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Cloning and Sequencing of *Escherichia coli ubiC* and Purification of Chorismate Lyase

BRIAN P. NICHOLS* AND JACALYN M. GREEN

Laboratory for Molecular Biology, Department of Biological Sciences, University of Illinois at Chicago, P.O. Box 4348, Mailcode 067, Chicago, Illinois 60680

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In *Escherichia coli*, chorismate lyase catalyzes the first step in ubiquinone biosynthesis, the conversion of chorismate to 4-hydroxybenzoate. 4-Hydroxybenzoate is converted to 3-octaprenyl-4-hydroxybenzoate by 4-hydroxybenzoate octaprenyltransferase. These two enzymes are encoded by *ubiC* and *ubiA*, respectively, and have been reported to map near one another at 92 min on the *E. coli* chromosome. We have cloned the *ubiCA* gene cluster and determined the nucleotide sequence of *ubiC* and a portion of *ubiA*. The nucleotide sequence abuts with a previously determined sequence that encodes a large portion of *ubiA*. *ubiC* was localized by subcloning, and overproducing plasmids were constructed. Overexpression of *ubiC* allowed the purification of chorismate lyase to homogeneity, and N-terminal sequence analysis of chorismate lyase unambiguously defined the beginning of the *ubiC* coding region. Although chorismate lyase showed no significant amino acid sequence similarity to 4-amino-4-deoxychorismate lyase (4-amino-4-deoxychorismate → 4-aminobenzoate), the product of *E. coli pabC*, chorismate lyase overproduction could complement the growth requirement for 4-aminobenzoate of a *pabC* mutant strain. Of the several enzymes that convert chorismate to intermediates of *E. coli* biosynthetic pathways, chorismate lyase is the last to be isolated and characterized.

Chorismate is the branch point precursor for the synthesis of many aromatic compounds in *Escherichia coli*. The seven major end products of chorismate anabolism are phenylalanine, tyrosine, tryptophan, ubiquinone, 4-aminobenzoate, menaquinone, and enterobactin. Chorismate itself undergoes five different conversions that result in those seven products. The tyrosine and phenylalanine pathways diverge following the isomerization of chorismate to prephenate, but two distinct, homologous chorismate mutase activities channel chorismate toward either phenylalanine or tyrosine (10). The menaquinone and enterobactin pathways diverge following the conversion of chorismate to 2-hydroxy-4-deoxychorismate (isochorismate) by isochorismate synthase (28, 30). For 4-aminobenzoate synthesis, chorismate is first aminated to form 4-amino-4-deoxychorismate, which then undergoes β-elimination of the enol-pyruvyl moiety to form 4-aminobenzoate (1, 8, 21, 29). The first step in ubiquinone biosynthesis is the conversion of chorismate to 4-hydroxybenzoate by chorismate lyase (5, 15), in a reaction apparently similar to the second step of 4-aminobenzoate synthesis, catalyzed by 4-amino-4-deoxychorismate lyase. Finally, the first step in tryptophan synthesis is chorismate amination and β-elimination of the enol-pyruvyl group to form anthranilate, or 2-aminobenzoate (2).

With the exception of the genes involved in ubiquinone biosynthesis, all of the *E. coli* genes encoding enzymes that use chorismate or 4-amino-4-deoxychorismate as substrates have been cloned and sequenced. These include *pheA* (chorismate mutase-prephenate dehydratase), *tyrA* (chorismate mutase-prephenate dehydrogenase) (10), *entC* (isochorismate synthase) (23), *pabA* and *pabB* (4-amino-4-deoxychorismate synthase) (6, 12), *pabC* (4-amino-4-deoxychorismate lyase) (7), and *trpE* and *trpD* (anthranilate synthase-5-phosphoribosyl-1-pyrophosphate phosphoribosyl transferase) (20, 22).

Comparisons of the chorismate-utilizing enzymes have shown that the two chorismate mutase activities encoded by *pheA* and *tyrA* are homologous (10). In addition, both subunits of anthranilate synthase and 4-amino-4-deoxychorismate synthase share sequence similarity (6, 12), while the chorismate-utilizing subunit of each also shares sequence similarity with isochorismate synthase (23). These data suggest that several of the chorismate-utilizing enzymes originated from a common ancestor and additionally suggest some commonality in reaction mechanisms (27). 4-Amino-4-deoxychorismate lyase, on the other hand, does not show sequence similarity to the other known chorismate-utilizing enzymes but is similar in sequence to several amino acid aminotransferases (7), suggesting a different ancestral relationship and, likely, a different catalytic mechanism.

We have undertaken the cloning and sequencing of *E. coli ubiC* to enhance our understanding of the relationships among the enzymes that use chorismate as a substrate. *ubiC* and *ubiA* have been reported to lie near one another at 92 min on the *E. coli* chromosome (15, 31). *ubiC* encodes the soluble chorismate lyase, while *ubiA* encodes the membrane-bound 4-hydroxybenzoate octaprenyltransferase. Starting from previously published information on the location of *ubiA* (16, 17), we have identified and cloned *ubiC*. In addition to determining the nucleotide sequence of *ubiC* and the beginning portion of *ubiA*, we have constructed chorismate lyase-overproducing plasmids and purified chorismate lyase to homogeneity.

MATERIALS AND METHODS

Bacterial strains, phages, and plasmids. The genotypes and sources of the strains used in this work are as follows: AN244 (*ubiC thi-1 rel*), Jeremy Knowles (15); LE392 (*trpR55 metB1 hsdR514 galK2 galT22 lacY1 supE44 supF58*), laboratory collection; BN117 [*pheA1 tyrA4 ΔtrpEA2 pabA1 pabB::Kn trpR*(Tn*10*) *his-4 proA2 argE3 rpsL704*] (21); BN1044 (*pabC::kan*) (7); DH5αF' [ϕ80*lacZΔM15 endA1*

* Corresponding author.

recA1 hsdR17(r_K^- m_K^-) *supE44 thi-1 gyrA relA1* Δ(*lacZYA-argF*)*U169* λ^-], laboratory stock; and MC1000 [Δ(*araAIOC-leu*)*7697 apaD139* Δ(*lacIPOZY-lacIPOZA*)*X74 strA galU galK*], Malcolm Casadaban. The λ phages containing *E. coli* DNA were obtained from Y. Kohara. pGP1-2 (25) was obtained from Stan Tabor, and pBS(+) and pBSII K+ were purchased from Stratagene.

