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Transcription Control of the *aroP* Gene in *Escherichia coli* K-12: Analysis of Operator Mutants

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The nucleotide sequence of the region containing the promoter-operator for the *aroP* gene was determined. The start site of *aroP* transcription was identified by using S1 nuclease mapping and primer extension techniques. Examination of the nucleotide sequence revealed the presence of two "TYR R" boxes which are similar to those identified in the regulatory regions of other genes in the *tyrR* regulon. Bisulfite-induced *aroP* operator-constitutive mutants were analyzed, and the base-pair changes responsible for alterations in *aroP* regulation were located within these boxes.

Previous studies have shown that transport of the aromatic amino acids phenylalanine, tyrosine, and tryptophan is carried out in *Escherichia coli* K-12 and *Salmonella typhimurium* by a general transport system (2, 6, 31) or by systems specific for each of the three amino acids (2, 6). Mutants deficient in the general aromatic amino acid transport system have been isolated, and their mutations have been mapped at the *aroP* locus, at minute 3 on the *E. coli* chromosome (6). The *aroP* gene has been cloned, and its product has been shown to be a protein associated with the cytoplasmic membrane (12). Whipp and Pittard (35) showed that the general aromatic amino acid transport system involving the *aroP* product is subject to repression control by the TyrR protein acting in conjunction with phenylalanine, tyrosine, or tryptophan. In addition to regulating its own expression, the TyrR protein also regulates the expression of genes involved in tyrosine-specific transport, tryptophan-specific transport, and aromatic biosynthesis (7–9, 17, 21, 23, 32, 34). These genes together constitute the *tyrR* regulon. Nucleotide sequence analysis of the regulatory regions of some of these genes (13, 14, 16, 18, 20, 22) has allowed the identification of putative operator loci which contain variations of a consensus sequence referred to as the "TYR R" box (16). Operator mutants have been sequenced in the cases of *aroF* (18) and *tyrP* (22). In each case the mutations have been located within the TYR R boxes.

In this paper we describe the construction of *aroP-lac* and *aroP-cat* fusions in which the level of *aroP* transcription can be readily measured, the cloning and sequencing of the *aroP* promoter-operator region, and the isolation and sequencing of *aroP* operator-constitutive mutants.

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophages. The bacterial strains used were all derivatives of *E. coli* K-12 and, along with the plasmids used in this study, are described in Table 1. The bacteriophages Mu dl(Ap *lac*), λ pl(209), M13mp8, and M13mp9 have been described previously (10, 11, 26). The promoter-cloning vectors pkk232.8 and pMU530 have also been described (5, 22).

Chemicals and media. The chemicals used were obtained commercially and were not further purified. L-[U-^{14}C]tyrosine (500 mCi/mmol), [α-^{32}P]dATP (~2,000 to 3,000 Ci/mmol; 10 mCi/ml), [α-^{32}P]dCTP (~2,000 to 3,000 Ci/mmol; 10 mCi/ml) and [α-^{35}S]dATP (1,220 Ci/mmol; 7.9 mCi/ml) were purchased from Amersham Corp.

The minimal medium used was half-strength medium 56 (29) supplemented with appropriate growth requirements. To study repression, minimal medium was supplemented with aromatic amino acids at the following concentrations: L-phenylalanine, 10^{-3} M; L-tyrosine, 10^{-3} M; and L-tryptophan, 5×10^{-4} M.

Antibiotics were used at the following final concentrations (micrograms per milliliter): ampicillin, 25; chloramphenicol, 25; kanamycin, 20; tetracycline, 5 in minimal medium and 15 in nutrient medium; and trimethoprim, 10 in minimal medium and 40 in nutrient medium. 5-Bromo-4-chloro-3-indolyl-β-galactopyranoside (X-Gal) was used at a final concentration of 25 μg/ml.

Transport and enzyme assays. The assays for tyrosine transport, β-galactosidase, and chloramphenicol acetyltransferase were carried out as previously described (22, 28, 33, 35).

