Cloning and Expression of the *Acinetobacter calcoaceticus* Mutarotase Gene in *Escherichia coli*

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This article describes the cloning of the mutarotase gene from *Acinetobacter calcoaceticus* and its expression in *Escherichia coli*. Purification of mutarotase (EC 5.1.3.3) led to a single polypeptide of 40 kilodaltons. The sequences of 27 N-terminal and 76 C-terminal amino acids were determined. From six amino acids of the N-terminal and seven amino acids of the C-terminal portion of the protein, the sequences of two oligonucleotides were deduced. These were synthesized and used as gene probes. Completely restricted chromosomal DNA from *A. calcoaceticus* was size fractionated, and only fractions hybridizing with the gene probes were used to construct gene banks enriched for the mutarotase determinant. With the N-terminal gene probe, a bank of 6- to 7-kilobase-pair *Bcl*I fragments in pBR327 was obtained. A total of 1,200 candidates were screened by colony hybridization followed by dot-blot analysis of purified plasmids from positive candidates and subsequent Southern blot analysis of the respective restricted plasmids, and 500 base pairs (bp) from the 5' end of the mutarotase gene were isolated by this procedure. The 3' portion of the gene was isolated from a gene bank containing 1,500-bp-long *Hind*III fragments inserted in M13mp11. This bank was screened by dot-blot analysis of single-stranded phage DNA with the C-terminal gene probe. The isolated gene fragments were fused at a common restriction site in their overlapping region to yield the complete mutarotase gene. High-level expression of mutarotase in *E. coli* was achieved when the gene was placed under transcriptional control of the phage lambda promoter *p_L*. More than 90% of the mutarotase activity was found in the culture medium. The *E. coli*-derived mutarotase was purified and shown to be identical to the *A. calcoaceticus*-derived product with respect to the molecular weight and N-terminal amino acid sequence. The expression of mutarotase in *E. coli* was increased 200-fold in comparison to that in the wild-type *A. calcoaceticus*.

Mutarotase (aldose-1-epimerase, EC 5.1.3.3) catalyzes the anomeric interconversion of D-glucose and other aldoses (10). It has been identified in various species, including mammals, plants, fungi, and bacteria (20, 21, 28, 36). High concentrations of mutarotase were found in the kidney, liver, and small intestine of mammals (24, 37). Little knowledge exists regarding its function in vivo. It is speculated that mutarotase is important for active transport of D-glucose across membranes (1, 11).

Mutarotase is frequently used as an accelerator of mutarotation for quantitative assays of D-glucose in clinical and laboratory applications (2, 19, 22, 23). The sources of mutarotase for these purposes were usually hog or bovine kidneys (20, 36). In a screening program of microorganisms, *Acinetobacter calcoaceticus*-encoded mutarotase was found to have higher stability and a greater affinity for D-glucose than the mutarotase from other sources. However, the poor expression of mutarotase in *A. calcoaceticus* was not sufficient for production of the enzyme on a technical scale. To increase its yield, we decided to clone the gene encoding mutarotase (*mro*) from *A. calcoaceticus* and study its expression under transcriptional control of a strong promoter in *Escherichia coli*.

Since no screening method for mutarotase expression based on selection or complementation was possible, we isolated the gene by using synthetic oligonucleotides as gene

probes. This article describes the complete scheme for cloning the *mro* gene. It starts with the first purification of this mutarotase to homogeneity. Then the partial amino acid sequence of the enzyme was determined, leading to the design and synthesis of oligodeoxyribonucleotides as gene probes. With these probes, the *mro* gene was isolated and expressed in *E. coli*. Finally, the *E. coli*-derived mutarotase was purified and shown to be identical with that obtained from *A. calcoaceticus*.

MATERIALS AND METHODS

Bacterial strains, phages, and plasmids. *A. calcoaceticus* DSM30007 was obtained from W. Ebeling, Merck, Darmstadt. *E. coli* R/R1 was generally used as a host for transformations (5). *E. coli* K-12 ΔH1 Δ*trp* is M72 Str^r *lacZ*(Am), Δ(*bio uvrB*) Δ*trpEA2* (λ Nam7 Nam53 *cI857* ΔH1) (4) and was used for expression of mutarotase under transcriptional control of the lambda promoter *p_L*. *E. coli* K-12 BMH71-18 (17, 18) was used for infection with M13 derivatives. *E. coli* WH1372 is *E. coli* K-12 ΔH1 Δ*trp* transformed with pWH1372 (see Results). M13mp11 (18) was used to construct the gene bank of 1,500-base-pair (bp) *Hind*III fragments (see Results). pBR327 (31) and pBR322 (35) were used for cloning, and pLc236 (26) was used to fuse the *mro* gene to the phage lambda promoter *p_L*.