DNA manipulations. Restriction endonuclease digestion, ligations, transformations, and plasmid preparations were performed by procedures described by Maniatis et al. (19). The two-plasmid T7 expression system described by Tabor (25) was used to produce chorismate lyase from pBS(+) derivatives. Nested deletions in plasmids were prepared by the method of Henikoff (9), and DNA sequence analysis was performed with a Sequenase 2.0 kit purchased from United States Biochemicals and a reverse primer (New England BioLabs).

Preparation of extracts and enzyme assays. Crude extracts of strains were prepared from mid-log-phase cultures grown in a medium containing 1% NZamine, 0.5% yeast extract, and 0.5% NaCl. Overnight cultures were diluted 1/100 in 50 ml of fresh broth and grown to 100 Klett units. The cells were harvested by centrifugation, resuspended in 0.5 volume of 0.85% NaCl, and harvested again. The cell paste was resuspended in 2 ml of 50 mM Tris-HCl (pH 7.5)–10 mM 2-mercaptoethanol and subjected to sonic disruption (twice for 30 s each time) in a Branson Sonifier. The sonicate was centrifuged at 30,000 × *g* for 30 min, and the supernatant was passed through a 0.45-μm-pore-size filter. For large preparations of enzyme, cultures (4 liters) were grown overnight and treated similarly.

Stock solutions (6 mM) of barium chorismate (Sigma Chemical Co.) were extracted several times with diethyl ether to remove contaminating 4-hydroxybenzoate prior to use. Chorismate lyase assays were carried out in a volume of 0.5 ml containing 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 10 mM 2-mercaptoethanol, 60 μM chorismate, and 0.2 to 4 U of chorismate lyase. After incubation at 37°C for 30 min, 4-hydroxybenzoate was detected and quantitated by high-pressure liquid chromatography (HPLC). Fifty microliters of each reaction mixture was applied to an HPLC system (Waters 625) equipped with a Nova-Pak C_{18} column equilibrated in 5% acetic acid and monitored at 240 nm. The height of the 4-hydroxybenzoate peak was compared with those of standard curves generated by treating known amounts of 4-hydroxybenzoate in a similar manner. One unit of chorismate lyase activity was defined as the amount of enzyme required to produce 1 nmol of 4-hydroxybenzoate in 30 min at 37°C.

Assays for 4-aminobenzoate and 4-amino-4-deoxychorismate were performed as described previously (7, 21).

Purification of chorismate lyase and determination of the N-terminal sequence. A crude extract from 2 g of cell paste was diluted with equilibrating buffer (50 mM Tris-HCl [pH 7.5], 100 mM NaCl, 10 mM 2-mercaptoethanol) to a protein concentration of 2 mg/ml. The diluted extract (100 ml) was applied to a Cibacron Blue 3GA-agarose column (10-ml bed volume; Sigma) in equilibrating buffer at a flow rate of 1 ml/min. The column was washed with 100 ml of equilibrating buffer, and chorismate lyase was eluted with a 100-ml gradient of chorismate (0 to 3 mM) prepared in equilibrating buffer. Fractions showing substantial chorismate lyase activity were pooled and concentrated in a Centriprep-10 concentrator (Amicon).

Chorismate lyase samples (12 μg) were purified by high-performance electrochromatography (HPEC) on an 8% acrylamide gel (0.25 by 10 cm) with an Applied Biosystems model 230 HPEC. The enzyme eluted as a relatively broad peak. Fractions containing the highest-molecular-weight shoulder of the peak were adsorbed to a Pro-Blot polyvinylidene difluoride (PVDF) membrane (Applied Biosystems), and the N-terminal sequence was determined in an Applied Biosystems model 477 amino acid sequencer.

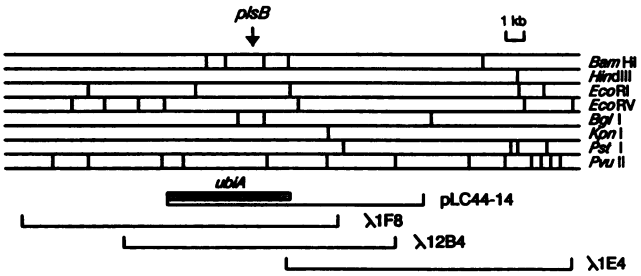

FIG. 1. Restriction map of the *E. coli* chromosome at 92 min. The restriction map and location of the λ clones were taken from reference 13. The restriction map of pLC44-14 and the location of *ubiA* were taken from reference 17.

Nucleotide sequence accession number. The nucleotide sequences of *ubiC* and *ubiA* have been deposited in GenBank and has been assigned accession number M93413.

RESULTS

Cloning of *ubiC*. Attempts to select *ubiC* or *ubiA* clones from plasmid libraries or from λ subclones by complementation were unsuccessful, apparently because of the leaky phenotype of the host strains. We therefore decided to construct subclones from a limited region of the *E. coli* chromosome and to detect *ubiC*-containing plasmids by assaying culture lysates for chorismate lyase activity. The conversion of chorismate to 4-hydroxybenzoate was detected by HPLC (see Materials and Methods).

ubiA lies at 92 min on the *E. coli* genetic map, and *ubiC* has been reported to map very near *ubiA* (15). pLC44-14, a member of the Clarke-Carbon *E. coli* library (3a), has been reported to contain *ubiA* (17). *ubiA* had been delimited to a 7-kb region upstream of an *Eco*RI site. We compared the restriction map of pLC44-14 to the restriction map of the *E. coli* chromosome (13) near the 92-min region (Fig. 1) and found a close match between pLC44-14 and λ clones λ1F8, λ12B4, and λ1E4. Since λ1F8 harbored the largest region containing the *ubiA* locus and upstream material, this phage was chosen for constructing subclones of the area.