Nuclease S1 mapping. The 5′ ends of in vivo transcripts were mapped by the nuclease S1 procedure, with RNA prepared from mid-log-phase cells (1, 4). Derivatives of M13mp8 were used to prepare single-stranded [α-^{32}P]dATP-labeled probes by methods previously described (22). Probe 1 was prepared from an mp8 derivative containing a 599-base-pair (bp) *Bgl*II-*Bam*HI fragment from pMU1409 cloned into the *Bam*HI site of mp8. Probe 1 included nucleotides 1 to 585 of the sequence of the *aroP* regulatory region (see Fig. 4). Probe 2 was prepared from an mp8 containing a 568-bp *Bgl*II-*Hin*cII insert and includes nucleotides 1 to 555 of the *aroP* sequence (see Fig. 4). The individual steps in the S1 mapping were as described before (22).

Primer extension technique. The 5′ end of in vivo transcripts was also mapped by using the primer extension technique described by Hudson and Davidson (20). A 120-base *Sfa*NI single-stranded DNA primer was generated from the M13mp8 clone used in the preparation of probe 1. In this case [α-^{35}S]dATP was used as a label, and following de novo synthesis the enzyme *Sfa*NI was used for digestion. This *Sfa*NI site lies downstream from the start site of *aroP* transcription (see Fig. 4). After digestion the DNA was denatured at 95°C in 100% formamide. The single strands were separated in a 6% sequencing gel, and the single-stranded DNA probe was purified as described previously (22). After hybridization to mRNA from JP3561(pMU1409),

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TABLE 1. *E. coli* strains and plasmids used in this study

Strain or plasmid	Characteristics[a]	Source or reference
Strain	*ung-1 relA1 spoT1*	B. Weiss
BW310		
JM101	F′ *traD36 proAB*$^+$ *lacI*^{q} Δ(*lac-pro*) *supE*	J. Messing
JP3060	*aroG aroH argE his* Δ*lacU169*	P. Kasian
JP3561	*thr-1 leu-1* Δ*lacZM15 supE44 fhuA2 gyrA379*	P. Kasian
JP3744	JP3060 *aroP*::Mu dl(Ap *lac*)	This study
JP3876	λ *imm*^{434} lysogen of JP3060	This study
JP3877	JP3876 (λ *paroP-lac*)	This study
Plasmid		
pkk232.8	Ap^r, ColE1 replicon	5
pMU512	Tp^r, R388 replicon, Lac$^-$	P. Kasian
pMU516	Tp^r Lac$^+$	P. Kasian
pMU530	Tp^r, R388 replicon	22
pMU1065	Km^r *tyrR*$^+$, p15A replicon	E. Cornish (unpublished data)[b]
pMU1406	Tp^r Lac$^+$, *aroP* regulatory region on 1.7-kb *Hin*dIII fragment	This study
pMU1407	Tp^r Lac$^+$, *aroP* regulatory region on 0.75-kb *Bgl*II-*Pst*I fragment	This study
pMU1408	Tp^r Lac$^+$, *aroP* regulatory region on 0.6-kb *Bgl*II-*Bam*HI fragment	This study
pMU1409	Tp^r Km^r Lac$^+$, *aroP* regulatory region on 0.6-kb *Bgl*II-*Bam*HI fragment	This study
pMU1443	Tp^r Lac$^+$, *Bal* 31 derivative of pMU1407	This study
pMU1444	Tp^r Km^r Lac$^+$, *Bal* 31 derivative of pMU1409	This study
pMU1445	Km^r Lac$^+$, *Bal* 31 derivative of pMU1409	This study
pMU1448	Tp^r Km^r Lac$^+$, *Bal* 31 derivative of pMU1409	This study
pMU1449	Tp^r Km^r Lac$^+$, *Bal* 31 derivative of pMU1409	This study
pMU1621	Tp^r Lac$^+$, *aroP* regulatory region on 0.3-kb *Sma*I-*Bam*HI fragment	This study
pMU1631	Ap^r Cm^r, *aroP* regulatory region on 0.3-kb *Sma*I-*Bam*HI fragment	This study
pMU1663	Ap^r Cm^r, pMU1631 derivative carrying *aroP* operator mutation	This study
pMU1674	Ap^r Cm^r, pMU1631 derivative carrying *aroP* operator mutation	This study
pMU1676	Ap^r Cm^r, pMU1631 derivative carrying *aroP* operator mutation	This study
pMU1677	Ap^r Cm^r, pMU1631 derivative carrying *aroP* operator mutation	This study
pMU1678	Ap^r Cm^r, pMU1631 derivative carrying *aroP* operator mutation	This study