General methods. Restriction endonucleases were purchased from Boehringer Mannheim, PL Biochemicals, or Bethesda Research Laboratories. T4 DNA ligase, T4 polynucleotide kinase, calf intestine phosphatase, and *E. coli* DNA polymerase (Klenow fragment) were from Boehringer Mannheim. All reactions were done as described previously

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(8). Polyacrylamide and agarose gel electrophoresis (8) and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was done as described previously (14). Analysis of native mutarotase on a polyacrylamide gel was carried out as described previously (25). DNA sequence determination was done by the methods of Maxam and Gilbert for plasmid DNA (16) and of Sanger et al. for M13 derivatives (29). The respective sequencing kits and radioactive chemicals were purchased from Amersham, Braunschweig. The N-terminal amino acid sequence determination of mutarotase was done in the liquid phase, and mutarotase-derived peptides were sequenced by using a solid-phase amino acid sequencer from LKB exactly as described (34). Peptides were coupled at their C terminus with 1-ethyl-3-(3-diethylaminopropyl)carbodiimide hydrochloride to 3-amino-propyl glass (27). Trypsin was from Merck, Darmstadt, and was purified prior to use as described (34). Endoproteinase ArgC was from Boehringer. All other chemicals were from Merck.

Purification of mutarotase from *A. calcoaceticus*. *A. calcoaceticus* DSM30007 was grown for 10 h at 34°C in 100 liters of broth containing 0.5% tryptone, 1% yeast extract, 0.3% cornsteep, 0.8% dipotassium hydrogen phosphate, 0.04% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 1.2% glucose at pH 6.8, which had been inoculated with 1 liter of an 18-h culture. The mutarotase activity was 340 U/liter of culture at that time. The cells were sedimented, suspended in 60 liters of 100 mM KCl, and ruptured at 500 bar in a high-pressure homogenator under continuous cooling. The mixture was centrifuged for 3 h at 2,700 rpm, and the supernatant was made 20% in polyethylene glycol (PEG). After incubation for 30 min at 4°C, the mixture was recentrifuged and the supernatant was dialyzed against water until the conductivity was 3×10^{-3} A/V. Then, 15 g of dry carboxymethyl (CM)-Sephadex (Pharmacia, Uppsala, Sweden) was stirred into this solution and sedimented by centrifugation. The resin was washed repeatedly with 25 mM potassium phosphate, pH 5.5, until the supernatant was clear. The resin was then placed into a column, and washing was continued until the absorption at 260 and 280 nm dropped below 0.04. Then the mutarotase was eluted with 1 M KCl in the above buffer.

A 110-ml amount containing 2.42 g of protein and 34,800 U of mutarotase was loaded on a Sephadex G100 (Pharmacia) column (20 by 100 cm) and eluted with 20 mM Tris hydrochloride, pH 7.2, at 70 ml/h. The mutarotase activity eluted in the void volume. The fractions were analyzed by SDS-polyacrylamide gel electrophoresis and for mutarotase activity. Mutarotase-containing fractions were pooled, avoiding a 21-kilodalton (kDa) contaminating protein, which otherwise coeluted with the enzyme in the following steps.

A 360-ml amount of this solution was applied to a CM-Sephadex column (2 by 20 cm) preequilibrated with 20 mM potassium phosphate, pH 9.0. The column was washed with 100 ml of the above buffer and then eluted with a gradient of 350 ml of 20 mM to 170 mM potassium phosphate, pH 9.0, at a flow rate of 20 ml/h. Mutarotase activity eluted at 100 mM.

Mutarotase-containing fractions were combined, diluted fourfold with water, and applied to a hydroxylapatite column (4 by 10 cm, DNA grade; obtained from BioRad Laboratories) which had been preequilibrated with 20 mM potassium phosphate, pH 9.0. The column was washed with three volumes of that buffer and eluted with 300 ml of a gradient from 20 to 300 mM potassium phosphate, pH 9.0, at a flow rate of 9.2 ml/h. Mutarotase eluted at 85 mM.

Fragmentation of mutarotase for partial amino acid sequencing. Purified mutarotase (30 mg) was desalted by chromatography on a Biogel P10 column (2.5 by 25 cm) in

70% acetic acid. Protein-containing fractions were pooled and dried in vacuo.

Cleavage with cyanogen bromide was performed as described previously (7). Mutarotase (15 mg) was cleaved in the dark for 7 h at ambient temperature, followed by evaporation in vacuo. The reaction products were dissolved in 3 ml of 70% acetic acid and separated by chromatography on a Biogel P10 column (2.5 by 50 cm) in 70% acetic acid. The cyanogen bromide cleavage of mutarotase yielded five peptides, named CB1 through CB5 according to their position in the primary structure of the protein. The separation on P10 yielded a fraction containing uncleaved mutarotase and CB2, which was rechromatographed on Biogel P60 (2.5 by 50 cm) under identical conditions. Another fraction contained a mixture of CB3 and CB5, which were separated by chromatography on Biogel P30 (2.5 by 50 cm). Due to its small size, CB4 was not detected in this procedure.

