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Genetic and Molecular Analysis of *aroL*, the Gene for Shikimate Kinase II in *Escherichia coli* K-12

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The gene *aroL* in *Escherichia coli* K-12, specifying shikimate kinase II, was contransduced with *proC* at a frequency of 99%. The gene order is *lac proC aroL*. A 2.7-kilobase *Bam*HI fragment containing *aroL*$^+$ was cloned into pBR322. This plasmid conferred highly elevated levels of shikimate kinase synthesis which were subject to repression control by *tyrR*. The *aroL* gene was localized within a 730-base-pair region by both subcloning and insertional mutagenesis with Tn*1000*. A second gene, designated *aroM* and encoding a protein of molecular weight 26,000, is cotranscribed with *aroL*. Transcription proceeds in the order *aroL aroM* in a clockwise direction on the chromosome. The function of *aroM* remains unknown.

It was Berlyn and Giles (2) who first demonstrated that *Escherichia coli* contains two enzymes able to convert shikimate to shikimate 3-phosphate, step 5 in the biosynthesis of aromatic compounds. Ely and Pittard (15) confirmed this finding and isolated a mutant strain in which one of these isoenzymes (shikimate kinase II) was nonfunctional. The structural gene for this isoenzyme, *aroL*, was located close to but was not cotransducible with *purE*, and its expression was shown to be under the control of the regulator gene *tyrR* (15). This gene has been shown to control the expression of a number of genes coding for biosynthetic and transport functions associated with the aromatic amino acids (5–7, 19, 37, 38). This paper describes an extension of these studies in which a more precise map location is established for *aroL*, the gene is cloned on multicopy plasmids, and the transcriptional unit containing this gene is examined in more detail.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used in this investigation are listed in Table 1. The bacterial strains were *E. coli* K-12 derivatives. CA7027 was obtained from J. Beckwith, and W3110 was obtained from C. Yanofsky. All other strains were from this laboratory. An *E. coli* genomic library (40) was a gift from I. Young. This pool of chimeric plasmids consisted of *Bam*HI fragments inserted into the *Bam*HI site of pSF2124 (36) and was present in the host strain C600 (F$^+$). Plasmid pMU371 was maintained in *recA* host strains to enhance its stability.

Chemicals. The chemicals used were obtained commercially and not further purified. Shikimic acid and X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) were from Sigma Chemical Co., and D-[^{14}C]shikimic acid (84 mCi/mmol) and L-[^{35}S]methionine were from the Radiochemical Centre, Amersham, England.

Media. Unless otherwise specified, the minimal medium used was half-strength medium 56, as described by Monod et al. (29), supplemented with 0.2% glucose, thiamine (10 μg/ml), and appropriate growth factors. When the minimal medium was supplemented with repressing concentrations of the aromatic amino acids and vitamins (end products), they were added at the following concentrations: L-phenylalanine, 10^{-3} M; L-tyrosine, 10^{-3} M; L-tryptophan, 5×10^{-4} M; *p*-aminobenzoic acid, 10^{-6} M; *p*-hydroxybenzoic acid, 4×10^{-6} M; and 2,3-dihydroxybenzoic acid, 5×10^{-5} M.

Ampicillin concentrations used were between 25 and 300 μg/ml, tetracycline was used at a final concentration of 5 μg/ml in minimal medium and 15 μg/ml in nutrient medium, kanamycin was used at 25 μg/ml, and X-gal was used at 30 μg/ml.

Genetic methods. Transductions using P1 *kc* were carried out by the method previously described (32). The pSF2124-derived plasmids of the genomic library were introduced into strain JP1664 by conjugation (28), with a donor/recipient ratio of 1:1. Ap^r recipients in the conjugation mixture were enriched by growth in Luria broth containing ampicillin (200 μg/ml), with tetracycline (15 μg/ml) present to select against the donors. The enriched mixture was plated onto minimal agar containing ampicillin and tetracycline (5 μg/ml) at a cell density of approximately 10^7 cells per plate.

Plasmid pMU309 (16) was introduced into strains by conjugation (28), selecting for resistance to 50 μg of kanamycin per ml. It was necessary to use recipients which were Mu 1 lysogens to avoid zygotic induction of the Mu prophage present on pMU309. Strains were lysogenized with Mu 1 by the method of Casadaban (8).

Lysates of the phage Mu dl (*lac*Ap) were prepared and used as described by Casadaban and Cohen (9).

