JOURNAL OF BACTERIOLOGY, Aug. 1993, p. 4729–4737
0021-9193/93/154729-09$02.00/0

Vol. 175, No. 15

The *aroQ*-Encoded Monofunctional Chorismate Mutase (CM-F) Protein Is a Periplasmic Enzyme in *Erwinia herbicola*†

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Received 28 January 1993/Accepted 3 May 1993

Enteric bacteria possess two species of chorismate mutase which exist as catalytic domains on the amino termini of the bifunctional PheA and TyrA proteins. In addition, some of these organisms possess a third chorismate mutase, CM-F, which exists as a small monofunctional protein. The CM-F gene (denoted *aroQ*) from *Erwinia herbicola* was cloned and sequenced for the first time. A strategy for selection by functional complementation in a chorismate mutase-free *Escherichia coli* background was devised by using a recombinant plasmid derivative of pUC18 carrying a *Zymomonas mobilis tyrC* insert which encodes cyclohexadienyl dehydrogenase. The *aroQ* gene is 543 bp in length, predicting a 181-residue protein product having a calculated molecular mass of 20,299 Da. The *E. herbicola aroQ* promoter is recognized by *E. coli*, and a putative sigma-70 promoter region was identified. N-terminal amino acid sequencing of the purified CM-F protein indicated cleavage of a 20-residue signal peptide. This was consistent with the monomeric molecular mass determined for the enzyme of about 18,000 Da. The native enzyme is a homodimer. The implied translocation of CM-F was confirmed by osmotic shock experiments which demonstrated a periplasmic location. Immunogold electron microscopy indicated a polar localization within the periplasm. Polyclonal antibody raised against *E. herbicola* CM-F did not cross-react with the CM-F protein from the closely related *Serratia rubidaea*, as well as from a number of other gram-negative bacteria. Furthermore, when the *E. herbicola aroQ* gene was used as a probe in Southern blot hybridizations with *Eco*RI digests of chromosomal DNA from *S. rubidaea* and other enteric organisms, no hybridization was detected at low stringency. Thus, the *aroQ* gene appears to be unusually divergent among closely related organisms. The deduced CM-F amino acid sequence did not exhibit compelling evidence for homology with the monofunctional chorismate mutase protein of *Bacillus subtilis*.

Gram-negative bacteria possess three distinct species of chorismate mutase (21, 33). All organisms within superfamilies A and B (synonymous with subdivisions beta and gamma of reference 32) possess CM-P, a catalytic domain located at the N terminus of the bifunctional PheA (P protein) (20). Some organisms such as *Pseudomonas stutzeri* and *Azotobacter* species possess only CM-P, which therefore must be committed to the production of prephenate precursor molecules for both tyrosine and phenylalanine biosynthesis (10).

The most common arrangement is typified by an organism like *Pseudomonas aeruginosa* which possesses, in coexistence with CM-P, a monofunctional chorismate mutase protein denoted CM-F (27). A second two-isozyme system is illustrated by that present in *Escherichia coli* in which CM-P coexists with CM-T, a catalytic domain at the N terminus of the *tyrA*-encoded T protein (20).

A number of enteric bacteria have recently been found to express all three species of chorismate mutase (2, 4, 33). This was surprising since CM-F was assumed to be dedicated to tyrosine biosynthesis in systems lacking CM-T (1), and it was hypothesized (3) that fusion of genes encoding CM-F and cyclohexadienyl dehydrogenase produced the bifunctional T protein. If this were correct, one would not expect to find CM-F (or cyclohexadienyl dehydrogenase) in the lineage radiating from the ancestor carrying the putative gene fusion.

The unexpected periplasmic location of the CM-F species reported here, in conjunction with the recently reported (35) location of cyclohexadienyl dehydratase in the periplasm as well, indicates that the primary function of CM-F is not to act as an enzyme of tyrosine biosynthesis in the cytoplasm. Its compartmentation in the periplasm indicates that it, probably in concert with cyclohexadienyl dehydratase, is dedicated to some as yet unknown function.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are described in Table 1 and the legend to Fig. 4. Plasmid constructs relevant to the subcloning analysis are diagrammed in Fig. 1.

Media and growth conditions. Luria-Bertani (LB) broth (14) was used as a complete medium, and the M9 formulation (23) was used for minimal medium as indicated in the text. Ampicillin (50 μg/ml), thiamine hydrochloride (17 μg/ml), and L-phenylalanine (50 μg/ml) were added as appropriate. Solid medium was obtained with 2.0% agar. X-Gal plating medium contained 50 μg of 5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside (X-Gal) per ml.

Construction of the pJX-H vector. A 1.1-kb DNA fragment having blunt termini and containing the *Zymomonas mobilis* structural gene for CDH (38) as well as the ribosome-binding site, but not the promoter, was cloned into pUC18 at the *Sma*I restriction site. Selection for plasmids having *tyrC* inserts in the same orientation as *lacZ* of pUC18 was accomplished by transformation of *E. coli* AT2471, a *tyrA* auxotroph. Transformed populations were incubated in LB medium for 10 h at 30°C, centrifuged and washed twice with M9 medium, and plated out at 37°C on M9 medium. The