To gain more sequence information from the C terminus of mutarotase, CB5 was subfragmented by using endoproteinase ArgC. For that purpose, 2 mg of CB5 was dissolved in 1 ml of 1 M ammonium bicarbonate and digested with 20 μg of endoproteinase ArgC for 2 h at 37°C. This procedure was repeated once. The reaction products were dried in vacuo, dissolved in 2 ml of 90% formic acid, diluted with 1 ml of water, and separated on a Biogel P10 column (2.5 by 100 cm), which was eluted with 70% formic acid in water. This yielded three peptides, called ARG1 through ARG3 according to their occurrence in CB5.

For cleavage with purified trypsin, 2 mg of desalted mutarotase was dissolved in 1 ml of 1 M ammonium bicarbonate, pH 8.8, containing 0.04% SDS. Trypsin (20 μg) was added, and the mixture was incubated at 37°C for 2 h. Addition of trypsin and incubation was repeated three more times. Separation of the tryptic peptides was achieved by high-pressure liquid chromatography (HPLC) of 100- μl portions from the reaction mixture on a Nucleosil 5 C₁₈ column (obtained from Macherey und Nagel, Düren), which was eluted with a gradient from 0.05% trifluoroacetic acid in water to 0.025% trifluoroacetic acid in 50% acetonitrile over 50 min at 50°C with a flow rate of 1.6 ml/min. For sequencing, the peptides from 7 to 10 runs were collected and combined.

Synthesis of oligodeoxynucleotides. The oligodeoxynucleotides were synthesized by the phosphoramidite method essentially as described previously (3, 15) and purified by HPLC. After the 5' ends were labeled with [γ -³²P]ATP (9), the phosphorylated oligonucleotides were separated from the nonphosphorylated ones by denaturing polyacrylamide gel electrophoresis (20% acrylamide, 0.8% bisacrylamide, 8 M urea, 60 mM Tris borate, 1 mM EDTA, pH 8.3) at 1,600 V for 6 h on 40-cm gels which were 1 mm thick. The phosphorylated probe was located by autoradiography and eluted from the gel as described previously (16).

Construction of gene banks. *A. calcoaceticus* cells (5 g) were suspended in 50 ml of 50 mM NaCl–5 mM EDTA–30 mM Tris hydrochloride (pH 7.9) containing 200 mg of lysozyme and incubated at 37°C for 30 min. The mixture was then made 1% (wt/vol) in SDS, 5 mg of proteinase K was added, and incubation at 37°C was continued for 30 min, followed by six phenol extractions, four extractions with chloroform-isoamyl alcohol (20:1, vol/vol), and ethanol precipitation. The pellet was dried and suspended in 5 ml of 10 mM Tris hydrochloride (pH 8.0)–0.1 mM EDTA. RNase A (50 μg ; previously heated to 100°C for 10 min in 0.1 M sodium acetate, pH 5.5) was added, and the mixture was incubated for 30 min at 37°C. Then 250 μg of proteinase K

was added, and incubation was continued for 30 min, followed by six phenol extractions and three ether extractions. The DNA was then precipitated by adding 1/2 volume of 30% PEG in 1.5 M NaCl and incubating it on ice for 30 min, followed by centrifugation. The pellet was rinsed with ethanol, dried in vacuo, and suspended in 2 ml of 10 mM Tris hydrochloride (pH 8.0)–0.1 mM EDTA. This procedure yielded 2 mg of DNA.

DNA (1 mg) was digested twice with 5,000 U of *Bcl*I for 5 h at 50°C. This restricted DNA (60 µg per 2-cm slot) was electrophoresed on a 3% polyacrylamide gel (0.8% bisacrylamide) at 80 V for 24 h and stained with ethidium bromide. Under these conditions a 3,850-bp marker fragment migrated to the bottom of the 20-cm gel. The gel matrix above this marker was cut into seven slices of 2 mm each, and the DNA from each slice was eluted as described previously (16), except that the gel matrix was crushed by pressing it through a 0.5-mm syringe. Ten percent of the eluted DNA was spotted on nitrocellulose and hybridized with the N-terminal gene probe. The size range 6 to 7 kilobase pairs (kbp) gave the strongest signal and was used to construct the *mro*-enriched gene bank. Five percent of the eluted DNA was mixed with *Bam*HI-linearized pBR327, which had been dephosphorylated and eluted from a 3% polyacrylamide gel by the procedure given above. After ligation and transformation into *E. coli* R/RI, 3,000 ampicillin-resistant colonies were obtained, and 156 of these were screened for tetracycline sensitivity; 3 were tetracycline resistant. Twenty-two candidates were analyzed for plasmid size. All of them contained insertions of 6 to 7 kbp.

Essentially the same procedure was followed to construct a gene bank from the 1,500-bp size range of an *Hind*III digest, which hybridized with the C-terminal gene probe. These DNAs were inserted into the single *Hind*III site of M13mp11, resulting in 541 recombinant phages.