Two methods were developed to distinguish *aroL* and *aroL*$^+$ strains. In an *aroF aroG* background, a mutation in *aroL* dramatically affects growth rate on minimal medium lacking the aromatic amino acids. Strains that are *aroL*$^+$ grow quite well on this medium, which can therefore be used to screen for aroL phenotype. In the second method, which was discovered fortuitously, strains are plated on to medium containing supplements of leucine, isoleucine, valine, and adenine. On this medium, growth of *aroL* strains is inhibited relative to isogenic *aroL*$^+$ strains. The reason for this growth inhibition is not understood. AroL phenotypes of strains were generally confirmed by shikimate kinase assays.

Recombinant DNA techniques. Chromosomal DNA was isolated by the method of Marmur (26). Plasmid DNA was amplified in growing cultures by the addition of chloram-

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TABLE 1. Bacterial strains and plasmids

Bacterial strains	Relevant genotype[a]
CA7027	HfrH Δ(*lacIOZYA*)*U169*
JP1634	*aroL476 proC14 tyrR366 lacY1 rpsL109*
JP1640	*ara leu proC14 aroL476 purE357 gal trp his argG ilv metA* or *metB*
JP1656	*aroF363* Δ*aroG395 aroL476 tyrR366 proC14 proA2 argG6 thr-352 leu-351 his-4*
JP1657	JP1656 *srl-300*::Tn*10 recA56*
JP1658	JP1656 *srl-300*::Tn*10 recA56 gyrA*
JP1662	*aroF363* Δ*aroG395 aroL476 proA2 proC14 argG6 thr-352 leu-351*
JP1663	*aroF363* Δ*aroG395 aroL476 tyrR366 proA2 proC14 argG6 thr-352 leu-351 srl300*::Tn*10 recA56*
JP1664	JP1662 *srl300*::Tn*10 recA56*
JP1666	JP1663(pMU370) F^+
JP1667	JP1664(pMU370) F^+
JP1669	JP1664 ΔTn*10*
JP1670	JP1662(pMU371)
JP1672	JP1669(pMU371)
JP1681	F^+ *purE trp his argG ilv leu met*(pMU371)
JP1694	JP1663(pMU371)
JP1713	JP1672)pMU309), lysogenic for Mu 1
JP1717	*aroF363* Δ*aroG395 proC14 argG6* Δ(*lacIOZYA*)*U169 zaj-2*::Tn*10* (Mu 1)
JP1732	JP1717 *proC+ aroM500*:: Mu dl (*lac* Ap)
JP1733	JP1732 *tyrR366 zci-2*::Tn*10*
JP2167	*aroF363* Δ*aroG395* Δ(*gal nadA*) *AroH367 argG6 thi-1 thr-352 leu-351 proA2 his-4 pyrF40 lacY1*
JP2230	*aroF363* Δ*aroG395 tyrR366 lacY1 argG6 thi-1 thr-352 leu-351 proA2 his-4*
W3110	Prototroph

[a] Gene designations are according to Bachmann (1).

phenicol (18) and isolated from cleared lysates (10) on CsCl-ethidium bromide gradients (35). Small-scale plasmid DNA isolations were by the method of Klein et al. (21). The restriction endonuclease *Rsa*I, purified by the method of Lynn et al. (24), was a gift from B. Davidson. Other restriction endonucleases and T4 DNA ligase (Boehringer Mannheim Biochemicals) were used as recommended by the manufacturers. DNA fragments were analyzed by agarose gel electrophoresis (12) or polyacrylamide gel electrophoresis (25). Transformation of $CaCl_2$-induced competent cells was by the method of Morrison (30).

Maxicell labeling of plasmid-encoded proteins. Plasmid-encoded proteins were labeled with L-[^{35}S]methionine in maxicells essentially as described by Sancar et al. (34). The proteins were separated on sodium dodecyl suflate-polyacrylamide gels (12.5 or 17%) by the method of Laemmli (22) and visualized by fluorography (3).

Enzyme assays. Shikimate kinase activity in cell extracts was assayed by measuring the rate of conversion of ^{14}C-labeled shikimate to shikimate 3-phosphate. Cultures were grown and cell extracts were prepared as described previously (15). Suitable dilutions of the cell extracts were added to the following in a total reaction volume of 1 ml: 25 mM veronal buffer (pH 9.0), 5 mM $MgCl_2$, 10 mM NaF, 5 mM ATP, 1 mM dithiothreitol, 1 mM shikimic acid, and 0.1 μCi of D-[^{14}C]shikimic acid. Samples (0.1 ml) were removed at 5-min intervals for 25 min, and each sample was pipetted into 0.4 ml of *n*-butanol–glacial acetic acid (100:6) to stop the reaction and then filtered through 25-mm DEAE-cellulose disks (Whatman DE81). The filters were then washed four times with 1 ml of *n*-butanol–glacial acetic acid–water (100:6:25) each time and dried, and the radioactivity retained on the filters was counted by liquid scintillation counting. Activities were expressed as nanomoles of product formed per minute (milliunits) per milligram of protein in the cell extracts.