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or description	Source or reference
E. herbicola ATCC 33243	Prototroph	American Type Culture Collection
E. coli		
K12	Prototroph	CGSC 4401
JM83	*ara* Δ(*proAB-lac*) *rpsL thi-1* φ80d*lacZ*ΔM15	GIBCO/BRL
AT2471	*thi-1 tyrA*	CGSC
JP2255	*aroF363 pheA361 pheO352 tyrA382 thi-1 strR712 lacY1 xyl-15*	6
Plasmids		
pUC18	Ap^r *lacI'*POZ'	36
pUC19	Ap^r *lacI'*POZ'	36
pGEM-5Zf(+)	Ap^r *lacZ*	Promega
pJX-H	Derivative of pUC18 carrying *Z. mobilis tyrC* (33) cloned into the *Sma*I site	This study
pJX-Ma	Original clone of *aroQ* selected from pJX-H:*Sau*3A genomic library of *E. herbicola*	This study
pJX-Mb	1.9-kb *Sph*I fragment subcloned from pJX-Ma into the *Sph*I site of pUC18	This study
pJX-Mc	1.7-kb *Hin*dIII-*Sph*I fragment subcloned from pJX-Ma into the *Hin*dIII-*Sph*I sites of pUC18	This study
pJX-Md	1.7-kb *Hin*dIII-*Sph*I fragment subcloned from pJX-Ma into the *Hin*dIII-*Sph*I sites of pUC19	This study
pJX-Me	1.2-kb *Sph*I-*Hin*dIII-*Pvu*II fragment subcloned from pJX-Mb into the *Sph*I-*Sma*I sites of pUC18	This study
pJX-Mf	0.7-kb *Pvu*II-*Nde*I-*Sph*I fragment subcloned from pJX-Mb into the *Sma*I-*Sph*I sites of pUC18	This study

recombinant vector, denoted pJX-H, was amplified by retransformation of *E. coli* JM83.

Construction of gene library. Chromosomal DNA was isolated from *Erwinia herbicola* and other organisms in this study by the method described by Silhavy et al. (30). Chromosomal DNA from *E. herbicola* was partially digested with *Sau*3A. DNA Fragments (2 to 4 kb) isolated from agarose gel after electrophoresis were ligated into the dephosphorylated *Bam*HI site of pJX-H. The ligation mixture was used to transform *E. coli* JM83, and ampicillin-resistant transformants were selected. Recombinant plasmids containing heterologous inserts derived from *Z. mobilis* and *E. herbicola* were purified from transformed populations as referenced (15).

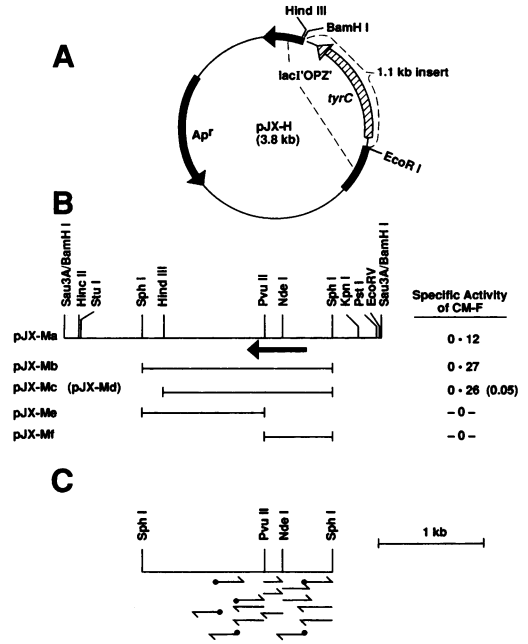

FIG. 1. (A) Construction of cloning vector pJX-H. The *tyrC*-containing fragment from *Z. mobilis* is shown by shading. (B) Restriction cleavage map of the 3-kb *Sau*3A fragment containing *aroQ*. The arrow indicates the location and 5'-to-3' orientation of *aroQ*. pJX-Mc and pJX-Md possess identical inserts but have opposite orientations with respect to the *lac* promoter in pUC18 and pUC19, respectively. (C) Strategy for sequencing the *Sph*I-*Pvu*II-*Nde*I-*Sph*I fragment from pJX-Mb.

Molecular genetic procedures. Restriction analysis, subcloning, and other standard procedures were performed as described by Sambrook et al. (29). Southern blot hybridization was done with a biotinylated probe according to the manufacturer's (Promega) instructions. Low-stringency conditions used entailed the use of 2× to 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) to wash the nitrocellulose membrane at room temperature after hybridization.

DNA sequencing and data analysis. The pJX-Mb restriction fragments were subcloned into pUC18 or pGEM-5Zf(+), and these subclones were purified by the method developed by Applied Biosystems, Inc. (User Bulletin 18: DNA Sequencing Model 373A, 1991). Double-stranded plasmid DNA was sequenced in both directions by methodology using fluorescent chain-terminating dideoxy nucleotides (28) at the DNA Core Facility of the University of Florida. The pUC18 and pGEM-5Zf(+) sequencing primers were obtained from Promega or Bethesda Research Laboratories; other primers binding to portions of the *E. herbicola* insert were synthesized in the DNA Core Facility.

The nucleotide sequence of DNA and the deduced peptide sequence were analyzed by the 1991 GCG software package of Genetics Computer Group, Inc. (University Research Park, Madison, Wis.).

Crude extract preparation. *E. coli* JP2255 derivatives carrying the indicated plasmids were grown at 37°C in 400 ml of LB medium containing ampicillin and harvested by centrifugation during the exponential phase of growth. The cells were suspended in buffer A (50 mM potassium phosphate buffer containing 1 mM dithiothreitol, pH 7.0) and disrupted by sonication. Cell debris was removed by 150,000 × *g* ultracentrifugation at 4°C for 40 min. The supernatant was passed through a PD-10 Sephadex column to remove small molecules, and the eluate was saved for enzyme assay.

Enzyme assays. Chorismate mutase was assayed by the method of Cotton and Gibson (13). One unit of enzyme activity is defined as the formation of 1 μmol of prephenate (assayed as phenylpyruvate after acidification) per min at 37°C. Alkaline phosphatase (7) and prephenate dehydrogenase (11) were assayed as referenced. Protein concentration was measured as described by Bradford (8).