Hybridizations. For hybridization analysis, 1,200 colonies were grown overnight on nitrocellulose placed on a 12- by 24-cm agar plate containing 60 mg of ampicillin per liter. The nitrocellulose was removed from the agar and soaked with each of the following solutions: 250 mM HCl for 5 min; 500 mM NaOH for 5 min; 500 mM NaOH–1.5 M NaCl for 5 min; 1 M Tris hydrochloride (pH 7.5) for 2 min; 500 mM Tris hydrochloride (pH 7.5)–1.5 M NaCl for 4 min; and 6× SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate) for 5 min. The nitrocellulose was then wrapped in chromatography paper, baked for 2 h at 80°C in vacuo, and sealed in Saran Wrap. The nitrocellulose was incubated for 3 h at 65°C in 1× Denhardt solution (0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone 40, 0.02% Ficoll) with 5× SSC and 0.5% SDS (1 ml/40 cm²). The nitrocellulose was washed three times with 10 ml of 900 mM NaCl–90 mM Tris hydrochloride (pH 8.3)–6 mM EDTA–10× Denhardt solution–0.1% SDS and then hybridized in 5 ml of the same buffer containing 2.4×10^7 cpm of the N-terminal gene probe for 5 h at 40°C. Afterwards the nitrocellulose was washed twice with 100 ml 6× SSC for 15 min at 4°C and then for 8 min at 40°C, 1 min at 44°C, 2 min at 44°C, and finally 1 min at 46°C. After each of the last three washes an autoradiogram was taken. The best resolution was obtained after the last wash. Of 1,200 colonies screened that way, 25 were positive.

Dot-blot hybridizations were performed on gel-eluted DNA, single-stranded recombinant M13 DNA, and plasmid DNA from candidates of the colony hybridization. Gel-eluted DNA and plasmid DNA were heated to 90°C for 3 min, chilled on ice, spotted on nitrocellulose in 1-µl portions, and dried by aeration. Recombinant M13 phages were cul-

tured for 6 to 7 h in 1 ml of medium, the cells were spun down, and 400 µl of the supernatants was mixed with 100 µl of 20% PEG in 2.5 M NaCl, precipitated for 15 min on ice, and centrifuged. The pellets were suspended in 80 µl of 10 mM Tris hydrochloride (pH 8.0)–0.1 mM EDTA, and 5 µl of this solution was spotted on nitrocellulose in 1-µl portions and dried by aeration. The nitrocellulose was soaked in the solutions described above and then baked at 80°C for 2 h in vacuo. Prehybridization of these filters was done in 900 mM NaCl–90 mM Tris hydrochloride (pH 8.3)–6 mM EDTA–10× Denhardt solution–tRNA (50 µg/ml) (1 ml/40 cm²) for 3 h at 65°C. Then 2×10^6 cpm of the respective gene probe was added, and hybridization was performed for 5 h at 40°C. Afterwards the nitrocellulose was washed twice with 6× SSC for 15 min at 4°C, once for 1 min at 40°C, and once for 1 min at 44°C. Hybridization with pWH1318 was done as described previously (38).

Southern blot analysis was done with restricted chromosomal DNA from *A. calcoaceticus* and restricted plasmid DNA from *E. coli* candidates. The amounts were 7 µg of chromosomal and 1.5 µg of plasmid DNA. Transfer of the DNA to nitrocellulose was done as described previously (32), and the hybridization and washing conditions were as given for the dot-blot analysis above, except that the stringency for the hybridization of plasmid DNA was increased from 1 min at 44°C to 1 min at 52°C in 2°C steps, which were interrupted by autoradiography (see Results).

Assay of mutarotase activity. Mutarotase activity was assayed in *A. calcoaceticus*, in *E. coli*, in the culture medium, and in the various eluates during the purification procedures. The latter solutions were used without further processing. From cultures of bacteria, 1 ml was removed and the cells were spun down. The supernatant was tested for mutarotase activity in the medium. The pelleted cells were washed with 1 ml of 50 mM Tris hydrochloride (pH 7.5) and suspended in 1 ml of the same buffer. Cells were broken by sonication with a 4-mm horn at 100 W four times for 15 s each, interrupted by 45-s cooling periods in ice water. The cell debris was spun down for 15 min in a microfuge. The supernatant was incubated at 45°C for 5 min to inactivate NADH oxidases and then used to determine mutarotase activity without further purification.

The reaction mixture of the mutarotase assay contained 3 mM NAD, 37.5 U of glucose dehydrogenase (EC 1.1.1.47; Merck), and 100 mM Tris hydrochloride pH 7.2; 950 µl of this mixture was pipetted into both cuvettes of a dual-beam spectrophotometer. After the mutarotase-containing solution was added to the sample cuvette, the absorption at 366 nm was followed until it remained constant. Then, 50 µl of a freshly prepared α-D-glucose solution (15 mg/25 ml, giving 166 µM in the reaction mixture) was added to the sample and reference cuvettes. The increase in absorption was recorded for several minutes. The amount of mutarotase in the assay was adjusted to give an absorption change between 0.01 and 0.06 per min under these conditions. One unit of mutarotase is defined as the amount of enzyme converting 1 µmol of α-D-glucose to β-D-glucose at 22°C, pH 7.2, in 1 min in addition to the nonenzymatic rate.