β-Galactosidase activities were determined by using toluene-treated cells by the method of Miller (28). Activities are expressed as units, which are defined as follows:

$$\text{units} = 1{,}000 \times \{[OD_{420} - (1.75 \times OD_{550})]/(t \times v \times OD_{600})\}$$

where t is time of assay in minutes, v is the volume in milliliters of the cell culture used in the assay, and OD_{600} is the optical density at 600 nm of the cell culture used.

Protein estimations. Protein concentrations in cell extracts were determined by the method of Lowry et al. (23).

RESULTS

Transductional mapping of *aroL*. Previous work located *aroL* in the region of the chromosome including *proC* and *purE* (15). No cotransduction by bacteriophage P1 was observed between *aroL* and *purE*, and linkage with *proC* had not previously been tested. To test this linkage, strain JP1634 carrying both the *aroL476* and *proC14* mutations was constructed. When P1 grown on the prototroph W3110 was used to transduce JP1634 to Pro$^+$, 113 of 114 transductants were found to be *aroL*$^+$. In a second transduction involving a *proC*$^+$ *aroL476* donor and a *proC14 aroL*$^+$ recipient, 40 of 40 Pro$^+$ transductants had the donor *aroL476* allele.

With strains in which transposon Tn*10* was inserted into the *aroL* gene, further transductions involving the markers Lac, Pro, and Tc^r indicated that the likely gene order was *aroL*::Tn*10 proC lac* (data not shown).

Cloning of *aroL*. Because *aroL* mutants are not aromatic auxotrophs (15), we had no simple selection for the AroL$^+$ phenotype. This problem was overcome when we investigated the growth properties of a strain which, in addition to a mutation in *aroL*, had mutations in *aroF* and *aroG* that inactivated two of the three 3-deoxy-D-arabinoheptulsonic acid 7-phosphate (DAHP) synthase enzymes, i.e., DAHP synthase (tyr) and DAHP synthase (phe). This strain, JP1656, grows poorly in minimal medium in the absence of aromatic amino acids. The corresponding *aroL*$^+$ strain JP2230 grows well on this medium. The difference in growth rates was found to be sufficient to allow selection of *aroL*$^+$ clones from a background of *aroL aroF aroG* cells.

To isolate a high-copy-number plasmid containing the *aroL*$^+$ gene, chimeric plasmids from an *E. coli* genomic library (40) were introduced into JP1664 by conjugation, and selection was made for Aro$^+$ Ap^r colonies. Eight such clones were purified, and when plasmid DNAs from these clones were digested with *Bam*HI, all were shown to contain plasmids with identical 2.7-kilobase *Bam*HI inserts in pSF2124. The insert from one of these plasmids, designated pMU370, was recloned into the *Bam*HI site of pBR322. Plasmids were obtained containing the insert in both orientations in pBR322; one of each kind was retained, and these two plasmids were designated pMU371 and pMU372. A restriction enzyme cleavage map of the insert in pMU371 is shown in Fig. 1. The insert was not cleaved by the enzymes *Bgl*I, *Eco*RI, *Hin*dIII, *Pst*I, *Pvu*I, *Sal*I, *Sma*I, *Xba*I, or *Xho*I.

Shikimate kinase activities of strains with *aroL*$^+$ plasmids. The presence of pMU370 or pMU371 in the *tyrR* host JP1663 (JP1666 and JP1694) conferred levels of shikimate kinase activity that were about 7- or 40- to 60-fold higher, respectively, than that of the haploid *aroL*$^+$ strain JP2230 (Table 2).

TABLE 2. Comparison of shikimate kinase activities of strains containing *aroL*$^+$ plasmids or haploid for *aroL*$^+$

Strain[a]	Plasmid	Shikimate kinase activity (mU/mg) of:			
		Strain with chromosomal genotype		Strain grown in[b]:	
		aroL	*tyrR*	MM	MM + EP
JP1657		476	366	NT	0.8
JP1666	pMU370	476	366	263	163
JP1667	pMU370	476	+	101	15
JP1694	pMU371	476	366	NT	1,520
JP1672	pMU371	476	+	NT	760
JP1713	pMU371, pMU309	476	+ (Multicopy)	NT	110
JP2230		+	366	38	25
JP2167		+	+	24[c]	3.6

[a] All strains are *aroF aroG*.
[b] MM, Minimal medium; MM + EP, minimal medium supplemented with the aromatic end products; NT, not tested.
[c] Shikimic acid was added to the minimal medium for growth of JP2167.

