gene *macA* (for maleylacetate conversion) starts at position 3311 of our sequence, is preceded by a putative ribosome binding site, and encodes a protein of 357 amino acids with a calculated molecular mass of 37,798 Da, which is similar to the molecular masses of previously purified maleylacetate reductases (23, 37). The predicted gene product shows significant similarities with known maleylacetate reductases: in the alignment in Fig. 2, MacA shares 43.5% identical positions with ClcE of *Pseudomonas* sp. strain B13, 42.6% with TcbF of pP51, 42.1% with TfdF of pJP4, 42.6% with TfdF<sub>II</sub> of pJP4, and 44.3% with TftE of *Burkholderia cepacia* AC1100. With each other, the known proteobacterial maleylacetate reductases share somewhat higher ratios of identical positions (45.8 to 59.4%). Corresponding to the homology of maleylacetate reductases to type III (iron-containing) alcohol dehydrogenases (46), signature sequences of this protein family could also be detected in MacA of *R. opacus* 1CP (Fig. 2). However, the sequence similarity to various type III alcohol dehydrogenases tested, i.e., less than 30% identical residues, was considerably lower than to the other maleylacetate reductases.

After subcloning of *macA* into the expression vector pET11a\*, yielding pMARRE10, induction by IPTG resulted in an extract with a specific maleylacetate reductase activity of 0.72 U/mg. By comparison, the same strain, but with just the vector, under inducing conditions showed an activity of less than 0.02 U/mg. Thus, although according to SDS-electrophoretic analysis most of the MacA protein occurred in an insoluble form (even after some optimization of the inducing conditions), this activity proves that a functional gene had been cloned.

Cell extracts prepared from the overexpressing *E. coli* cells after induction with IPTG were used for the purification of MacA. The enzyme turned out to be very difficult to purify (the Red and the Blue Sepharose steps being especially detrimental), and in eight steps only 13-fold purification with a 0.07% yield was achieved. The final preparation on an SDS-polyacrylamide gel showed one major band of ca. 38 kDa, in agreement with the mass predicted for MacA.

The purified MacA preparation converted maleylacetate and 2-chloromaleylacetate with similar apparent  $K_m$  values (100 and 120  $\mu$ M, respectively) and with similar  $k_{cat}$  values (1,210 and 718  $\text{min}^{-1}$ , respectively). Since the MacA preparation might have contained inactivated protein, the absolute kinetic constants of a fully active protein might differ from those determined. However, the relative values for the two substrates are less likely to be influenced by inactivation. During conversion of 2-chloromaleylacetate, maleylacetate was detected by HPLC as an intermediate. In addition, the NADH consumption with 2-chloromaleylacetate was twice as high as with maleylacetate. These results showed that MacA of *R. opacus*, like the maleylacetate reductases of *Pseudomonas* sp. strain B13 (ClcE) (22), *Pseudomonas aeruginosa* RHO1 (28), and *R. eutropha* JMP134 (TfdF<sub>II</sub>) (37), is able not only to convert 2-chloromaleylacetate with a relatively high turnover rate and affinity but also to bring about a reductive dehalogenation of 2-chloromaleylacetate to maleylacetate. Thus, it appears that the dehalogenation is catalyzed by an enzyme which is (i) relatively distant from other maleylacetate reductases and (ii) apparently not part of a gene cluster dedicated to chlorocatechol conversion (see below). These findings support the hypothesis that no special adaptation of maleylacetate reductases is necessary to effect dehalogenation, but that halide elimination either is the result of hydride addition in the normal reaction mechanism (24), which is a reaction gratuitously