Purification of CM-F. Crude extract was prepared from 16 g (wet weight) of *E. coli* JP2255(pJX-Mc) in buffer A and applied to a DEAE-cellulose (DE52) column (4.5 by 20 cm) equilibrated in buffer A; the column was washed with the same buffer. CM-F eluted in the void volume without retardation. Fractions containing CM-F were pooled and loaded onto a hydroxyapatite column (2.5 by 24 cm) equilibrated with buffer A, and the column was then eluted with a 700-ml linear gradient run between 50 and 400 mM potassium phosphate at pH 7.0 containing 1 mM dithiothreitol. Eluate fractions containing high CM-F activity were pooled and concentrated to 1.5 ml with an Amicon YM10 membrane. The enzyme solution was applied to a Sephadex G-100 column (2.5 by 96 cm), and the column was eluted with buffer A. Fractions having high CM-F activity were pooled, concentrated to 6 ml with the YM10 membrane, and dialyzed against buffer B (20 mM potassium phosphate at pH 8.3 containing 1 mM dithiothreitol). The enzyme solution at pH 8.3 was applied to a DE52 column (2.0 by 9.0 cm) equilibrated in buffer B, and the column was eluted with a 500-ml linear gradient run between 0 and 300 mM KCl in buffer B. The fractions exhibiting high CM-F activity were saved.

N-terminal amino acid sequencing. The N-terminal sequence of the purified CM-F was determined with an Applied Biosystems 470A Protein Sequencer with an on-line 120A PTH-Analyzer at the Protein Core Facility of the University of Florida.

Antibody preparation and Western blotting (immunoblotting) analysis. Antiserum against CM-F was prepared from a New Zealand rabbit by Kel-Farms (Alachua, Fla.). The Western blot procedure was performed as described by Towbin et al. (31).

Isolation of CM-F in periplasmic fractions of *E. coli*. *E. coli* JP2255(pJX-Mc) was grown in 1,000 ml of LB medium containing ampicillin. The culture was divided into two 500-ml parts and harvested by centrifugation during the late exponential phase of growth. One of the cell pellets (1.5 g of wet cells) was subjected to osmotic shock (24). After centrifugation, the supernatant was collected and designated as the periplasmic fraction. The extract prepared from the cell pellet remaining after osmotic shock (using the method described above for crude extract preparation) was designated as the cytoplasmic fraction. The unshocked cells making up the second cell pellet were used directly for crude extract preparation.

Demonstration of CM-F in periplasmic fractions of *E. herbicola*. Populations of *E. herbicola* were grown at 37°C in 1,200-ml amounts of LB medium and M9 medium. Each culture was divided into three 400-ml portions and harvested by centrifugation during the late exponential phase of growth. One portion was used to prepare crude extract, and a second portion was treated with chloroform (5). The remaining portion was subjected to osmotic shock as described above for *E. coli*, and periplasmic and cytoplasmic fractions were generated. All three preparations were used for Western blotting analysis of CM-F and for assays of alkaline phosphatase (periplasmic control) and prephenate dehydrogenase (cytoplasmic control).

Molecular mass determination. The molecular mass of native CM-F was estimated by gel filtration on a Sephadex G-200 column as described previously (34), and subunit molecular size was determined (22) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Electron microscopy. Exponential-phase populations of *E. coli* JP2255(pJX-Mc) were grown in M9 medium containing ampicillin and were fixed with 2.5% formaldehyde–0.5% glutaraldehyde (9) and then dehydrated and embedded in Lowicryl K4M by the progressive-lowering-of-temperature electron microscopy protocol (12). After incubation overnight with primary antibody and secondary labeling with gold-goat anti-rabbit antiserum, the thin sections were poststained and photographed under a JEOL 100-CX electron microscope.

Biochemicals and chemicals. T4 DNA ligase, calf intestinal phosphatase, nick translation kit, and restriction enzymes were obtained from Bethesda Research Laboratories (GIBCO/BRL) or Promega and used according to protocols provided by the suppliers. Sephadex G-100, the molecular weight standards for gel filtration, and the alkaline phosphatase-linked goat antibody (against rabbit immunoglobulin G) were obtained from Sigma. DEAE-cellulose and hydroxyapatite were from Whatman and Bio-Rad, respectively. The molecular weight standards for SDS-PAGE were purchased from Pharmacia. *p*-Nitroblue tetrazolium, the *p*-toluidine salt of 5-bromo-4-chloro-3-indolyl phosphate, and nitrocellulose membranes were obtained from GIBCO/BRL. Unless indicated otherwise, all other biochemicals and chemicals were obtained from Sigma. Chorismate was isolated from the accumulation medium of triple auxotroph 62-1 of *Klebsiella pneumoniae* (18).

RESULTS

Cloning of *aroQ* from *E. herbicola*. The strategy of cloning a gene encoding chorismate mutase in *E. coli* by functional complementation poses a special problem. Double mutants which are deficient in the *pheA* and *tyrA* gene products are required to give a genetic background lacking chorismate mutase activity, i.e., lacking the two isoenzymes (CM-P and CM-T) expressed from these genes. Introduction of a heterologous chorismate mutase into this background, however, will not give a selectable phenotype (i.e., growth in the presence of phenylalanine alone or tyrosine alone) because the double mutant lacks the dehydratase and dehydrogenase activities of the bifunctional proteins which are necessary to complete the overall conversion of chorismate to phenylalanine or tyrosine, respectively.