RESULTS

Purification of mutarotase from *A. calcoaceticus*. The first step in the cloning scheme for the *mro* gene was the purification of mutarotase from *A. calcoaceticus* to homogeneity. This was accomplished by a protocol involving four separation steps. Fractionation of the total proteins from *A.*

FIG. 1. Polyacrylamide gel analysis of purified mutarotase. Lanes S and D show the result of denaturing polyacrylamide gel electrophoresis (14). Lane S shows molecular weight standards of 66K, 44K, 29K, and 14K. Lane D shows the resulting band when 30 μ g of purified mutarotase was loaded. The apparent molecular weight is 40K. Lane N shows the result with 30 μ g of purified mutarotase electrophoresed under native conditions on a polyacrylamide gel (25).

calcoaceticus was achieved by a batch procedure with CM-Sephadex. Purification was then done by successive column chromatography on three resins. From Sephadex G100 the mutarotase eluted in the void volume, indicating that the native enzyme is an oligomer. From CM-Sephadex the mutarotase eluted at 80 mM potassium phosphate. After this step the denaturing SDS gel analysis revealed the presence of two polypeptides of 40 and 33 kDa, respectively (not shown). These were separated by chromatography on hydroxylapatite, from which the mutarotase activity eluted at 85 mM potassium phosphate. Denaturing polyacrylamide gel electrophoresis indicated that the mutarotase was pure after this step and had an apparent molecular weight of 40,000 (40K) per subunit (Fig. 1). The purification of mutarotase is quantitatively summarized in Table 1. The final confirmation that mutarotase activity was associated with the 40-kDa polypeptide was achieved by electrophoresis of the enzyme on a nondenaturing polyacrylamide gel, where it also migrated as a single band (Fig. 1). Elution of the protein from this gel yielded mutarotase activity.

Partial amino acid sequence of the mutarotase. The general use of amino acid sequence information to devise gene probes is hampered by the degeneracy of the genetic code. It is therefore important to identify sequences consisting mostly of amino acids which are encoded by only one or two triplets. Therefore, the protein sequencing was only pursued to the point where suitable probes spanning the entire *mro* gene could be designed.

Figure 2 shows the strategy and results of the protein sequencing. The N-terminal amino acid sequence was determined by repetitive Edman degradation of mutarotase in the liquid phase. This gave the sequence of 24 amino acids and allowed the localization of the first two of the five cyanogen bromide fragments (see Materials and Methods). CB5 was identified as the C-terminal cyanogen bromide fragment by the lack of homoserine lactone in its amino acid composition. Repetitive Edman degradation of CB5 yielded the sequence of 42 amino acids.

To obtain more sequence information from the C-terminal portion, CB5 was cleaved with the endoprotease ArgC at the arginine positions, and the resulting oligopeptides were purified by gel exclusion chromatography (see Materials and Methods). Sequence analyses of these peptides revealed the C-terminal amino acid sequence of mutarotase (Fig. 2).

Mutarotase was also cleaved with trypsin, and the resulting oligopeptides were separated by HPLC (see Materials and Methods). The amino acid compositions of purified tryptic fragments were determined, and one of them, called F38, was sequenced because it contained two methionine residues. It overlapped with CB3, CB4, and CB5, yielding the order of the remaining two cyanogen bromide fragments (Fig. 2).

Inspection of that sequence information with respect to the design of gene probes revealed two suitable strings of amino acids. These are shown enlarged in Fig. 2. One of them was located in the N-terminal region and the other consisted of the C-terminal seven amino acids of mutarotase.

Synthesis of oligodeoxyribonucleotides as gene probes. Two mixtures of oligonucleotides were synthesized as gene probes. The mixture designated probe I was complementary to the nucleotide sequence encoding amino acids 12 to 17 of mutarotase (Fig. 2). This sequence was 64-fold degenerate. To limit the mixed probe to 16 oligonucleotide sequences, the Gly codon was represented by GGT. When two nucleotides were possible at wobble positions, both nucleotides were incorporated in the respective position. The resulting mixture of oligonucleotides is indicated in Fig. 2.

The same strategy was used to design a probe from the last seven amino acids of the mutarotase. This resulted in a mixed probe containing 16 oligonucleotides, called probe II, the sequences of which are also shown in Fig. 2.

Cloning of the *mro* gene from *A. calcoaceticus*. To reduce the number of candidates which had to be screened by colony hybridization, a gene bank enriched for the *mro* gene was constructed. Chromosomal DNA from *A. calcoaceticus* was digested with *Eco*RI, *Hind*III, and *Bcl*II. Southern blot analyses of these digests with probe I are shown in Fig. 3. Positive signals were obtained from the *Hind*III digest at 1,500 bp, the *Eco*RI digest at 2,000 bp, and the *Bcl*II digest at

TABLE 1. Purification of mutarotase from *A. calcoaceticus* and *E. coli* WH1372

Mutarotase	Purification step	Mutarotase activity (U)	Protein (mg)	Sp act (U/mg)	Yield ^a (%)
<i>A. calcoaceticus</i>	Batch CM-Sephadex	34,800	2,420	16.5	100
	G100	27,800	410	68	80
	CM-Sephadex	19,800	185	107	57
	Hydroxylapatite	15,600	67	233	45
<i>E. coli</i>	Culture broth	4,700	ND ^b	ND	ND
	(NH ₄) ₂ SO ₄ (90%)	4,600	69	68	90
	Dialysis	2,800	52	54	60
	CM-Sephadex	2,400	212	200	52

^a The overall yield varied between 52 and 75%. Highest yields were obtained when mutarotase was isolated 9 h after induction.