Digestion of λ1F8 with *Eco*RI yielded the two λ arms (23 and 9 kb) and four fragments derived from the *E. coli* DNA insert. The *Eco*RI fragments derived from the insert were predicted to be 5.6, 5.1, 4.6, and 1.9 kb in length. DNA prepared from λ1F8 was digested with *Eco*RI, and the fragments were ligated with *Eco*RI-digested pBS(+) DNA. Following transformation of *E. coli* DH5αF', 10 Ap^r Lac^- colonies were characterized. Restriction analysis of the plasmids indicated that the 5.1- and 4.6-kb *Eco*RI fragments had been cloned in both orientations in pBS(+), while the 5.6- and 1.9-kb *Eco*RI fragments had been captured in only a single orientation.

The plasmids were introduced into *E. coli* AN244 (*ubiC thi*), and lysates were prepared for chorismate lyase assays. Of the six plasmids tested, only lysates from pBN162 and pBN163 showed detectable chorismate lyase activity.

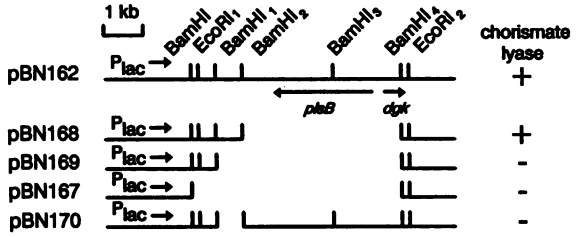

FIG. 2. Localization of *ubiC* by subcloning. pBN162 is shown with relevant restriction sites and the locations of *plsB* and *dgk*. Derivatives (pBN167 to pBN170) were produced by deletion of various *Bam*HI DNA fragments. The ability of each plasmid to produce chorismate lyase is indicated at the right.

pBN162 and pBN163 contained the 5.1-kb *Eco*RI fragment in opposite orientations.

To localize *ubiC* further, we took advantage of the five *Bam*HI sites present in pBN162 and pBN163. Each plasmid was partially digested with *Bam*HI, religated, and introduced into *E. coli* DH5αF'. The products obtained from pBN162 are shown in Fig. 2, and a similar series of products were obtained from pBN163. Chorismate lyase assays were performed with lysates prepared from *E. coli* AN244 transformed with pBN162 and pBN167-170 (Fig. 2). The smallest plasmid derivative that produced chorismate lyase contained about 1 kb of DNA comprising a 450-bp *Eco*RI$_1$-*Bam*HI$_1$ fragment and the adjacent 600-bp *Bam*HI$_1$-*Bam*HI$_2$ fragment (pBN168). Removal of the 600-bp *Bam*HI fragment resulted in a loss of chorismate lyase activity (pBN169 and pBN170). These data suggested that *ubiC* (or sequences required for its expression) either lay within the 600-bp *Bam*HI$_1$-*Bam*HI$_2$ fragment or spanned the *Bam*HI$_1$ site linking the 600- and 450-bp fragments.

A portion of the 5.1-kb *Eco*RI fragment from the *Bam*HI$_2$ to the *Eco*RI$_2$ sites had been sequenced previously and shown to encode *plsB* and *dgk* (16). The remaining portion of the *Eco*RI fragment encoded *ubiC*, and a previous characterization had suggested that *ubiA* also lay in this region (17).

Nucleotide sequence analysis of *ubiC*. pBN162 and pBN173 (like pBN168, but with the *Eco*RI fragment inverted) were cleaved with *Sph*I and *Sal*I, and deletions were made with exonuclease III and S1 nuclease by the method of Henikoff (9). Appropriately spaced deletions were chosen for sequence analysis with double-stranded plasmid DNA and a reverse primer (Fig. 3). The sequence of the 1,034-bp *Eco*RI$_1$-*Bam*HI$_2$ fragment was determined on both strands, and all junctions were overlapped, including the sequence across the *Bam*HI$_2$ site of pBN162. The sequence across the *Bam*HI$_2$ site is identical to that previously reported (16), indicating that the sequence reported here is contiguous with that reported previously.

Figure 4 shows the nucleotide sequence of approximately 1,950 bp from the *Eco*RI$_1$ site through the end of *plsB*. Two open reading frames of significant length were found within this sequence. The first (translated from bp 380 to 874 in Fig. 4) lay within the *Eco*RI$_1$-*Bam*HI$_2$ fragment and spanned the *Bam*HI$_1$ site, consistent with the localization of *ubiC*. The second open reading frame began 12 bp downstream of the *ubiC* termination codon, extended across the *Bam*HI$_2$ site, and terminated 154 bp prior to the termination codon of *plsB* (oriented oppositely). This open reading frame likely corresponds to *ubiA*.

Correlation of gene products with the nucleotide sequence. pBN163, pBN173, and pBN173 deletions were also used to characterize the product of *ubiC*. For the following experiments, T7 RNA polymerase-dependent expression (25) was driven from the T7 promoter of pBS(+). *E. coli* MC1000 was transformed with pGP1-2 (carrying the temperature-inducible T7 RNA polymerase gene). A Kn^r colony was chosen and transformed with pBN163, pBN173, and pBN173 deletions. Kn^r Ap^r transformants were chosen for expression experiments. Cultures were grown at 32°C and divided into two portions. One portion (1 ml) was induced at 42°C, treated with rifampin, and allowed to incorporate [^{35}S]methionine specifically into the products of genes transcribed from the T7 promoter. The second portion (45 ml) was induced at 42°C for 20 min and incubated at 37°C for an additional 90 min to express genes under the control of the T7 promoter. Lysates prepared from strains containing pBN173 and pBN173Δ4 produced chorismate lyase, while lysates from strains containing pBN173Δ6, pBN173Δ8, pBN173Δ13, pBN173Δ14, and pBN173Δ16 did not (Fig. 3). The correlation between the deletion endpoints and the expression of chorismate lyase activity was consistent with the position of the *ubiC* open reading frame indicated in Fig. 4.