This problem was resolved by use of recombinant vector pJX-H, a derivative of pUC18 carrying *tyrC* from *Z. mobilis* (Fig. 1A). *Z. mobilis tyrC* encodes an allosterically insensitive type of cyclohexadienyl dehydrogenase (38). A genomic library of *E. herbicola*, consisting of approximately 15,000 colonies obtained after transformation of *E. coli* JM83 with the ligation mixture as detailed under Materials and Methods, was constructed in pJX-H. Plasmids isolated from this library were used to transform *E. coli* JP2255. The transformed population was incubated in LB medium at 25°C overnight, washed twice with M9 medium, and then plated on M9 agar containing phenylalanine and ampicillin. After incubation at 37°C for 3 days, colonies were picked and screened by enzymatic analysis to eliminate $PheA^+$ or $TyrA^+$ transformants. Crude extracts prepared from cultures derived from two transformants possessed the desired combination of activities: chorismate mutase and allosterically insensitive cyclohexadienyl dehydrogenase (from *Z.*

TABLE 2. Purification of CM-F expressed in *E. coli* JP2255(pJX-Mc)

Purification step	Total protein (mg)	Sp act (units/mg)	Purification (fold)	Yield (%)
Crude extract	1,161	0.29	1	100
DEAE-cellulose (pH 7.0)	108	2.28	7.8	73
Hydroxyapatite	30.3	6.00	21	54
Gel filtration	3.86	29.7	102	34
DEAE-cellulose (pH 8.3)	0.95	63.8	220	18

mobilis), but not prephenate dehydratase (i.e., PheA) or tyrosine-inhibited cyclohexadienyl dehydrogenase (i.e., TyrA). Plasmid DNA purified from the colonies had *E. herbicola* inserts of about 3.0 and 3.5 kb in size. The plasmid containing the 3.0-kb insert was designated pJX-Ma and was used for further study.

A high level of chorismate mutase activity was found in crude extracts prepared from *E. coli* JP2255(pJX-Ma) as shown in Table 2. Cyclohexadienyl dehydrogenase, the gene product of *Z. mobilis tyrC*, was also present in the crude extract and possessed the particular properties described by Zhao et al. (38).

Expression of *aroQ* in subclone derivatives of pJX-Ma. Subclones derived from the 3.0-kb insert present in pJX-Ma (Table 1) are shown in Fig. 1B. Restriction fragment analysis indicated that the orientation of the *Sph*I insert in pJX-Mb is pUC18 *lac* promoter-*Sph*I-*Pvu*II-*Hin*dIII-*Sph*I. An identical *Hin*dIII-*Pvu*II-*Sph*I fragment was joined at the *Hin*dIII-*Sph*I sites of pUC18 and pUC19 to yield pJX-Mc and pJX-Md, respectively. Although CM-F activity levels were fivefold higher when driven from the *lac* promoter in the JP2255(pJX-Mc) construct, the activity obtained in the JP2255(pJX-Md) construct indicates the presence of an *E. herbicola* promoter that is recognized by *E. coli*.

Since *E. coli* JP2255 carrying either pJX-Me or pJX-Mf failed to express chorismate mutase activity (Fig. 1B), the *Pvu*II restriction site must be located within the *aroQ* structural gene (also see Fig. 2).

Nucleotide sequence of *aroQ*. The strategy used for sequencing the *Sph*I-*Pvu*II-*Nde*I-*Sph*I fragment spanning *aroQ* is shown in Fig. 1C. Sequencing was done in both directions as shown. Using the GCG computer program software, an open reading frame of 546 bp was identified, starting with an ATG codon and ending with a TAA codon. The deduced amino acid sequence of CM-F shown in Fig. 2 yields a 181-residue protein with a calculated molecular mass of 20,299 Da and an isoelectric point of 6.80. The G+C ratio (52.2%) and the codon usage of *aroQ* were similar to those reported previously (35) for *pheA*, *tyrA*, and *aroF* cistrons of *E. herbicola*. However, an unusual feature of the protein is its high content (12.7%) of serine residues.

Figure 2 shows the location of a likely ribosome-binding site 7 bp upstream of the start codon. Within the span of 285 bp upstream of the start codon, the −35 and −10 regions of two possible promoters which overlap are shown in the vicinity of the *Hin*fI restriction site.

Downstream of CM-F, a portion of a flanking open reading frame was sequenced. The deduced peptide sequence of 93 residues at the N terminus (data not shown) was used to search the GenBank and EMBL data bases, but no resemblance to any recorded sequences was found. This open reading frame is preceded with the appropriate spacing by the possible ribosome-binding site marked in Fig. 2. Since no

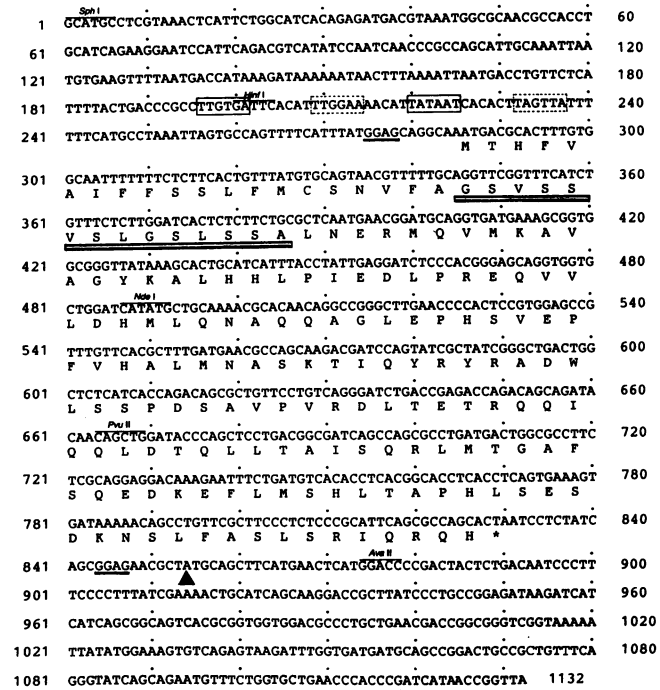

FIG. 2. Nucleotide sequence of *aroQ* and immediately flanking regions. Deduced amino acid sequences are shown in the single-letter code. Recognition sequences for various restriction endonucleases are marked, as are potential ribosome-binding sites. The translation start for a downstream open reading frame is shown (▲). Two potential sigma-70 promoter regions which overlap are boxed. The 14 N-terminal amino acids sequenced for purified CM-F protein are shown with a double underline.

termination structure is apparent between *aroQ* and the downstream open reading frame, these genes might share a common promoter.