[a] The nomenclature for genetic symbols follows that described by Bachmann (3). Allele numbers are given where known. Ap^r, Ampicillin resistance; Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance; Tp^r, trimethoprim resistance.

[b] pMU1065 is a 5.5-kb *tyrR*$^+$ plasmid constructed by cloning a 1,771-bp *Eco*RV-*Pvu*II fragment containing the *tyrR* gene (13) in the *Hin*dII site of pACYC177 (E. Cornish, unpublished results).

TABLE 2. Specific activities of β-galactosidase of *aroP-lac* fusion strains

Strain	Sp act of β-galactosidase in[a]:			
	MM	MMP	MMT	MMA
JP3744	118	7	73	6
JP3744 *tyrR*	106	111	111	111
JP3877	92	8	42	6

[a] The units of β-galactosidase specific activity are those described by Miller (28). Growth occurred in minimal medium only (MM) or in MM supplemented with 10^{-3} M phenylalanine (MMP), 5×10^{-4} M tryptophan (MMT), or both of these aromatic amino acids plus 10^{-3} M tyrosine (MMA).

the *Sfa*NI primer was extended with reverse transcriptase. The product of extension was electrophoresed against the sequencing reactions of the identical mp8 clone used to generate the primer.

Recombinant DNA techniques. Standard procedures were used essentially as described elsewhere (15, 26, 27). Purification of the 1.7-kilobase (kb) *Hin*dIII restriction fragment from pMU516 was performed by the method of Langridge et al. (25). Nucleotide sequences of cloned fragments were determined by the dideoxy nucleotide termination method of Sanger et al. (32). In vitro mutagenesis with sodium bisulfite was based on the method of Peden and Nathans (30) (see Fig. 7). Treatment with 0.75 M sodium bisulfite for 20 min produced mutant clones with single base-pair changes. A more drastic treatment with 3 M bisulfite for 2 h produced clones with multiple base-pair changes.

RESULTS

Isolation of *aroP-lac* fusions. To clone the promoter-operator region of *aroP*, operon fusions were constructed between *aroP* and *lacZ*. Bacteriophage Mu dl(Ap *lac*) was used to lysogenize strain JP3060 (*aroP*$^+$ *aroG aroH*), and putative *aroP*::Mu dl(Ap *lac*) clones were selected on medium containing ampicillin and 3-fluorotyrosine (2×10^{-6} M). Previous experiments had shown that *aroP* mutant derivatives of JP3060 but not JP3060 itself would grow on medium containing this level of analog (P. Kasian, unpublished results). A number of clones were purified, and their identity was confirmed by β-galactosidase and transport assays. One of these clones (JP3744) was chosen for further study. With λp1(209) and the method of Komedo and Iino (24), λ *aroP-lac* phage was isolated and used to lysogenize a λ *imm*^{434} derivative of JP3060, designated JP3876, to form JP3877. Enzyme studies with JP3744, its *tyrR* derivative, and JP3877 establishing that β-galactosidase expression occurs from the *aroP* promoter in strains JP3744 and JP3877 are shown in Table 2. Because these strains possessed only the tyrosine-inhibitable and -repressible 3-deoxy-D-arabino-heptulsonic acid 7-phosphate synthase enzyme, it was not possible to test the effect of tyrosine alone on β-galactosidase expression. However, phenylalanine alone or the three aromatic amino acids together almost completely repress β-galactosidase expression in strains JP3744 and JP3877 but not in the *tyrR* derivative of JP3744 (Table 2). Tryptophan causes about 50% inhibition in *tyrR*$^+$ cells. It is also worth noting that the level of β-galactosidase expression in *tyrR*$^+$ cells grown in minimal medium is about the same as that observed in the *tyrR* mutant. Control by *tyrR* is therefore only evident when the aromatic amino acids are added to the medium. These results are in agreement with previous studies on the control of *aroP* expression involving measurement of tyrosine transport (35) and establish that the TyrR-mediated control of *aroP* occurs at the level of transcription.