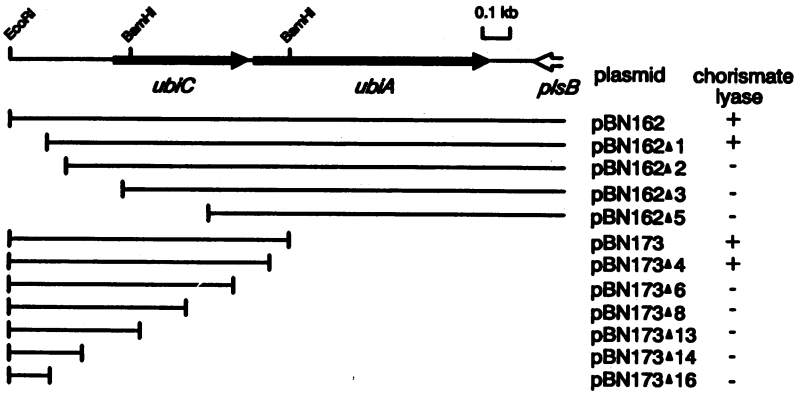

FIG. 3. Deletions in pBN162 and pBN173 used for DNA sequence analysis and localization of *ubiC*. The top line shows the locations of *ubiC*, *ubiA*, and *plsB*. The remainder of the lines show DNA remaining in the deletion plasmids. Each of the deletion derivatives was used to determine the nucleotide sequence of *ubiC* and a portion of *ubiA* with a reverse primer. The ability of each plasmid to produce chorismate lyase is indicated at the right.

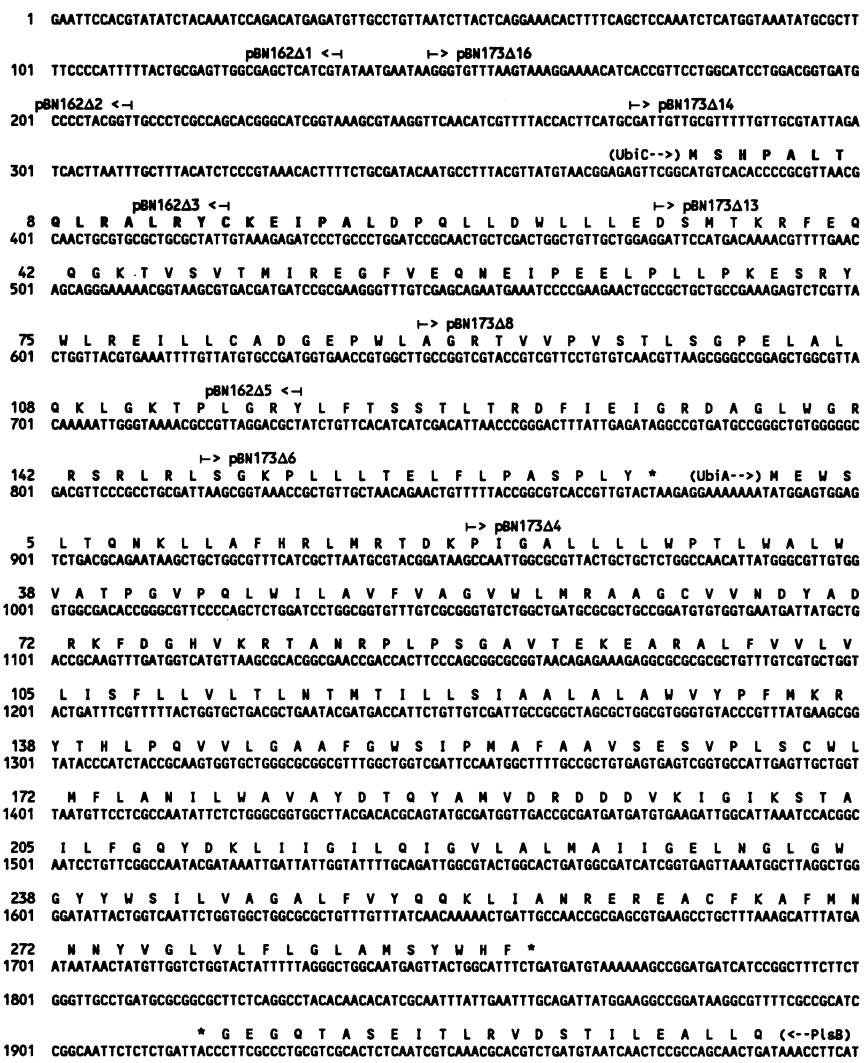

FIG. 4. Nucleotide and deduced amino acid sequences of *ubiC* and *ubiA*. Numbering of the nucleotide sequence begins at the beginning of the *Eco*RI site. Amino acids are indicated above the nucleotide sequence at the first base of each codon. Amino acids shown in boldface type were determined by amino acid sequence analysis of purified chorismate lyase. Arrows above the sequence indicate the limits of the deletions in pBN162 and pBN173 used to determine the sequence. The sequence of nucleotides 1 to 1080 was determined in this work, and the remainder of the sequence was taken from reference 16.

Expression analysis of deletions to determine the 5′ end of the open reading frame was complicated by the fact that the removal of the sequences necessary for *ubiC* expression may also have led to the loss of chorismate lyase activity. Deletions in pBN162 were introduced into *E. coli* AN244, and lysates were assayed for chorismate lyase activity (Fig. 3). pBN162 and pBN162Δ1 included the entire *ubiC* open reading frame, and both produced chorismate lyase activity. Furthermore, pBN162Δ3 and pBN162Δ5 contained deletions of portions of the *ubiC* translational frame and did not produce chorismate lyase activity. In contrast, pBN162Δ2 contained the entire open reading frame but did not produce chorismate lyase activity. The open reading frame could not be extended to include material removed in pBN162Δ2, and the lack of *ubiC* expression was probably due to the removal of sequences essential for transcription. For this reason, additional data needed to assign the 5′ terminus of *ubiC* were sought (see below).