Purification of CM-F from JP2255(pJX-Mc). To obtain a purified source of CM-F for antibody preparation and for N-terminal amino acid sequencing, the purification steps summarized in Table 2 were done. The starting level of CM-F activity in JP2255(pJX-Mc) was about 100-fold greater than the level present in crude extracts of wild-type *E. herbicola*. After the final fractionation step, a single protein band was obtained after SDS-PAGE (Fig. 3). The subunit

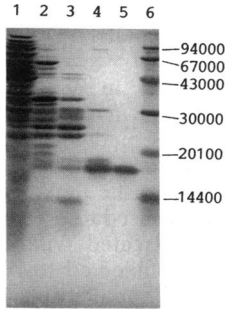

FIG. 3. Purification of *E. herbicola* CM-F from JP2255(pJX-Mc). SDS-PAGE of the preparations obtained after the various steps of purification. Lanes 1 to 5 correspond to the five stages of purification detailed in Table 2. Lane 6 contains the protein standards for calibration of molecular mass.

TABLE 3. Release of CM-F from *E. coli* JP2255(pJX-Mc) cells by osmotic shock

Fraction	Total protein (mg) (%)	Total CM-F activity (U[a]) (%)
Periplasmic[b]	33 (22)	34.3 (93)
Cytoplasmic[c]	122 (81)	1.48 (4)
Crude extract	151 (100)	36.8 (100)

[a] One unit of CM-F equals 1 μmol of prephenate produced per min.
[b] Contained 99% of total alkaline phosphatase activity.
[c] Contained <1% of total prephenate dehydrogenase activity.

molecular mass was about 18 kDa. Since a native molecular mass of about 34 kDa was estimated following elution from a calibrated gel filtration column, CM-F exists as a homodimer.

The purified CM-F exhibited Michaelis-Menten kinetics, and a K_m for chorismate of 0.17 mM was determined. Prephenate was a strictly competitive inhibitor, and a K_i value for prephenate of 0.20 mM was determined (data not shown).

N-terminal amino acid sequencing. The sequence of the 14 amino acids at the N terminus of purified CM-F was determined. It was a perfect match with the deduced sequence, but began with the 21st amino acid (as underlined in Fig. 2), rather than with the first amino acid, of the deduced sequence. This indicated that a 20-residue signal peptide might have been cleaved to yield the mature CM-F. Such a cleavage was consistent with the approximate 2-kDa discrepancy in calculated subunit size and subunit size estimated by SDS-PAGE. The putative signal peptide is phenylalanine rich (5 of 20), and the N terminus of the mature CM-F is serine rich (7 of the first 13 residues). The 20-amino-acid amino-terminal peptide possesses an appropriate size and other features (basic terminus, hydrophobic core region, and processing site motif) which typify periplasmic signal sequences (25).

Southern blot hybridization. The 674-bp *Hin*fI-*Ava*II restriction fragment spanning the entire *aroQ* structural gene (Fig. 2) was isolated and biotin labeled by nick translation. This DNA probe hybridized very well with *Hin*fI-*Ava*II digests of either pJX-Ma or chromosomal DNA from *E. herbicola*, and the hybridization bands were the same size. Hybridization with the larger fragment generated following digestion of chromosomal DNA with *Eco*RI was readily shown for *E. herbicola*, but not for *E. coli*, *Salmonella typhimurium*, *Erwinia carotovora*, *Serratia rubidaea*, *Enterobacter cloacae*, or *P. aeruginosa* (even under conditions of low washing stringency).

Location of CM-F in periplasmic fractions of *E. coli*. The possibility that CM-F might be translocated to the periplasmic space was tested by an osmotic shock procedure. Alkaline phosphatase was used as a positive control for periplasmic enzymes, and prephenate dehydrogenase was used as a positive control for cytoplasmic proteins. The data given in Table 3 show that CM-F was indeed located in the periplasmic space. Chloroform treatment has been used as an alternative to osmotic shock for obtaining periplasmic fractions which contained almost all the cloned cyclohexadienyl dehydratase in *E. coli* (37). In contrast, this relatively gentle technique released only 3.85% of the total CM-F activity (data not shown), indicating that CM-F may be more tightly associated with other structures in the periplasmic compartment than is cyclohexadienyl dehydratase or alkaline phosphatase.

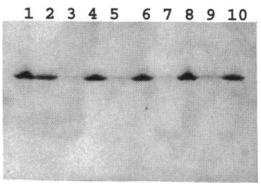

FIG. 4. Detection of CM-F in periplasmic space by Western blotting. Lane 1 contained 2 μg of protein from the osmotic shock fraction of *E. coli* JP2255(pJX-Mc) grown in LB medium; lane 2 contained 300 μg of protein from the crude extract of *E. herbicola* grown in LB medium; lane 3 contained 300 μg of protein from the cytoplasmic fraction of *E. herbicola* grown in LB medium; lane 4 contained 100 μg of protein from the osmotic shock fraction of *E. herbicola* grown in LB medium; lane 5 contained 100 μg of protein from the chloroform fraction of *E. herbicola* grown in LB medium; lane 6 contained 300 μg of protein from crude extract of *E. herbicola* grown in M9 medium; lane 7 contained 300 μg of protein from the cytoplasmic fraction of *E. herbicola* grown in M9 medium; lane 8 contained 100 μg of protein from osmotic shock fraction of *E. herbicola* grown in M9 medium; lane 9 contained 100 μg of protein from the chloroform fraction of *E. herbicola* grown in M9 medium; lane 10 contained 4 μg of protein from the crude extract of *E. coli* JP2255(pJX-Mc) grown in LB medium.