These changes in enzyme levels roughly reflect the proposed copy numbers for pSF2124 (35) and pBR322 (11), although in the latter case the levels are slightly higher than would have been predicted. When pMU370 or pMU371 was present in an isogenic *tyrR*$^+$ strain, it could be shown that after growth of the strain in the presence of the aromatic amino acids, the levels of shikimate kinase activity were lower than those observed in *tyrR* strains grown under similar conditions. The observed difference was 11-fold between JP1667 and JP1666, but only 2-fold between JP1672 and JP1694 (Table 2). The latter result suggested that in JP1672, the TyrR protein molecules were being titrated by the large number of *aroL* operators, thus allowing considerable escape synthesis of shikimate kinase. This possibility was proved to be true by the introduction into JP1711 (a Mu 1 lysogen of JP1672) of the plasmid pMU309, a derivative of RP4 which carries

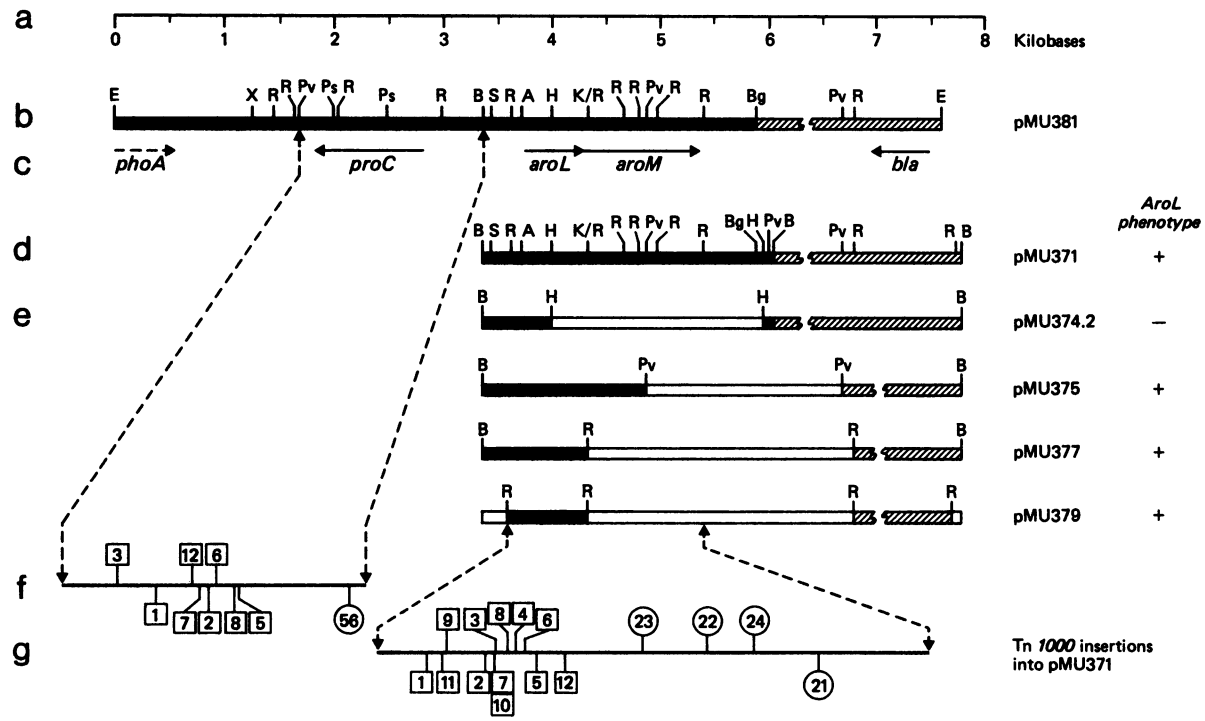

FIG. 1. Physical and genetic maps of the region of the *E. coli* chromosome containing *aroL*. (a) Kilobase scale. (b) Restriction enzyme cleavage map of pMU381. Chromosomally derived DNA is indicated by the solid bar, and pBR322 DNA (not drawn to scale) is represented by the striped bar. Cleavage sites for the enzymes are designated as follows: A, *AvaI*; B, *Bam*HI; Bg, *Bgl*II, E, *Eco*RI; H, *Hpa*I, K, *Kpn*I; Ps, *Pst*I; Pv, *Pvu*II; R, *Rsa*I; S, *Sst*II; X, *Xho*I. (c) Locations of genes which have been identified. The dashed arrow indicates the 3′ end of the *phoA* gene. Gene designations are according to Bachmann (1). *bla* indicates the β-lactamase gene of pBR322. (d) Restriction enzyme cleavage map of pMU371. (e) Deletion derivatives of pMU371. Open bars indicate the extent of the deletions. (f) Sites of Tn*1000* insertions into pMU381. The insertional derivatives are designated pMU382.01 to pMU382.12. Boxes indicate Pro$^-$ insertions, and the circle indicates a Pro$^+$ insertion. Numbers inside the boxes correspond to the plasmid designations, e.g., 1 refers to pMU382.01, etc. (g) Sites of Tn*1000* insertions into pMU371. The insertional derivatives are designated pMU378.01 to pMU378.24. Boxes indicate AroL$^-$ insertions, and circles indicate AroL$^+$ insertions. Numbers correspond to the plasmid designations, e.g., refers to pMU378.01, etc. (f and g) Numbers above and below the line represent opposite orientations of Tn*1000*.