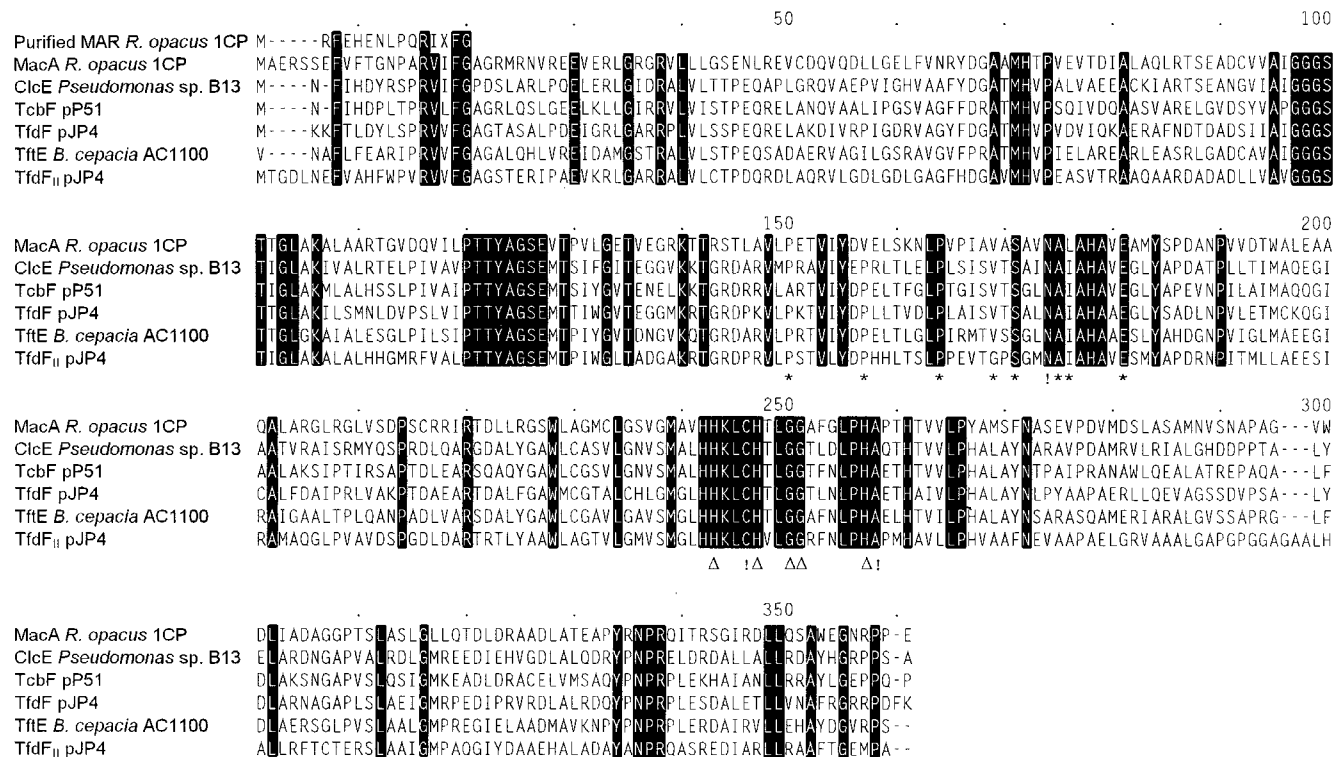


FIG. 2. Multiple sequence alignment of various maleylacetate reductases as calculated by the CLUSTAL program. Amino acids identical in all sequences are highlighted. Numbers above the sequences refer to positions in the alignment, not in a single sequence. Asterisks indicate one pattern typical for type III (iron-containing) alcohol dehydrogenases, [AP]-x(6)-[STAP]-x(5,6)-P-x(4)-[AIV]-x-[GST]-x(2)-D-[AI]-[LIVM]-x(4)-E (Prosite entry PDOC00059; last update, November 1995). A second pattern ( $\Delta$ ) typical for this enzyme family could be important for binding ferrous iron: H-x(2)-[SAE]-[HY]-x(2)-[SGA](2)-x(5)-H-G. Interesting differences from these patterns are marked by exclamation marks. Accession numbers and references for the published sequences (order as in the alignment): AF019038 (21), M57629 (45), M35097 (29), U19883 (8), U16782 (44). MAR, maleylacetate reductase.

catalyzed by the enzyme, or is not enzyme catalyzed at all but instead is the result of a spontaneous decomposition of the initial reaction product.