Location of CM-F in periplasmic fractions of *E. herbicola*. The use of direct enzyme assays to demonstrate the presence of CM-F in periplasmic fractions of *E. herbicola* is complicated by the chorismate mutase activities associated with the cytoplasmic prephenate dehydrogenase (*tyrA*) and prephenate dehydratase (*pheA*) proteins. Western blotting was therefore used for the selective detection of CM-F, with antibody raised against purified CM-F. A control was probed with preimmune serum. The results given in Fig. 4 illustrate the relative levels of CM-F in wild-type *E. herbicola* compared with the levels obtained following cloning in *E. coli*. Consistent with the enzymatic results shown above, CM-F was found in periplasmic fractions obtained by osmotic shock, but not by chloroform treatment. In contrast, more than 90% of total alkaline phosphatase activity was found in periplasmic fractions obtained by either osmotic shock or chloroform treatment. As a control for possible contribution of cell lysis to apparent periplasmic location, the cytoplasmic prephenate dehydrogenase (*tyrA*) was assayed in all fractions. No prephenate dehydrogenase activity was found in osmotic shock or chloroform treatment preparations, and 99% of the crude extract activity was accounted for in the cytoplasmic fraction obtained following osmotic shock. Osmotic shock and chloroform treatment were found to release about 20 and 13% of total protein, respectively, regardless of whether enriched or minimal medium was used to support growth. (Alkaline phosphatase activity, of course, was substantially lower following growth in M9 medium than in LB medium.)

Immunogold labeling. Ultrathin sections of *E. coli* JP2255(pJX-Mc) labeled with rabbit polyclonal anti–CM-F antibody and then with secondary antibody labeled with 10-nm gold confirmed the periplasmic location of CM-F by electron microscopic visualization (Fig. 5). Not only was the CM-F verified to have a periplasmic location, but the antibody appeared to have a subperiplasmic location within the polar caps. The thin sections shown in Fig. 5B and C are both longitudinal, and both show an inclusion body at one pole. However, it is unknown whether these are unipolar. In Fig. 5C, the periplasmic location of the polar body is clearly visualized since the plasma membrane is between the inclu-