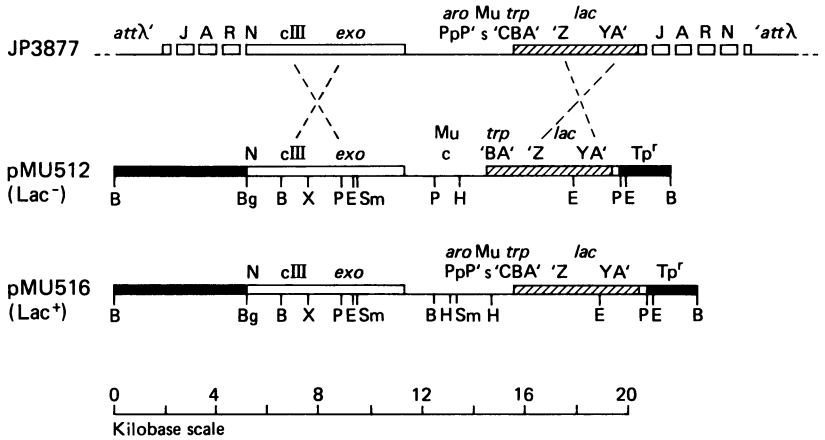

FIG. 1. Physical map of pMU512 (P. Kasian, personal communication) and a diagrammatic representation of the transfer of the chromosomal *aroP-lac* fusion from JP3877 to this plasmid by in vivo recombination, via *lac* and λ homologies, to form pMU516. pMU512 carries a 14.5-kb *Bgl*II-*Pst*I insert in a low-copy-number IncW-derived Tp^r vector, pREG151 (22). The construction of pMU512 was carried out in a series of steps (data not shown), but essentially the 14.5-kb insert consists of DNA derived from λ p1(209) (10). The pREG151-derived region is denoted by solid bars, the *trp'BA'-lac'ZYA'* region by cross-hatched bars and λ DNA by open bars. The event of crossing-over can occur within the region of λ DNA (~6 kb) located upstream from the *aroP* promoter and in the *trp'BA'-lac'ZYA'* region (~5 kb; located downstream from this promoter) which are homologous in pMU512 and the λ p*aroP-lac* fusion. pMU516 has acquired each of a *Bam*HI, *Hin*dIII, and *Sma*I site from the *aroP* promoter region and a *Hin*dIII site from the *trp'CBA'* region (11), but has lost each of a *Pst*I and *Hin*dIII site from the Mu *c* region. The λ p*aroP-lac* phage was not physically mapped, and therefore restriction sites on the λ p*aroP-lac* fusion in JP3877 are not shown. Abbreviations: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; Sm, *Sma*I; X, *Xho*I; Tp^r, trimethoprim resistance.