The molecular weight of chorismate lyase was estimated by two different methods and correlated with the nucleic acid sequence. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) analysis of labelled proteins showed a band migrating with an M_r of 17,000 in extracts derived from cells containing pBN163, pBN173, and pBN173Δ4 (Fig. 5), in reasonable agreement with the predicted value (M_r, 18,800) for the translated open reading frame indicated in Fig. 4. The 17-kDa protein was absent from a strain carrying pBN173Δ6, and a smaller protein was visible, consistent with the truncation of the *ubiC* open reading frame of pBN173Δ6. Neither the 17-kDa protein nor the truncated derivatives were detected in strains harboring pBN173Δ8, pBN173Δ13, pBN173Δ14, or pBN173Δ16 (Fig. 5 and additional data not shown). pBN163 produced an additional radiolabelled protein with an M_r of 24,000, in marked disagreement with the molecular weight of 4-hydroxybenzoate octaprenyltransferase predicted from DNA sequence analysis (data not shown). However, a truncated *ubiA* product present in Fig. 5, lane 2, is consistent with translation

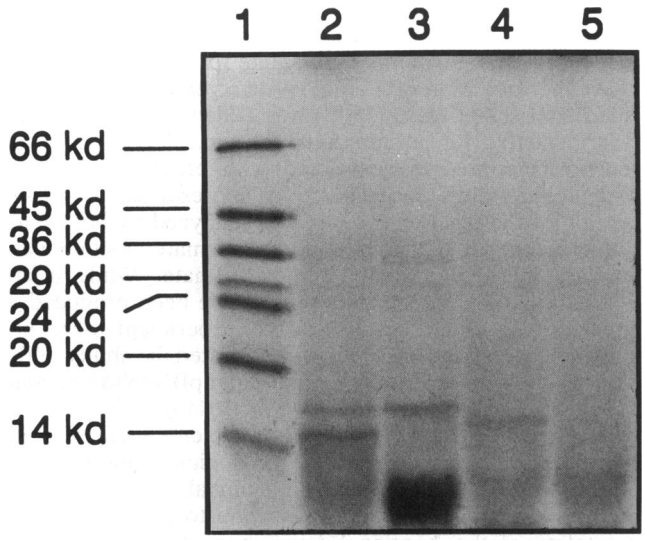

FIG. 5. Protein production by T7 RNA polymerase. ^{35}S-labelled proteins were prepared by the method of Tabor (25) and separated on an SDS–12.5% polyacrylamide gel. Lanes: 1, ^{14}C-labelled molecular mass markers; 2, pBN173; 3, pBN173Δ4; 4, pBN173Δ6; 5, pBN173Δ8.

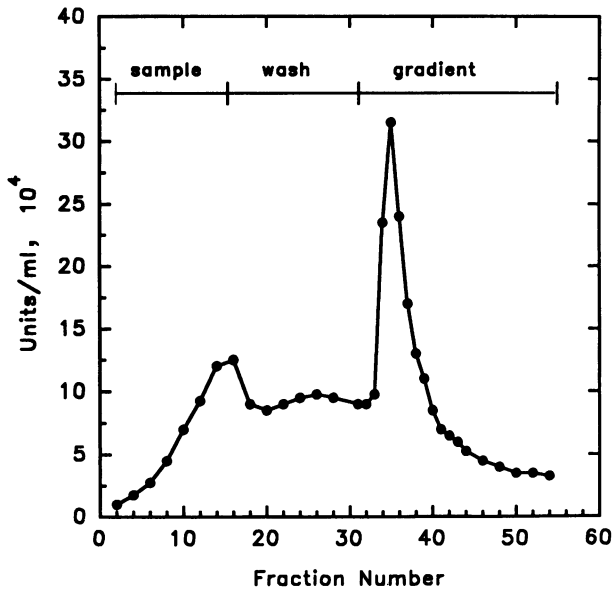

FIG. 6. Elution profile of overproduced chorismate lyase from a Cibacron Blue 3GA-agarose column. Experimental details are given in Materials and Methods and Results. Seven-milliliter fractions were collected during the sample application and wash, and 4-ml fractions were collected during the elution with a chorismate gradient.

initiation at the position indicated in Fig. 4. Similar labelling experiments with pBN162, with the T7 promoter oriented in the direction opposite of that for *ubiC*, revealed the expression of a polypeptide with a molecular weight consistent with the molecular weight predicted for the product of *plsB* (data not shown).

To estimate the size of native chorismate lyase, we chromatographed a portion of the T7-induced pBN173 lysate over a Superose 12 gel permeation column and compared its elution with the elution of molecular weight standards (cytochrome *c*, carbonic anhydrase, bovine serum albumin, and alcohol dehydrogenase). Chorismate lyase activity eluted with an M_r of 17,000, matching precisely the molecular weight determined by SDS-PAGE and indicating that the native enzyme is active as a monomer.

The *ubiC* open reading frame shown in Fig. 4 contained three possible initiating codons, at residues 269, 380, and 472. Polypeptides terminating at bp 874 had predicted molecular weights of 23,100, 18,800, and 14,800. The molecular weight of chorismate lyase was most consistent with initiation at position 380, although rigorous confirmation of this initiation codon assignment required determination of the N-terminal amino acid sequence of the purified enzyme. To this end, we undertook the overproduction and purification of chorismate lyase.

Overproduction and purification of chorismate lyase. Throughout the course of the above-described experiments, we noticed that chorismate lyase activity could be best detected in strains that lacked other enzymes that use chorismate as a substrate. The host strain used for the overproduction studies was *E. coli* BN117 (*pheA tyrA ΔtrpEA2 pabA pabB*). We also noted that chorismate lyase production was enhanced when plasmids lacked the majority of *ubiA* and when certain portions of the 5′-flanking region of *ubiC* were deleted. To obtain a plasmid with high overproduction potential, we digested pBN168 (Fig. 2) with *Pst*I and *Sal*I and made short deletions by using exonuclease III and S1 nuclease. Following transformation of *E. coli* LE392 with the plasmid mixture, 40 colonies were grown and assayed for chorismate lyase activity. The plasmid producing the highest amount of chorismate lyase (pBN168Δ3) was chosen for additional experiments. *E. coli* BN117 was transformed with pBN168Δ3, and a series of pilot experiments showed that chorismate lyase production was maximal following overnight growth in rich medium and reached levels 250-fold higher than those in host cells grown under similar conditions.