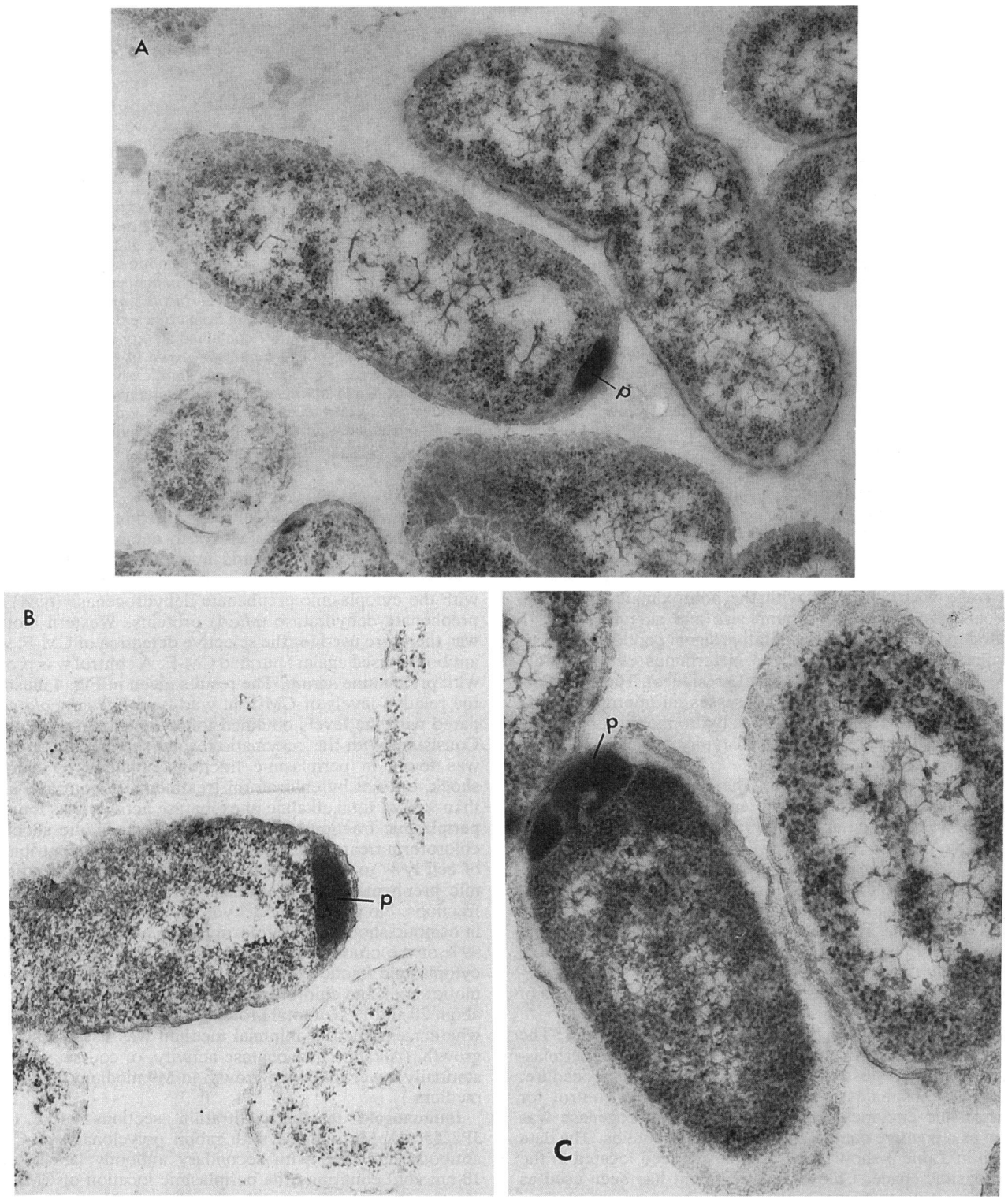

FIG. 5. Immunocytological localization of CM-F in the periplasm. Exponential-phase cells of *E. coli* JP2255(pJX-Mc) were grown in M9 medium. Thin sections of these cells were incubated with antibody (1:100) prepared against purified CM-F, maintained overnight at 4°C, labeled with gold particles, and examined under the electron microscope. Polar body inclusions are indicated (p) in each panel. The black dots seen within the polar bodies are immunogold-labeled molecules of CM-F. Panel A (magnification, ×35,000) shows a control treated exactly as in panels B and C (magnification, ×46,000), but with polyclonal antibody raised against *E. coli* signal peptidase instead. The longitudinal sections of panels B and C exhibit gold labeling within the polar inclusion bodies which is rather light, but distinct against a background of negligible nonspecific gold deposition.

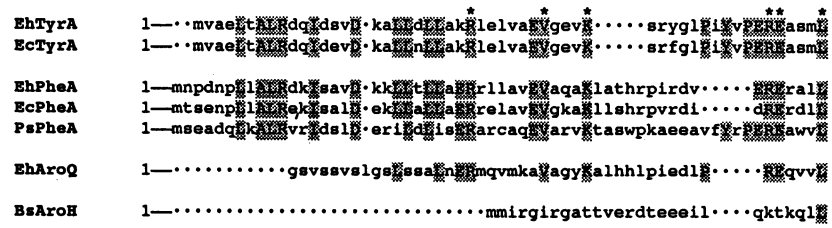

FIG. 6. Multiple alignment (Pileup Program) of chorismate mutase amino acid sequences of TyrA, PheA, AroQ, and AroH from *E. herbicola* (Eh), *E. coli* (Ec), *P. stutzeri* (Ps), and *B. subtilis* (Bs). Residues that are highly conserved in the six gram-negative proteins are shown with shading and capital letters. Asterisks denote residues common to all six (in one case, seven) proteins. Although only 3 of the 28 BsAroH residues shown are identical with the corresponding EhAroQ residues, 13 of the remaining 25 residues are conservative amino acid matches. References for the sequences shown are, from top to bottom, 35, 20, 35, 20, 16, this study, and 19.

sion and the cytoplasm. The use of the progressive-lowering-of-temperature technique for dehydration and embedding was used to minimize the possible leaching of contents from the polar periplasmic inclusion bodies. This technique (12), which has been reported to preserve ultrastructure and enzyme integrity, visualized periplasmic inclusion bodies which incorporated the staining reagents more heavily than has been observed with standard fixation and embedding techniques. Similar visualization of CM-F by immunogold electron microscopy in *E. herbicola* was unsuccessful, probably owing to its very low abundance (2).

DISCUSSION

The deployment of chorismate mutase for biosynthesis of phenylalanine and tyrosine in microorganisms and higher plants exhibits exceedingly high diversity in terms of variable numbers of isoenzymes, regulatory properties, and coexistence with other catalytic domains on multifunctional proteins (21). Two of the three major superfamilies of gram-negative eubacteria possess in common the bifunctional PheA protein having CM-P as one catalytic domain. Included in this grouping is *E. coli* and other enteric bacteria which possess the bifunctional TyrA protein that has CM-T as one catalytic domain. This appealing symmetry of competing channeling systems at a metabolic branchpoint leaves little doubt that CM-P is dedicated to phenylalanine biosynthesis and CM-T is dedicated to tyrosine biosynthesis.

However, within the two superfamilies, the *P. aeruginosa* type of arrangement is much more widely distributed. No bifunctional TyrA exists, and therefore no CM-T exists. In such organisms, it has been assumed that a monofunctional chorismate mutase protein (CM-F) initiates precursor flow toward tyrosine. In fact, a compelling basis seemed to exist (3) to suggest that fusion of *aroQ* and *tyrC* in an immediate ancestor of enteric bacteria resulted in *tyrA*, i.e., CM-F is the precursor of CM-T.

Uncertainty about the latter evolutionary scenario centered on three findings. First, a phylogenetic cluster closely related to *P. aeruginosa* (containing *P. stutzeri*, *Azomonas* sp., and *Azotobacter* sp.) lacks CM-F and therefore relies on CM-P for both phenylalanine and tyrosine biosynthesis (10). Second, CM-F was found to coexist with CM-T in some enteric bacteria. Fusion of *aroQ* should hypothetically have eliminated CM-F in any organisms possessing TyrA (i.e., CM-T). Third, although *aroQ* may share a common ancestor with the chorismate mutase-encoding portions of *pheA* and *tyrA* (Fig. 6), the newly evolved CM-T resembles CM-P much more (20, 21) than it resembles CM-F. Therefore, the genetic region encoding CM-T probably originated by recruitment of the N-terminal-encoding domain of *pheA*.