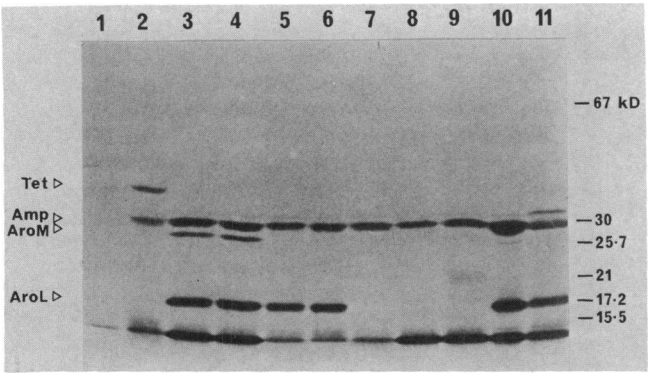

FIG. 2. Fluorograph of ^{35}S-labeled proteins from maxicell preparations, after electrophoresis on a sodium dodecyl sulfate–12% polyacrylamide gel. The lanes contain extracts from strain CSR603 (33) containing the following plasmids: lane 1, no plasmid; lane 2, pBR322; lane 3, pMU371; lane 4, pMU372 (contains the same chromosomal insert as pMU371 but in the opposite orientation); lane 5, pMU375; lane 6, pMU377; lane 7, pMU374.2; lane 8, pMU378.11; lane 9, pMU378.12; lane 10, pMU378.23; and lane 11, pMU378.22. kD, Kilodaltons.

tyrR$^+$ and has a copy number of 4 to 7 (16). When the copy number of *tyrR*$^+$ was increased in this way, there was a further sevenfold repression of shikimate kinase synthesis. (Table 2) These results also indicated that the *aroL* operator is intact in pMU370 and pMU371.

Localization of the *aroL* gene on pMU371. To determine the location of *aroL* on the 2.7-kb insert in pMU371, several derivative plasmids were constructed by the deletion of specific restriction endonuclease fragments (Fig. 1) and subsequent introduction of the deleted plasmids into the *aroL* strain JP1669. The presence or absence of a functional *aroL* gene on each of these plasmids was demonstrated by shikimate kinase assays. Plasmid pMU375 was constructed by the deletion of two *Pvu*II fragments from pMU371, and pMU377 was constructed by the deletion of five *Rsa*I fragments. Both of these plasmids remained AroL$^+$, indicating the *aroL* lay to the left of the *Kpn*I site on pMU371. When the *Hpa*I fragment was deleted, the resultant plasmid (pMU374.2) was AroL$^-$, thus localizing the right-hand boundary of *aroL* between the *Hpa*I and *Kpn*I sites. Plasmid pMU379 was constructed by deleting an *Rsa*I fragment from pMU377. pMU379 contains only 730 base pairs of chromosomally derived DNA, but remains AroL$^+$, indicating that *aroL* lies within this region.

The location of *aroL* was confirmed by the transposition of Tn*1000* (γδ) into pMU371 and the mapping of the sites of insertion which inactivated the gene. Transpositional derivatives of pMU371 were obtained by the F-mediated conduction (17) of pMU371 from JP1681 into JP1658 (*aroL aroF aroG*). Recipient cells which were resistant to 200 μg of ampicillin per ml were selected and screened for their Aro phenotype. The majority were Aro$^+$, but 12 of 1,020 of those screened were Aro$^-$. The sites of insertion in these derivatives were clustered within a 0.5-kb region spanning the *Hpa*I site (Fig. 1). All of the sites lay within the 0.73-kb *Rsa*I fragment shown by subcloning to contain *aroL*.