Purification of chorismate lyase was achieved by pseudo-affinity chromatography on a Cibacron Blue 3GA-agarose column. One hundred milliliters of an S-30 crude extract (2.1 mg of protein per ml) was applied to a 10-ml column equilibrated in 50 mM Tris-HCl (pH 7.5)–100 mM NaCl–10 mM 2-mercaptoethanol, and 7-ml fractions were collected. Following sample application, the column was washed with 100 ml of equilibrating buffer. A 100-ml chorismate gradient (0 to 3 mM in equilibrating buffer) was applied, and 4-ml fractions were collected. Not all of the chorismate lyase activity bound to the column, and some activity continued to trickle off during the wash (Fig. 6). However, the majority of the chorismate lyase activity eluted with chorismate, and the eluted enzyme was judged >95% pure by Coomassie blue staining of an SDS-polyacrylamide gel (Fig. 7). The purification protocol resulted in a 46-fold purification from the crude extract, with a 25% yield. Approximately 1 mg of purified enzyme was obtained from 210 mg of crude extract (derived from approximately 2 g of cell paste). For preparations used in kinetic analyses, the enzyme was passed over a Sephacryl S-200 column (1.5 by 30 cm) to remove the substrate.

Determination of the N-terminal amino acid sequence of chorismate lyase. Samples of purified chorismate lyase were further purified by HPEC and adsorbed to a PVDF membrane, and the N-terminal amino acid sequence was determined. Twenty cycles of automated analysis yielded the sequence NH_2-SHPALTQLRALRYCKEIPAL-. This se-

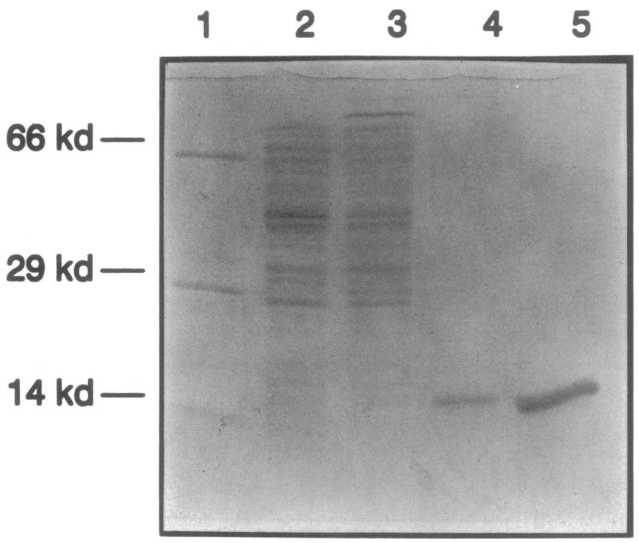

FIG. 7. SDS-PAGE of purified chorismate lyase. Lanes: 1, molecular mass standards; 2, S-30 extract of *E. coli* BN117 (10 μg); 3, S-30 extract of *E. coli* BN117/pBN168Δ3 (10 μg); 4, chorismate lyase (0.5 μg); 5, chorismate lyase (2.5 μg).

quence is consistent with the initiation of translation at the methionine residue indicated in Fig. 4, followed by the removal of the N-terminal formyl-methionine residue.

Characterization of chorismate lyase. To determine the kinetic constants of chorismate lyase, we performed initial velocity experiments at various substrate concentrations. Figure 8 shows a double-reciprocal plot of initial velocity versus chorismate concentration. From these data, the K_m for chorismate was determined to be 9.7 μM, and the V_{max} was determined to be 2.15 nmol of 4-hydroxybenzoate per 30 min. The turnover number for chorismate lyase was calculated to be 49 min^{-1}.

The capability of chorismate lyase to use 4-amino-4-deoxychorismate as a substrate was tested in vitro and in vivo. A reaction mixture containing purified 4-amino-4-deoxychorismate synthase components I and II was incubated with chorismate and glutamine for 60 min at 37°C to allow the formation of 4-amino-4-deoxychorismate (8). Purified chorismate lyase (35 U) was added, and the mixture was incubated for an additional 30 min. A portion of the reaction mixture was extracted with ethyl acetate, and 4-aminobenzoate was measured by fluorescence. A second portion of the reaction mixture was analyzed by HPLC for the presence of 4-amino-4-deoxychorismate, 4-aminobenzoate, 4-hydroxybenzoate, and chorismate. While it appeared that 4-aminobenzoate might have been produced in small amounts, the results of the experiment were not statistically convincing. We therefore tested the ability of the chorismate lyase-overproducing plasmid pBN168Δ3 to complement a *pabC* mutation. *E. coli* BN1044 (*pabC*::*kan*) was transformed with pBN168Δ3, and recipients were selected on rich medium supplemented with ampicillin and kanamycin. Colonies were streaked on minimal medium supplemented with ampicillin and kanamycin to test for complementation of the 4-amino-4-deoxychorismate lyase lesion. After several restreakings on minimal medium, BN1044 containing pBN168Δ3 continued to grow normally, while control strains containing pBSII SK+ failed to grow in the absence of 4-aminobenzoate.

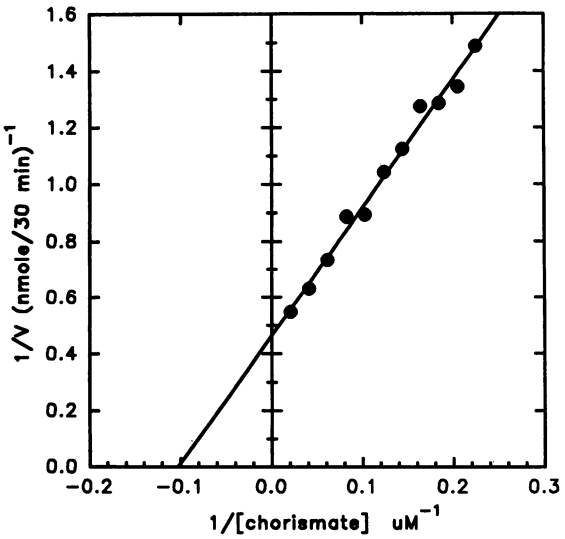

FIG. 8. Double-reciprocal plot of initial velocity versus chorismate concentration. The chorismate concentration ranged from 4.5 to 50 μM.