In vivo recombination was next used to transfer *aroP-lac* to the plasmid vector pMU512 as shown in Fig. 1. DNA was extracted from cells of JP3877(pMU512) and used to transform the Lac$^-$ strain JP3561. Selection was made for trimethoprim-resistant clones, and their Lac phenotype was determined by including X-Gal in the medium. The plasmid (pMU516) contained in one of the Lac$^+$ clones was shown to contain a new 1.7-kb *Hin*dIII fragment and to express β-galactosidase from the *aroP* promoter. That is, in *tyrR*$^+$ cells β-galactosidase synthesis was repressed by phenylalanine and tyrosine and to a lesser extent by tryptophan, whereas in *tyrR* mutant cells synthesis was constitutive (data not shown). Subcloning of this 1.7-kb *Hin*dIII fragment to the low-copy-number promoter-cloning plasmid pMU530 (22) confirmed that the *aroP* promoter-operator region was contained in this fragment (Table 3). Subsequent digestion of the purified 1.7-kb fragment with *Hae*III retained this region on an 0.75-kb fragment (pMU1407; Fig. 2). Digestion with *Bal* 31 from the unique *Pst*I site in pMU1407 produced plasmids with 0.6- and 0.45-kb inserts (pMU1408 and pMU1443, respectively). These constructions are summarized in Fig. 2, and the results of the enzyme assays are presented in Table 3. Two aspects of these results require special comment. First, regulation of β-galactosidase expression by the aromatic amino acids is retained in all constructs except for pMU1443, in which the insert has been reduced to 0.45 kb. The major feature of this control is the very strong repressing effect of either tyrosine or phenylalanine, which contrasts with the reproducible but less severe effect of tryptophan previously reported in Table 2. Second, the level of β-galactosidase expression under repressing conditions was not constant in the various constructs. The most likely explanation for this relates to a deficiency in the promoter cloning vector pMU530. This vector does not possess terminator sequences to prevent transcriptional readthrough from promoters upstream of the cloning site. When the *aroP* promoter-operator region present on pMU1408 was subsequently introduced into the promoter-cloning vector of Brosius (5) (see below), full repression by the aromatic amino acids was observed, confirming our conclusion that this region is retained intact on pMU1408.

TABLE 3. Specific activities of β-galactosidase of JP3561 derivatives bearing various *aroP-lac* plasmid fusions

Plasmid (size of *aroP* insert, kb)	Sp act of β-galactosidase in[a]:				
	MM	MMP	MMY	MMT	MMA
pMU516 (>1.7)[b]	164	25	29	95	22
pMU1406 (1.7)	195	35	40	84	35
pMU1407 (0.75)	219	35	47	90	48
pMU1408 (0.6)	341	75	85	172	68
pMU1443 (0.45)	96	97	93	88	101

[a] The units of β-galactosidase specific activity are those defined by Miller (28). Growth occurred in minimal medium only (MM) or in MM supplemented with 10^{-3} M phenylalanine (MMP), 10^{-3} M tyrosine (MMY), 5×10^{-4} M tryptophan (MMT), or all three aromatic amino acids (MMA).

[b] In pMU516 the size of the insert is not known exactly but is greater than the 1.7-kb *Hin*dIII fragment.

Nucleotide sequence determination of the *aroP* regulatory region. The strategy used for determining the nucleotide sequence of the *aroP* operator-promoter region is summarized in Fig. 3. To derive plasmids with shortened versions of the insert in pMU1408, a 1.4-kb *Sal*I fragment encoding kanamycin resistance was first cloned into the *Xho*I site of pMU1408 to make pMU1409. This plasmid was then linearized at the *Bgl*II site and digested with *Bal*31. After *Bal*31 digestion, *Bam*HI linkers were added and the molecules were religated. These were then cloned into M13mp8 and M13mp9 (Fig. 3) and used to determine the nucleotide sequence of the 585-bp region containing the *aroP* promoter-operator region.

This sequence (Fig. 4) was analyzed for the presence of putative promoters, TYR R boxes, and the Shine-Dalgarno sequence by using the TRLTIN computer program (13) and others of the MELBDBSYS suite of computer programs developed by A. P. Kyne (personal communication). Al-