E. herbicola CM-F exhibited no obvious resemblance to the *Bacillus subtilis* monofunctional chorismate mutase protein, a cytoplasmic enzyme (19). Thus, these latter two proteins either originated independently or diverged markedly through evolutionary time. It is perhaps surprising that EntC, an enzyme which rearranges chorismate to isochorismate, exhibits homology with enzymes such as TrpE and PabB (26), but not with the various chorismate mutases for which deduced amino acid sequences are available. Even more surprising than the latter example was the observation that CM-F in closely related enteric genera has diverged sufficiently so as to escape detection by either hybridization in Southern blots (with *E. herbicola aroQ*) or with anti-CM-F antibody from *E. herbicola*.

The compartmentation of CM-F in the periplasm, together with the recent appreciation that cyclohexadienyl dehydratase is also periplasmic (37), suggests that CM-F (plus the dehydratase and perhaps an aminotransferase) are spatially sequestered to form phenylalanine (or phenylpyruvate) for some as yet unknown function. Figure 7 shows a comparison of the overall pathway construction in *E. herbicola* and in *P. aeruginosa*. We conclude that *P. stutzeri* and *P. aeruginosa* (and indeed many gram-negative bacteria) utilize a common cytoplasmic pathway, but differ in that *P. stutzeri* lacks the periplasmic pathway. Thus, CM-P is commonly an enzyme which functions for both phenylalanine and tyrosine biosynthesis. The enteric cluster is exceptional in that CM-P and CM-T are specialized for directing precursor flow toward phenylalanine or tyrosine, respectively. The usual functional role of CM-F must be unrelated to the generation of amino acids which are needed for cytoplasmic protein synthesis, regardless of the presence or absence of CM-T.

E. coli is well appreciated as an efficient system for obtaining heterologous genes by means of functional complementation. This study illustrates that even when pathway differences create selection complications, sufficient knowledge of these differences can lead to a strategy for successful functional complementation. The functional complementation obtained in this study is essentially a phenomenon of extracistronic suppression. We suggested that CM-F does not normally participate in cytoplasmic phenylalanine or tyrosine biosynthesis in wild-type organisms. However, under conditions in which massive accumulation of chorismate occurs [as during tyrosine starvation of JP2255(pJX-Ma)], chorismate entering the periplasm is converted to prephenate, which regains entrance to the cytoplasm where the *Zymomonas*-derived cyclohexadienyl dehydrogenase

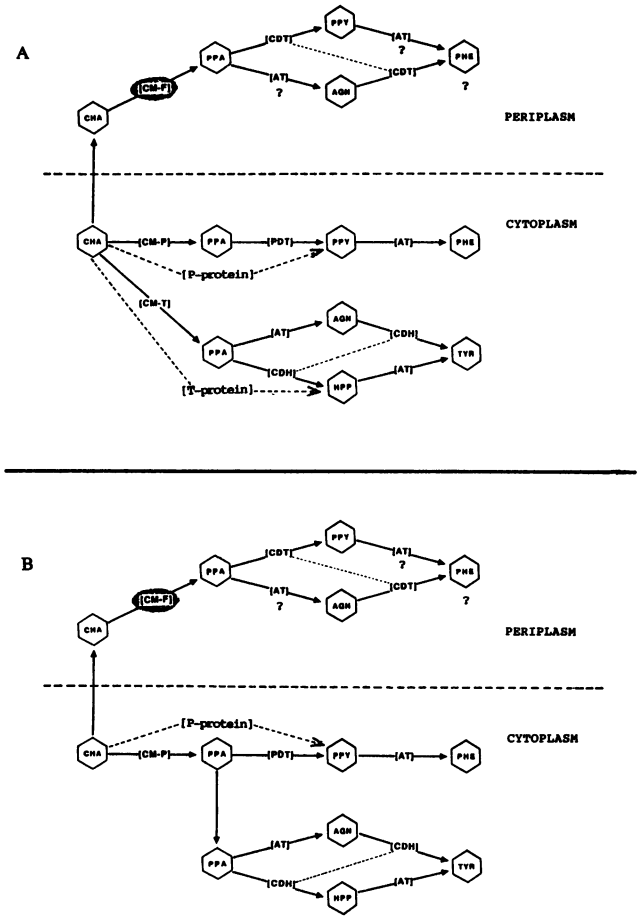

FIG. 7. Compartmentation of aromatic pathway enzymes in *E. herbicola* (A) compared with *P. aeruginosa* (B). Abbreviations: CHA, chorismate; PPA, prephenate; AGN, L-arogenate; PPY, phenylpyruvate; HPP, 4-hydroxyphenylpyruvate; PHE, L-phenylalanine; TYR, L-tyrosine; [CM-P], [CM-T], and [CM-F], isoenzymes of chorismate mutase as specified in the text; [PDT], prephenate dehydratase; [CDT], cyclohexadienyl dehydratase; [CDH], cyclohexadienyl dehydrogenase; [AT], aminotransferase. The dual-substrate capabilities of CDT and CDH are indicated by dashed lines. The bifunctional T protein (dashed arrows) has two catalytic domains designated [CM-T] and [CDH]; the bifunctional P protein (dashed arrows) has two catalytic domains designated [CM-P] and [PDT]. For simplicity, dehydrogenase and aminotransferase cofactors are not shown.

uses it as substrate. Essentially the same phenomenon occurs in *P. aeruginosa* when chorismate accumulation is greatly increased following early-pathway deregulation (17). In this case, cytoplasmic chorismate, largely unaccepted by the feedback-sensitive prephenate dehydratase and cyclohexadienyl dehydrogenase enzymes in the cytoplasm, is apparently forced to the periplasm where periplasmic CM-F and cyclohexadienyl dehydratase participate in the overall conversion to large quantities of phenylalanine.

ACKNOWLEDGMENTS

This research was supported by Public Health Service grant DK38309.

Rabbit polyclonal antibody raised against *E. coli* signal peptidase was the kind gift of Paul Wolfe (Johns Hopkins University).

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