DISCUSSION

We have determined a 1,034-bp nucleotide sequence containing all of the *E. coli ubiC* gene and a portion of what is in all probability the beginning of the *ubiA* gene. The sequence that we have determined abuts with a sequence previously determined by others (16), completes the sequence of the putative *ubiA* gene, and defines the gene order in the 91.8-min region of the *E. coli* chromosome as *ubiC-ubiA-plsB-dgk-lexA*. *ubiC* and *ubiA* appear to be organized in a single transcription unit with a 15-bp purine-rich intercistronic sequence. *ubiC*, *ubiA*, *dgk*, and *lexA* are transcribed in the clockwise direction, while *plsB* is transcribed counterclockwise.

The products of *ubiC* and *ubiA* catalyze the first two steps of ubiquinone biosynthesis, the conversion of chorismate to 4-hydroxybenzoate and the condensation of 4-hydroxybenzoate with farnesylfarnesylgeraniol to form 3-octaprenyl-4-hydroxybenzoate (31). 4-Hydroxybenzoate octaprenyltransferase has been determined to be a membrane-bound enzyme (31), and a similarity search of GenBank with the FASTA algorithm (24) found 21% identity between 4-hydroxybenzoate octaprenyltransferase and the product of the *E. coli cyoE* gene. *cyoE* lies in a gene cluster encoding the cytochrome *o* ubiquinol oxidase complex, which functions to oxidize ubiquinol-8 to ubiquinone-8 (3). While the precise function of the *cyoE* gene product is not known, similarity to a ubiquinone biosynthetic enzyme suggests that it may have a function involving an interaction with ubiquinol-8 or ubiquinone-8.

The construction of plasmids that overproduced chorismate lyase over 250-fold allowed us to devise a single-step purification scheme that yielded an additional 50-fold purification and resulted in a homogeneous enzyme. The final purification factor, compared with those for wild-type strains, was nearly 10,000-fold. The K_m for chorismate, 9.7 μM, is somewhat higher than those of anthranilate synthase (1 μM) (2) and 4-amino-4-deoxychorismate lyase (4 μM) (26), is significantly lower than those of chorismate mutase-prephenate dehydratase (45 μM) (4) and chorismate mutase-

prephenate dehydrogenase (92 μM) (11), but is approximately the same as that of isochorismate synthase (14 μM) (18).

It is clear that a major mechanism for the derivation of enzymes with similar catalytic capabilities is the duplication of an ancestral gene (perhaps one with a broad substrate specificity) and the divergence of sequences that would modify that specificity. Such a duplication-divergence model has been used to explain the relationship between the two chorismate mutases (10) and the relationship among 4-amino-4-deoxychorismate synthase, anthranilate synthase, and isochorismate synthase (6, 12, 23). A similar duplication-divergence mechanism might have been a logical expectation in an investigation of the relationship between chorismate lyase and 4-amino-4-deoxychorismate lyase. The substrates differ only in the presence of a hydroxy group versus an amino group at position 4 of the cyclodiene ring, and the products are derived by elimination of the enol-pyruvyl moiety concomitantly with aromatization of the ring structure. Furthermore, we have shown by complementation that chorismate lyase has the capability to use 4-amino-4-deoxychorismate as a substrate. However, a similarity search of GenBank with the translated *ubiC* sequence did not reveal a significant relationship with 4-amino-4-deoxychorismate lyase or any other known proteins. However, 4-amino-4-deoxychorismate lyase is more similar to the branched-chain amino acid transaminase encoded by *E. coli ilvE* than to any other known *E. coli* enzyme (7, 14). On the basis of this finding, Green et al. (7) have shown that 4-amino-4-deoxychorismate lyase uses a pyridoxal cofactor in the conversion of 4-amino-4-deoxychorismate to 4-aminobenzoate. Presumably, the enhanced efficiency imparted to 4-amino-4-deoxychorismate lyase by the pyridoxal cofactor has precluded the evolution of chorismate lyase and 4-amino-4-deoxychorismate lyase from a common ancestral lyase. Rather, the two enzymes appear to represent an evolutionary convergence towards similar reaction capabilities from different ancestral progenitors.

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REFERENCES

1. **Anderson, K. S., W. M. Kati, Q.-Z. Ye, J. Liu, C. T. Walsh, A. J. Benesi, and K. A. Johnson.** 1991. Isolation and structure elucidation of the 4-amino-4-deoxychorismate intermediate in the PABA enzymatic pathway. J. Am. Chem. Soc. **113**:3198–3200.
2. **Baker, T. I., and I. P. Crawford.** 1966. Anthranilate synthetase. Partial purification and some kinetic studies on the enzyme from *Escherichia coli*. J. Biol. Chem. **241**:5577–5584.
3. **Chepuri, V., L. Lemieux, D. C.-T. Au, and R. B. Gennis.** 1990. The sequence of the *cyo* operon indicates substantial structural similarities between the cytochrome *o* ubiquinol oxidase of *Escherichia coli* and the aa_3-type family of cytochrome *c* oxidases. J. Biol. Chem. **265**:11185–11192.

3a. **Clarke, L., and J. Carbon.** 1976. A colony bank containing synthetic ColE1 hybrid plasmids representative of the entire *E. coli* genome. Cell **9**:91–99.