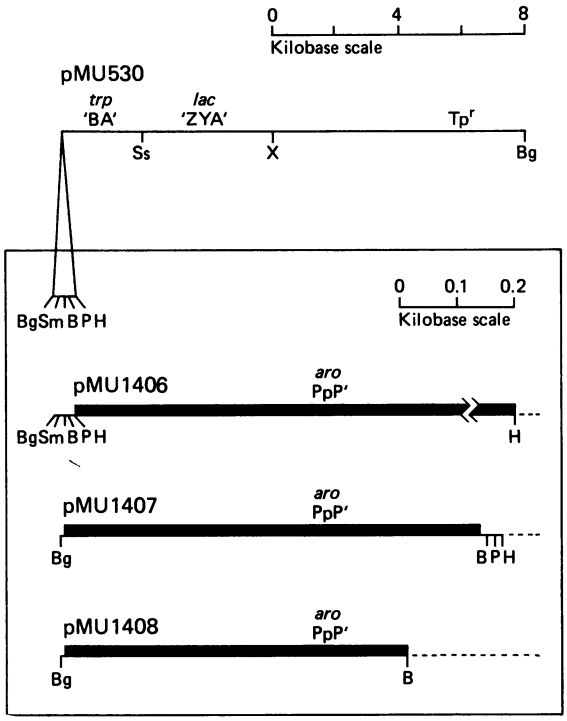

FIG. 2. Physical map of pMU530 and its derivatives, the *aroP-lac* plasmids pMU1406, pMU1407, and pMU1408. Only the relevant portions of the *aroP-lac* plasmids are shown, and these have been enlarged together with the polylinker site on pMU530. pMU1406 carries a 1.7-kb *Hin*dIII fragment containing the *aroP* promoter cloned into the *Hin*dIII site of pMU530. pMU1407 was obtained by ligating a *Hae*III digest of this 1.7-kb *Hin*dIII fragment to the *Sma*I site of pMU530. pMU1408 is a *Bal*31 derivative of pMU1407. The *aroP* promoter insert is denoted by solid bars. Abbreviations: B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; P, *Pst*I; Sm, *Sma*I; Ss, *Sst*I; X, *Xho*I; Tp^r, trimethoprim resistance.

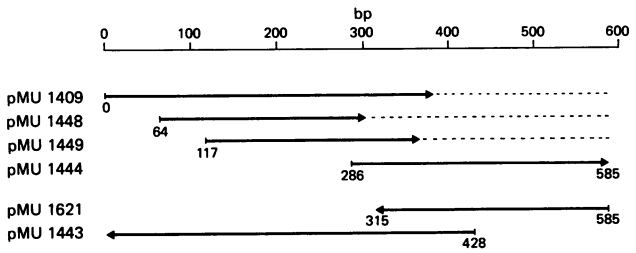

FIG. 3. Sequencing strategy for the *aroP* regulatory region by using M13 clones. These clones were derived from various plasmids as marked in the figure. Sequence determination of the antisense strand of the *aroP* regulatory region was carried out with M13mp9 derivatives carrying *Bgl*II-*Bam*HI fragments from pMU1409 and from its *Bal* 31 derivatives, pMU1444, pMU1448, and pMU1449, cloned into the *Bam*HI site of mp9. The sequence of the sense strand was determined by using M13mp8 clones carrying the *Bam*HI-*Pst*I insert from pMU1621 (a derivative of pMU1445) and the *Bgl*II-*Bam*HI insert from pMU1443 in the *Bam*HI site of mp8. The arrows indicate the direction and extent of the DNA sequencing runs. The dotted lines indicate the remaining part of the clone which was not sequenced. The nucleotide at which each clone starts on the sequence is numbered with the corresponding nucleotide number found in the nucleotide sequence of Fig. 4.