**Indications for the occurrence of different maleylacetate reductases in *R. opacus* 1CP.** A shoulder in the elution profile during anion-exchange chromatography of cell extracts had previously indicated that there might be more than one maleylacetate reductase induced in 4-chlorophenol-grown cells of *R. opacus* 1CP (27). To investigate whether the protein encoded by *macA* is in fact induced during growth of strain 1CP with 4-chlorophenol, we attempted to purify the maleylacetate reductases present under these conditions. However, the enzyme responsible for the shoulder or second activity peak during anion-exchange chromatography on a Q Sepharose High Performance column appeared to be unstable under the conditions used and could not be isolated. Only the enzyme responsible for the first peak could be further enriched by using a Blue Sepharose column. Active fractions from this chromatography on an SDS-polyacrylamide gel had a major band in a position corresponding to the molecular mass of other maleylacetate reductases. This band after excision from a Western blot was subjected to N-terminal amino acid sequencing and yielded the sequence MRFEHENLPQRIXFG. As shown in Fig. 2, on the one hand this sequence is clearly distinct from the predicted MacA sequence, but on the other hand four of the five amino acids conserved among the other maleylacetate reductases were also present in the partially purified maleylacetate reductase, thus proving that a maleylacetate reductase, not a contaminating protein, had been sequenced. The result that the maleylacetate reductase (partially) purified from 4-

chlorophenol-grown cells of *R. opacus* 1CP has an N-terminal sequence distinct from that of the predicted MacA supports the hypothesis, derived from anion-exchange chromatography, that strain 1CP has at least two different maleylacetate reductases. Whether, however, MacA is in fact the enzyme which gave rise to the second activity peak or shoulder during the chromatography or whether MacA represents yet a third iso-enzyme, not induced during growth with 4-chlorophenol, is not clear.

**MacA is a maleylacetate reductase not encoded by a major gene cluster specialized on catabolism of chloroaromatic compounds.** Sequence characterization of the cloned fragment (Fig. 1) revealed most upstream of *macA* an incomplete open reading frame (ORF1') and a region comprising several partial reading frames ("ORF2"), the products of which have significant sequence similarity to transposition-related proteins of IS117 or of IS1237 and IS402, respectively. ORF3, which might code for a protein homologous to flavin adenine dinucleotide synthetase, appears to be transcribed divergently from *macA* and thus not part of the same operon. This conclusion may also be drawn from ORF6, since it is relatively distant from *macA* (666 bp) and seems to code for a transcription factor-related protein, as well as for a downstream region ("ORF7") which, when translated in different frames, shows sequence similarity to *p*-aminobenzoate synthetases.

Thus, ORF4 (positions 2455 to 3264 of our sequence) is the only one which is likely to be cotranscribed with *macA*: it is relatively close (46 bp upstream) and apparently transcribed in the same direction. By comparison of ORF4 to database entries, the predicted gene product was shown to share significant

similarity with members of the family of short-chain (pyridine nucleotide-linked) dehydrogenases/reductases (19). Among the proteins with relatively high sequence similarity (ca. 28 to 33% identical positions) were 3-oxoacyl-(acyl carrier protein) reductases, tropinone reductases of solanaceous plants, hydroxysteroid dehydrogenases, and *cis*-1,2-dihydroxy-3,4-cyclohexadiene-1-carboxylate dehydrogenases. The functional divergence of these oxidoreductases as well as their limited sequence similarity to the ORF4 product did not allow any clear-cut prediction with respect to the actual function of the latter. If ORF4 is, in fact, cotranscribed with *macA*, one might assume its gene product to be an oxidoreductase which either forms or further converts maleylacetate. The *pnpD* gene of the maleylacetate forming 4-hydroxymuconic semialdehyde dehydrogenase (40) has recently been sequenced from 4-nitrophenol-utilizing *Pseudomonas fluorescens* ENV2030 and was shown to code for a protein of 494 amino acids with sequence similarity to aldehyde dehydrogenases (4). Since the predicted ORF4 product is only 269 amino acids long and related to different enzymes, it is unlikely to be similar to PnpD.

Whatever the function of the product of ORF4 turns out to be, *macA*, unlike other known maleylacetate reductase genes, appears not to be part of a specialized gene cluster encoding all enzymes between two major catabolic hubs, like chlorocatechols and 3-oxoadipate. Instead, the presumed ORF4-*macA* gene cluster could be assumed to code for only a small part of a major pathway or to have some auxiliary function. A similar situation may be found for maleylacetate reductase genes located on proteobacterial chromosomes (36).

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