4. **Dopheide, T. A. A., P. Crewther, and B. E. Davidson.** 1972. Chorismate mutase-prephenate dehydratase from *Escherichia coli* K-12. J. Biol. Chem. **247**:4447–4452.
5. **Gibson, M. I., and F. Gibson.** 1964. Preliminary studies on the isolation and metabolism of an intermediate in aromatic biosynthesis: chorismic acid. Biochem. J. **90**:248–256.
6. **Goncharoff, P., and B. P. Nichols.** 1984. Nucleotide sequence of *Escherichia coli pabB* indicates a common evolutionary origin of *p*-aminobenzoate synthetase and anthranilate synthetase. J. Bacteriol. **159**:57–62.
7. **Green, J. M., W. K. Merkel, and B. P. Nichols.** 1992. Characterization and sequence of *Escherichia coli pabC*, the gene encoding aminodeoxychorismate lyase, a pyridoxal phosphate-containing enzyme. J. Bacteriol. **174**:5317–5323.
8. **Green, J. M., and B. P. Nichols.** 1991. *p*-Aminobenzoate biosynthesis in *Escherichia coli*: purification of aminodeoxychorismate lyase and cloning of *pabC*. J. Biol. Chem. **266**:12971–12975.
9. **Henikoff, S.** 1987. Unidirectional digestion with exonuclease III in DNA sequence analysis. Methods Enzymol. **155**:156–165.
10. **Hudson, G. S., and B. E. Davidson.** 1984. Nucleotide sequence and transcription of the phenylalanine and tyrosine operons of *Escherichia coli* K12. J. Mol. Biol. **180**:1023–1051.
11. **Hudson, G. S., V. Wong, and B. E. Davidson.** 1985. Chorismate mutase-prephenate dehydrogenase from *Escherichia coli* K-12. Purification, characterization and identification of a reactive cysteine. Biochemistry **23**:6240–6249.
12. **Kaplan, J. B., and B. P. Nichols.** 1983. Nucleotide sequence of *Escherichia coli pabA* and its evolutionary relationship to *trp(G)D*. J. Mol. Biol. **168**:451–468.
13. **Kohara, Y., K. Akiyama, and K. Isono.** 1987. The physical map of the whole *E. coli* chromosome: application of a new strategy for rapid analysis and sorting of a large genomic library. Cell **50**:495–508.
14. **Kuramitsu, S., T. Ogawa, H. Ogawa, and H. Kagimiyama.** 1985. Branched-chain amino acid aminotransferase of *Escherichia coli*: nucleotide sequence of the *ilvE* gene and the deduced amino acid sequence. J. Biochem. **97**:993–999.
15. **Lawrence, J., G. B. Cox, and F. Gibson.** 1974. Biosynthesis of ubiquinone in *Escherichia coli* K-12: biochemical and genetic characterization of a mutant unable to convert chorismate into 4-hydroxybenzoate. J. Bacteriol. **118**:41–45.
16. **Lightner, V. A., R. M. Bell, and P. Modrich.** 1983. The DNA sequences encoding *plsB* and *dgk* loci of *Escherichia coli*. J. Biol. Chem. **258**:10856–10861.
17. **Little, J. W.** 1980. Isolation of recombinant plasmids and phage carrying the *lexA* gene of *Escherichia coli* K-12. Gene **10**:237–247.
18. **Liu, J., N. Quinn, G. A. Berchtold, and C. T. Walsh.** 1990. Overexpression, purification, and characterization of isochorismate synthase (EntC), the first enzyme involved in the biosynthesis of enterobactin from chorismate. Biochemistry **29**:1417–1425.
19. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. **Nichols, B. P., G. F. Miozzari, M. vanCleemput, G. N. Bennett, and C. Yanofsky.** 1980. Nucleotide sequences of the *trpG* regions of *Escherichia coli*, *Shigella dysenteriae*, *Salmonella typhimurium*, and *Serratia marcescens*. J. Mol. Biol. **142**:503–517.
21. **Nichols, B. P., A. S. Seibold, and S. Z. Doktor.** 1989. *para*-Aminobenzoate synthesis from chorismate occurs in two steps. J. Biol. Chem. **264**:8597–8601.
22. **Nichols, B. P., M. vanCleemput, and C. Yanofsky.** 1981. Nucleotide sequence of *Escherichia coli trpE*: anthranilate synthetase component I contains no tryptophan residues. J. Mol. Biol. **146**:45–54.
23. **Ozenberger, B. A., T. J. Brickman, and M. A. McIntosh.** 1989. Nucleotide sequence of *Escherichia coli* isochorismate synthetase gene *entC* and evolutionary relationship of isochorismate synthetase and other chorismate-utilizing enzymes. J. Bacteriol. **171**:775–783.
24. **Pearson, W. R., and D. J. Lipman.** 1988. Improved tools for

biological sequence comparison. Proc. Natl. Acad. Sci. USA **85:**2444–2448.

25. **Tabor, S.** 1990. Expression using the T7 RNA polymerase/promoter system, p. 16.2.1–16.2.11. *In* F. A. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology. Greene Publishing and Wiley-Interscience, New York.
26. **Viswanathan, V. K., J. M. Green, E. A. Rayl, and B. P. Nichols.** Submitted for publication.
27. **Walsh, C. T., J. Liu, F. Rusnak, and M. Sakaitani.** 1990. Molecular studies on enzymes in chorismate metabolism and the enterobactin biosynthetic pathway. Chem. Rev. **90:**1105–1129.
28. **Weische, A., and E. Leistner.** 1985. Cell free synthesis of *o*-succinylbenzoic acid from *iso*-chorismic acid, the key reaction in vitamin K (menaquinone) biosynthesis. Tetrahedron Lett. **26:**1487–1490.
29. **Ye, Q.-Z., J. Liu, and C. T. Walsh.** 1990. *p*-Aminobenzoate synthesis in *Escherichia coli*: purification and characterization of PabB as aminodeoxychorismate synthase and enzyme X as aminodeoxychorismate lyase. Proc. Natl. Acad. Sci. USA **87:**9391–9395.
30. **Young, I. G., L. Langman, R. K. J. Luke, and F. Gibson.** 1971. Biosynthesis of the iron transport compound enterobactin: mutants of *Escherichia coli* unable to synthesize 2,3-dihydroxybenzoate. J. Bacteriol. **106:**51–57.
31. **Young, I. G., R. A. Leppik, J. A. Hamilton, and F. Gibson.** 1972. Biochemical and genetic studies on ubiquinone biosynthesis in *Escherichia coli* K-12: 4-hydroxybenzoate octaprenyltransferase. J. Bacteriol. **110:**18–25.