Analysis of pMU371-encoded proteins in maxicells. The maxicell technique of Sancar et al. (34) was used to analyze the proteins encoded by pMU371 and several of its derivatives. At least two proteins, of 26 and 17.5 kilodaltons (kDa), were clearly encoded by pMU371 but not by pBR322 (Fig. 2). A third protein, which travelled with the front during electrophoresis on 12% polyacrylamide gels, might also be encoded by the chromosomal insert of pMU371 (compare lanes 2 and 3, Fig. 2). The 17.5-kDa protein was clearly shikimate kinase II, as it was the only protein apart from β-lactamases encoded by pMU375 and pMU377, both of which are *aroL*$^+$. This 17.5-kDa protein was absent from the maxicells containing pMU374.2 or the Tn*1000* insertion plasmids pMU378.11 or pMU378.12 (Fig. 2), all of which fail to confer an AroL$^+$ phenotype. Since it was not present in the maxicells containing pMU375, the 26-kDa protein is encoded in part or in full within the region to the right of the *Pvu*II site at coordinate 4.8 kb (Fig. 1). Surprisingly, the Tn*1000* insertions in pMU378.11 and pMU378.12 resulted in the loss of the 26-kDa protein as well as the 17.5-kDa shikimate kinase II (Fig. 2). This unexpected result indicated that these Tn*1000* insertions in *aroL* had a strong polar effect on the gene encoding the 26-kDa protein and suggested that this gene was expressed from the *aroL* promoter. This second gene is henceforth designated *aroM*.

Four plasmids were isolated that contained Tn*1000* insertions within the 0.6-kb region to the right of *aroL* (Fig. 1). When proteins encoded by two of these plasmids (Fig. 2, lanes 10 and 11) were examined in maxicells, it could be seen that the 26-kDa protein was absent, whereas shikimate kinase II was still produced. Hence it could be concluded that *aroL* and *aroM* are cotranscribed and that the direction of transcription of this operon is from left to right on pMU371.

Isolation of a Mu d1 (*lac* Ap) insertion into the *aroLM* operon. The existence of a second gene under *tyrR* control, closely linked to *aroL*, was confirmed by using the transposon Mu d1 (*lac* Ap) (9). The approach used to isolate *aroLM*::Mu d1 (*lac* Ap) lysogens made use of the high cotransduction frequency between *proC* and *aroL*. Strain CA7027 was lysogenized with Mu d1 (*lac* Ap), and Ap^r colonies were selected at 30°C. Approximately 80,000 colonies were pooled, and a P1 lysate grown on this pool was used to transduce JP1717 simultaneously to Pro$^+$ and Ap^r. One transductant (JP1732) was obtained that had all of the characteristics expected of a strain with Mu d1 (*lac* Ap) inserted on the downstream side of *aroL* but still within the *aroL* transcription unit. First, the Ap^r marker of the phage in JP1732 was tightly linked to *proC* in P1-mediated transductions with JP1717 as the recipient. When the primary selection was for Ap^r, 99% (126 of 127) of the transductants were Pro$^+$, whereas when the selection was for Pro$^+$, 83% (116 of 130) of the transductants were Ap^r. Second, the β-galactosidase activity in the *tyrR* derivative JP1733 was elevated sixfold compared with the activity in JP1732, indicating regulation by *tyrR* of *lacZ* expression in JP1732. Third, as shown by the repression patterns of shikimate kinase (Table 3), JP1732 retained the *aroL*-specified shikimate kinase activity, and hence the phage insertion may be presumed to be distal to *aroL* in the transcriptional unit. Whether it is within *aroM* or in the region between *aroL* and *aroM* (13) has not yet been established.

Cultures of JP1732 were grown in minimal media supplemented with various combinations of aromatic amino acids. β-galactosidase and shikimate kinase assays performed on these cultures demonstrated that the expression of *lacZ* in JP1732 showed a similar pattern of regulation to the expression of *aroL* (Table 3). Both enzymes showed significant repression when the strain was grown in the presence of either tyrosine or phenylalanine singly or all three aromatic amino acids. The regulation of *aroL* and *lacZ* in JP1732 by

tryptophan could not be monitored, as this strain has only the *aroH*-specified, tryptophan-inhibitable DAHP synthase isoenzyme and therefore would not grow if the medium were supplemented with tryptophan in the absence of tyrosine or phenylalanine.

What is the function of the *aroM* protein? The phenotype of strain JP1732 was identical to that of its parent, JP1717, in that JP1732 did not have any additional growth requirements, remained Aro$^+$, and grew at an identical rate in minimal medium either with or without aromatic amino acid supplementation. Strain JP3123, which carries an insertion of the transposon Tn*10* into *aroL*, also remained Aro$^+$ (S. Wilcox, unpublished observation). This insertion is expected to prevent the expression of more distal genes in the operon due to polarity effects. If the insertion of Mu dl (*lac* Ap) in JP1732 is within *aroM* or in the region between *aroM* and *AroL*, we would also expect *aroM* function to be abolished. Hence, we conclude that the function of *aroM*, if there be any, is not required for growth on minimal medium.