^b ND, Not determined.

FIG. 2. Partial amino acid sequence of mutarotase. The 27 amino acids (aa) determined from the N-terminal and 76 amino acids determined from the C-terminal portion of mutarotase are shown. The arrows indicate sequencing experiments beginning at the leftmost end towards the arrow. Mutarotase indicates the sequencing experiment from the N terminus of the complete enzyme. CB1, CB2, and CB5 indicate sequencing experiments with the respective cyanogen bromide fragments. F38 indicates an experiment with a tryptic peptide, and ARG1, ARG2, and ARG3 indicate the three peptides derived from CB5 by cleavage with the protease *ArgC*. The two regions of the sequence used for the deduction of gene probes are shown enlarged. The nucleotide sequences of the oligonucleotides contained in the mixed probes are indicated.

7,000 bp. The gene bank was constructed (see Materials and Methods) with the 7,000-bp size range of a *Bcl*I digest because (i) it gave a clear hybridization signal with probe I (Fig. 3), (ii) the large size of the DNA increased the probability of cloning the complete *mro* gene in one piece, and (iii) cloning of these fragments into the *Bam*HI site of pBR327 allowed the use of insertional inactivation of the tetracycline resistance gene to screen for recombinants (see Materials and Methods). Three thousand clones, with an insertion frequency of 98%, were obtained in this experiment, and 1,200 of these candidates were used for colony hybridization with probe I. This resulted in 25 positive candidates (not shown), which were picked, and the plasmids were purified and analyzed by dot-blot hybridization with the same probe. Ten of these 25 candidates remained positive in this screen (not shown). Southern blot analysis of *Hind*III and *Eco*RI digests of six candidates with probe I is shown in Fig. 4. At low stringency fragments from four of these six candidates

gave positive signals. Only the 1,500-bp *Hind*III and 2,000-bp *Eco*RI fragments of candidate 18 gave a signal at increased stringency (Fig. 3). This result indicated that the cloned DNA contained the fragments identified in the Southern blot analysis of chromosomal DNA digests shown in Fig. 3. The respective plasmid was called pWH1318 and restriction mapped. Analysis of the remaining four candidates from the dot-blot screen yielded another clone which was identical to pWH1318. The physical map of pWH1318 is shown in Fig. 5. The 1,500-bp *Hind*III fragment was subcloned into the *Hind*III site of pBR322 to facilitate further analysis. The resulting plasmid was called pWH1319 and is also shown in Fig. 5. A partial nucleotide sequence analysis of this DNA revealed an open reading frame coding for an amino acid sequence identical to the one determined by protein sequencing of the purified mutarotase. Unfortunately pWH1318 did not contain the entire *mro* gene but only the N-terminal 500 bp.

FIG. 3. Southern blot analysis of restricted chromosomal DNA from *A. calcoaceticus*. The left side shows an agarose gel of chromosomal DNA from *A. calcoaceticus* digested with *Bcl*I (B), *Hind*III (H), and *Eco*RI (E). Lane S contains radioactivity labeled molecular weight markers, with the sizes indicated on the left (in base pairs). The right side shows the result of the Southern blot analysis. The arrows on the right side indicate the size (in base pairs) of the fragments hybridizing with the N-terminal gene probe. A signal in the 7,000-bp size range was detected with the *Bcl*I digest (B), the 2,000-bp size range gave a signal in the *Eco*RI digest (E), and a signal at 1,500 bp was obtained with the *Hind*III (H) digest.

A similar approach was taken to isolate the C-terminal part of the *mro* gene with probe II. The analysis of Southern blots of restricted *A. calcoaceticus* DNA with probe II revealed C-terminal *mro* sequences in the 1,500-bp range of *Hind*III digest (data not shown). If this *Hind*III fragment is located directly adjacent to the *Hind*III fragment containing the N-terminal part of the gene, it must overlap the already cloned part of the *mro* gene in pWH1318 between the *Hind*III and *Bcl*I-*Bam*HI fusion sites (Fig. 5). In addition, they should share the *Eco*RI site located in this region. The 1,500-bp size range of an *Hind*III digest of *A. calcoaceticus* DNA was cloned into the *Hind*III site of M13mp11. Single-stranded phage DNA from 541 candidates was screened by hybridization with probe II and pWH1318 in a dot-blot analysis. Two candidates gave positive signals with both probes. The replicative form of these M13 derivatives was prepared and restriction mapped. They turned out to be identical and were called pWH1301. Their physical map and relevant restriction sites are also depicted in Fig. 5. pWH1301 contained all of the missing C-terminal *mro* gene sequence, as judged by two criteria: (i) pWH1301 and pWH1318 shared a 90-bp *Hind*III-*Eco*RI fragment in their overlapping sequence (see Fig. 5); (ii) the nucleotide sequence of respective parts of pWH1301 showed an open reading frame which agreed with the primary structure of mutarotase purified from *A. calcoaceticus*.

The N- and C-terminal parts of the *mro* gene were joined at their common *Hind*III site, and the complete gene was fused to the phage lambda promoter p_L . For that purpose pWH1319 was digested with *Sca*I and *Hind*III, and the 574-bp fragment containing the N-terminal part of the *mro* gene was isolated. It was inserted into pPLc236 which had been digested with *Eco*RI, filled in, and redigested with *Hind*III. The resulting plasmid, called pWH1307, was digested with *Hind*III and *Sal*I and ligated with the gel-eluted *Hind*III-*Sal*I fragment containing the C-terminal part of the *mro* gene from pWH1301 to yield pWH1372. The physical map of pWH1372 is also shown in Fig. 5. It contained the *mro* gene from *A. calcoaceticus* under transcriptional control of the phage lambda promoter p_L .

Expression of the *A. calcoaceticus*-derived mutarotase in *E. coli*. For controlled expression of the *mro* gene, pWH1372 was transformed into *E. coli* K-12 Δtrp $\Delta H1$ containing the *cI857* mutation. This renders the *cI* repressor, which controls transcription from the p_L promoter, active at 30°C and inactive at 40°C (4). Thus, expression of the *mro* gene can be induced by elevating the temperature of the culture from 30 to 40°C. Figure 6 shows a typical time course of the expression of mutarotase in *E. coli*. After induction the cells continued to grow for two more doublings and then entered the stationary phase. Afterwards the A_{650} of the culture remained constant for at least 16 h. During the first 3 h after induction the mutarotase activity was expressed at a low

FIG. 4. Southern blot analysis of six candidates. The upper part shows the agarose gel electrophoresis of plasmids prepared from six candidates. For this analysis each plasmid was digested with *Eco*RI (lanes E) or *Hind*III (lanes H). Lane S contains a standard of DNA fragments labeled with ^{32} P at their 5' ends. The respective molecular sizes are given on the left (in base pairs). The middle panel shows an autoradiograph of a Southern analysis with probe I for hybridization. The washing was done 4°C below the calculated denaturing temperature for 1 min. The lower panel shows the result after washing at 4°C above the calculated denaturing temperature for 1 min. Only candidate 18 showed a signal with probe I under these stringent conditions. The molecular sizes of the hybridizing bands were 1,500 bp for the *Hind*III and 2,000 bp for the *Eco*RI fragment.

FIG. 5. Physical maps of plasmids containing *mro* gene DNA. pWH1318 is a pBR327 derivative. The vector portion is denoted by the thin line. The *A. calcoaceticus*-derived insertion is drawn as a thick line. *n-mro* indicates the location of the 5' portion of the *mro* gene, and *bla* indicates the location of the β -lactamase gene. pWH1319 contains the *Hind*III fragment (heavy line) of pWH1318 cloned into the *Hind*III site of pBR322 (thin line). pWH1301 is an M13mp11 derivative containing a 1,500-bp *A. calcoaceticus*-derived *Hind*III fragment carrying the 3' portion of the *mro* gene (*c-mro*). *lacZ* indicates the N-terminal portion of the *lacZ* gene. pWH1372 is a derivative of pPLc236 (thin line) containing the complete *mro* gene (open arrow designated *mro*) flanked by *A. calcoaceticus* DNA (heavy line). The triangle indicates the location and direction of the promoter p_L from bacteriophage lambda. The approximate size of each plasmid is shown. Abbreviations for restriction sites: B, *Bam*HI; B', *Bcl*I; E, *Eco*RI; H, *Hind*III; N, *Nde*I; P, *Pst*I; S, *Sal*I; S', *Sca*I. ES' is a fusion of a filled-in *Eco*RI site to a *Sca*I site, and BB' denotes fusion sites of *Bam*HI-*Bcl*I ends.

level and remained cell associated. During the next 6 h leaking of mutarotase activity into the culture medium occurred, and the total expression of mutarotase was increased 10-fold. The cell-associated activity reached a maximum at 6 h after induction, whereas the activity located in the medium increased sharply until 9 h after induction. After that time the mutarotase activity in the medium remained constant for at least another 7 h. We conclude that the enzyme is stable under continuous shaking and aeration at 40°C. The total amount of mutarotase synthesized by *E. coli* WH1372 was between 5,000 and 6,000 U (20 mg) per liter of culture. This is approximately a 200-fold increase over the expression in the wild-type *A. calcoaceticus* strain under the same conditions.