In view of both the dispensability of *aroM* and its organization in an operon with *aroL*, it seemed possible that the *aroM* gene product was involved in the uptake of shikimate. This compound is actively taken up from the medium and utilized for the synthesis of aromatic compounds by most strains of *E. coli* (4, 33). However, an examination of shikimate uptake into strains containing pMU371 (*aroL*$^+$ *aroM*$^+$) or pMU377 (*aroL*$^+$) showed that the rates of uptake were not significantly different in these strains. In addition, shikimate transport was unimpaired in strain JP3123 (*aroL478*::Tn*10*). These results indicate that *aroM* is not involved in shikimate transport. The function of *aroM*, if there be any, remains unknown.

Isolation of a clone carrying both *proC* and *aroL*. Transductional mapping had indicated a close proximity of *aroL* to *proC* on the chromosome and a probable gene order in this region of *phoA proC aroL*. Since the introduction of pMU371 into JP1640 (*proC14*) did not render this strain Pro$^+$, this plasmid did not contain a functional *proC* gene. To confirm the transductional mapping, it was desirable to attempt to clone a fragment containing both *proC*$^+$ and *aroL*$^+$, utilizing the *Eco*RI site known to be present in *phoA* (20).

Chromosomal DNA from strain W3110 was digested with both *Eco*RI and *Bgl*II and ligated into the *Eco*RI-*Bam*HI site of pBR322. The mixture was used to transform JP1640 simultaneously to Pro$^+$ and Ap^r. The plasmid from one clone, designated pMU380, contained a 5.84-kb *Eco*RI-*Bgl*II fragment encoding both *proC*$^+$ and *aroL*$^+$ and an additional 2.1-kb *Bgl*II fragment. The *Eco*RI-*Bgl*II fragment was recloned into pBR322, yielding pMU381. The 5.84-kb insert was not cut by *Hin*dIII or *Sal*I.

TABLE 3. Regulation by tyrosine and phenylalanine of β-galactosidase and shikimate kinase expression in JP1732

Growth medium[a]	Shikimate kinase activity (mU/mg) in strain[b]:				β-Galactosidase activity (U)[c] in strain:	
	JP1717	JP1722	JP1732	JP1733	JP1732	JP1733
MM	36.4		55.0		18	
MM + Tyr	7.3		9.2		8	
MM + Phe	17.6		18.8		10	
MM + Tyr + Phe	10.0		11.1		11	
MM + EP	4.7	38.9	8.7	66.9	9	53

[a] MM, Minimal medium; MM + EP, minimal medium supplemented with the aromatic amino acids and vitamins as specified in Materials and Methods.
[b] JP1722 and JP1733 are *tyrR366* derivatives of JP1717 and JP1732, respectively.
[c] β-Galactosidase activity is expressed as described in Materials and Methods.

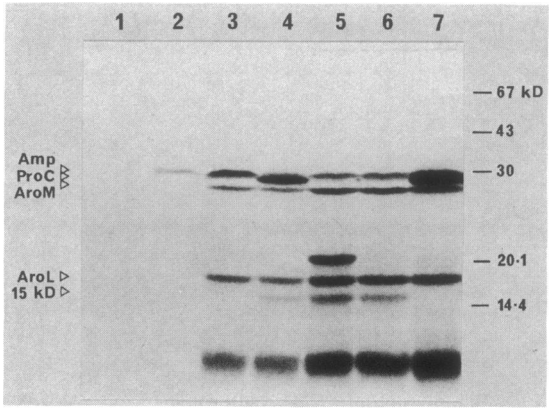

FIG. 3. Fluorograph of ^{35}S-labeled proteins from maxicell preparations, after electrophoresis on a sodium dodecyl sulfate–12% polyacrylamide gel. The lanes contain extracts from strain CSR603 containing the following plasmids: lane 1, no plasmid (CSR603 control); lane 2, pBR322; lane 3, pMU371; lane 4, pMU381; lane 5, pMU382.01; lane 6, pMU382.05; and lane 7, pMU382.56. The *proC* gene product and β-lactamase were not resolved on this gel but were resolved on other gels. kD, Kilodaltons.

The location of *proC* on pMU381 was determined by insertion of Tn*1000* into the plasmid and mapping of the sites of insertion which inactivated the gene, as described above for *aroL*. Unambiguous map locations were assigned to eight Pro$^-$ transpositional insertions (Fig. 1); all of these sites were clustered in a region of 0.7 kb spanning two *Pst*I sites in pMU381. One transpositional insertion, in the plasmid pMU382.56, which mapped between the *proC* and *aroL* genes, was also identified. This plasmid retained functional *proC*$^+$ and *aroL*$^+$ genes.