Isolation and analysis of mutarotase expressed in *E. coli*. The location of the mutarotase in the culture medium of *E. coli* WH1372 simplified the purification procedure considerably over the procedure necessary for the *A. calcoaceticus*-derived product. It involved only ammonium sulfate precipitation followed by dialysis and a single chromatography on CM-Sephadex. The respective data are also summarized in Table 1. The overall yield varied between 50 and 70%. The best yields were obtained when the preparation was started immediately after the expression of mutarotase had reached

its maximum. The apparent molecular weight of the product expressed in *E. coli* was indistinguishable from that of the product derived from *A. calcoaceticus*. The N-terminal amino acid sequence was determined by repetitive Edman degradation. It was Ala-Thr-Leu-Asn-Val-Lys-Ser-Tyr-Gly. Except for serine at position 7, this sequence was identical to the one determined for the mutarotase purified from *A. calcoaceticus* (Fig. 2). The partial nucleotide sequence analysis of the cloned gene revealed that serine was encoded by TCA. We therefore assume that the proline codon CCA was mutated to the serine codon TCA during the cloning procedures. The identity of both enzymes was confirmed by the following results: (i) they had the same specific activity (Table 1), (ii) they had the same apparent molecular weight (40K), (iii) they eluted at identical ionic strength from CM-Sephadex, (iv) they had identical amino acid compositions except for serine and proline (data not shown), and (v) their N-terminal amino acid sequence was identical except at position 7. These results provided the final evidence that the complete *mro* gene from *A. calcoaceticus* had been cloned.

DISCUSSION

The starting position for cloning of the *mro* gene was the observation that *A. calcoaceticus* contained mutarotase ac-

FIG. 6. Expression and localization of mutarotase in *E. coli*. The upper part of the figure shows the growth curve of *E. coli* WH1372. The arrow indicates the induction of transcription from phage lambda promoter p_L . The lower part shows the expression of mutarotase given as activity versus time. The solid line indicates mutarotase activity pelleted with the cell fraction, and the dashed line indicates mutarotase activity found in the culture medium.

tivity. The first step was to purify the enzyme to homogeneity. This was facilitated by the stability and the basic nature of the protein. Even at pH 9.0 it bound to CM-Sephadex, permitting separation in a batch procedure from the bulk of the proteins. Cloning of the *mro* gene could then be approached either in an expression system with antibodies for screening (13) or after partial sequencing of the protein with synthetic oligonucleotides for screening a gene bank (12). Both routes were pursued; however, while the antibodies were still being prepared, the alternate approach had yielded the sequence of a total of 103 amino acids containing regions which were suitable for the design of gene probes. The probes hybridized specifically to size fractions of restricted *A. calcoaceticus* DNA. Only these narrow size ranges of restricted DNA were used to construct the gene banks. When eluted from 3% polyacrylamide gels, about 1 μ g of DNA in the 7-kbp size range gave 6×10^4 recombinants (see Materials and Methods). In our hands this was much superior to the results obtained after elution of the DNA from agarose gels.

The frequency of the *mro* gene in a bank of 7-kbp fragments from *A. calcoaceticus* should be 1 in approximately 1,400 clones if the genome size is about 3×10^6 bp. Estimating that about 5% of the total *A. calcoaceticus* DNA is in the 7-kbp size range of the *Bcl*I digest, the *mro* gene frequency in the enriched bank should be 1 in 70 clones. The observed frequency was significantly smaller, 1 in about 600 clones. Since it was ascertained that the *Bcl*I digest of total *A. calcoaceticus* DNA was complete (data not shown), it was clear that the *Bcl*I fragment with the 5' part of the *mro* gene was underrepresented in the gene bank. No instability,

however, was noticed for pWH1372 containing the complete *mro* gene. Therefore, this effect must be due to portions of the DNA flanking the 5' side of the *mro* gene in pWH1318. The frequency of 1 in 270 candidates found for the *Hind*III fragment containing the C-terminal portion of the *mro* gene was deduced from the statistical approximation. Screening a total of 1,750 candidates resulted in the independent isolation of two copies of the complete *mro* gene. Thus, the complete *mro* gene was isolated by screening a very low number of candidates. Even considering misfortunes such as the underrepresentation of the N-terminal *mro* sequence discussed above or the cutting of the gene into two pieces by the restriction enzyme, the approach of constructing enriched gene banks seems to be an advantage over working with complete gene banks.

With the complete *mro* gene fused to the promoter p_L from coliphage lambda, the mutarotase activity was expressed in *E. coli* WH1372 at a 200-fold-higher level than from the wild-type gene in *A. calcoaceticus*. Furthermore, the mutarotase was found in the culture medium of *E. coli* WH1372. The procedure for purifying mutarotase from the broth is reduced to a single chromatography step on CM-Sephadex after ammonium sulfate precipitation. Both effects are improvements for the technical production of the enzyme.

Specific export of proteins into the culture medium has only been found for hemolysin and two other endotoxins in *E. coli* (6, 30, 33). A detailed analysis of mutarotase export will have to distinguish between specific secretion and nonspecific leakage.

Mutarotases from mammalian sources have been studied frequently in the past (21, 24). The ones isolated from hog kidney and bovine kidney have molecular weights of 41K and 36K, respectively (36). While the molecular weight of the *A. calcoaceticus*-encoded enzyme is similar, 40K, it remains to be elucidated whether other common features are shared by mutarotases from different sources.

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