The maxicell technique (Fig. 3) indicated that pMU381 encoded β-lactamase and at least five other proteins of 28, 26.5, 17, 15, and less than 11 kDa. From previous results the 17- and 26.5-kDa proteins could be identified as the *aroL* and *aroM* gene products, respectively. The 28-kDa protein was absent from the insertional derivatives of pMU381 that were Pro$^-$, and this protein is therefore the *proC* gene product. The insertion plasmid pMU382.01 encoded instead a polypeptide of 20 kDa. If this band represents a truncated *proC* polypeptide, then it can be deduced that the direction of transcription of *proC* was from right to left on pMU381 (Fig. 1) and hence counterclockwise on the *E. coli* chromosome. The data also indicated that the 15-kDa protein, which was absent from maxicells containing the insertion plasmid pMU382.56, was encoded within the region of pMU381 between *proC* and *aroL*.

DISCUSSION

Genetic experiments indicated that the gene *aroL* is tightly linked to *proC*, being coinherited at a frequency of 99% in P1-mediated transductions. The gene order relative to *lac* was *lac proC aroL*. When considered in conjunction with the results of Nakata et al. (31) and Yagil et al. (39), this fact

indicates that the gene order around *aroL* is *phoA proC aroL aroM phoB*. Subsequent cloning work established that *aroL* and *proC* are separated by not more than 0.9 kb, as measured by transpositional inactivation of the cloned genes. The analysis of plasmid-encoded proteins in maxicells showed that the region between the genes contained an unidentified gene that encoded a 15-kDa polypeptide.

The development of a method to distinguish *aroL*$^+$ and *aroL* strains allowed us to select directly for strains that contained *aroL*$^+$ plasmids. Presumably the strains lacking the two major DAHP synthase isoenzymes (*aroF* and *aroG*) cannot accumulate an internal pool of shikimate that is sufficient for shikimate kinase I alone to carry out the phosphorylation, but the reaction can be performed by shikimate kinase II. Preliminary studies on the affinity of the shikimate kinase isoenzymes for shikimate support this interpretation, the K_m of shikimate kinase I being approximately 2 orders of magnitude higher than that of shikimate kinase II (13a). The excretion of shikimate into the growth medium by *aroL* strains which have wild-type DAHP synthase levels (B. Ely, unpublished observations) also supports this notion.

The *aroL* gene was localized to within a 0.73-kb region of the cloned DNA by both subcloning and Tn*1000* insertional inactivation, and shikimate kinase II was identified as a 17.5-kDa protein in maxicells. The size of this subunit is similar to the estimate of 20 kDa previously obtained for the native isoenzyme (15), suggesting that the native enzyme is a monomer. Assuming an average residue weight of 110, this molecular weight corresponds to a coding region of 0.45 to 0.50 kb for *aroL*. The insertions in *aroL* spanned a region of 0.45 kb.

The observation that insertion of Tn*1000* into *aroL* also inactivated a second gene, designated *aroM*, which codes for a 26-kDa gene product, led to the model that *aroL* and *aroM* form a two-gene operon. The alternative explanation that the 26 kDa protein was a precursor of shikimate kinase or an artefact caused by translational readthrough was ruled out by the observation that four Tn*1000* insertions distal to *aroL* inactivated only the 26-kDa protein, leaving shikimate kinase intact. In addition, this observation indicated that the direction of transcription of the operon was from *aroL* to *aroM*. As reported in the accompanying paper (13), this model was confirmed by the nucleotide sequencing of the operon. Since strains containing a Tn*10* insertion (JP3123) or Mu d1 (*lac* Ap) insertion (JP1732) in the *aroLM* operon remain aromatic prototrophs and have not acquired new nutritional requirements, *aroM* is not essential for growth on minimal medium. The regulation of *aroM* by *tyrR*-mediated repression suggests that the function might be involved in the metabolism of the aromatic compounds. The only such known function for which a gene has not been identified is shikimate kinase I, but clearly this is not encoded by *aroM*, for several reasons: synthesis of shikimate kinase I is not regulated by *tyrR*, the molecular weight of shikimate kinase I is only 19,000 (15; R. DeFeyter, unpublished observation), and strain JP3123 (*aroL*::Tn*10*) retains shikimate kinase I activity. It was also shown that *aroM* is not involved in the uptake of shikimate. Hence the function of *aroM* remains unknown.

Of the eight transcriptional units that are known to be regulated by *tyrR*, the *aroLM* operon is only the second found to be multicistronic, the other being the *aroF tyrA* operon (27).

The observations that *proC* is transcribed counterclockwise on the chromosome and that it encodes a 29-kDa polypeptide are in agreement with the results of Deutch et al. (14).

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