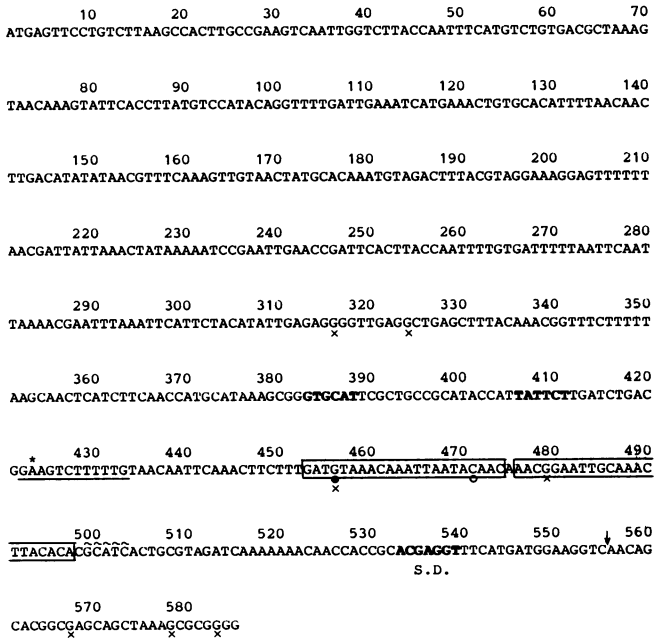

FIG. 4. The nucleotide sequence of the *aroP* regulatory region. The −35 and −10 hexamers and the putative Shine-Dalgarno sequence (S.D.) are in boldface type. The two putative TYR R boxes are boxed in. The start site region of *aroP* transcription mapped by the nuclease S1 technique is underlined, while the asterisk (*) denotes the start site obtained by primer extension. The *Hin*cII restriction site on the sequence used in the preparation of probe 2 is marked (↓). The *Sfa*NI recognition sequence ($\begin{smallmatrix}G&C&A&T&C\\C&G&T&A&G\end{smallmatrix}$ $(N)_5$ $(N)_9$) relevant to primer extension studies is marked with a wavy line (∿). The plasmid used in mutagenesis experiments, pU1631, carried the *aroP* regulatory region on a fragment including nucleotides 315 to 585. The mutations occurring in the mutant plasmids are marked: ○, the C→T single-base substitution in each of the plasmids pMU1674, pMU1676, and pMU1677; ●, the G→A single-base substitution of pMU1678; ×, each of the seven G→A substitutions of pMU1663.

though a double TYR R box was readily identified, no sequences corresponding to putative −10 or −35 promoter regions could be found closer than 41 bases from the putative operator. A strong Shine-Dalgarno sequence could also be identified with a potential ATG initiation codon within 5 to 9 bases (Fig. 4).

Identification of the start site of *aroP* transcription. The results from S1 mapping experiments are presented in Fig. 5. A 180-bp protected fragment (band A) was obtained when probe 1 was hybridized to mRNA from JP3561(pMU4300, pMU1409) grown in minimal medium (lane 5). This band was less intense when RNA was obtained from the same strain grown in the presence of aromatic amino acids (lane 6), indicating that the latter repress *aroP* at the level of transcription. This repression effect was relieved in a *tyrR* strain (lanes 7 and 8), confirming the role of the TyrR protein acting in conjunction with these aromatic amino acids in repressing transcription from the *aroP* promoter. Probe 2 gave a protected fragment of 152 bp (band B, lane 11). Taking into account the 27 and 18 bases of linker DNA on probes 1 and 2, respectively, which lie downstream from the *aroP* sequence and will hybridize to mRNA generated from pMU1409, we mapped the start site of *aroP* transcription between positions 442 and 433 (Fig. 4).

The primer extension method was used to map the start site of *aroP* transcription more precisely. The results pre-

sented in Fig. 6 show that the 5′ end of the *aroP* transcript was located at nucleotide 423 of the sequence shown in Fig. 4.

Isolation of operator mutants. To isolate operator mutants, an *aroP-cat* fusion plasmid was constructed by using the promoter-detecting plasmid pkk232.8 (5) and a 0.3-kb *aroP* promoter-operator fragment obtained from pMU1621 (Fig. 7). This fragment contains the TYR R boxes for *aroP* and includes nucleotides located up to 108 bp upstream from the start point of *aroP* transcription. Plasmids pMU1621 and pkk232.8 were each digested with *Sma*I and *Bam*HI and, after ligation, transformed into the *ung* mutant strain BW310 selecting on glucose minimal medium containing ampicillin and chloramphenicol (25 μg/ml). Chloramphenicol acetyltransferase synthesis was not repressed by the aromatic amino acids in these transformants, presumably because the increased number of copies of the *aroP* operator exceeded the availability of TyrR protein. However, the introduction of the high-copy-number *tyrR*$^+$ plasmid pMU1065 into this strain corrected this defect and produced a strain BW310 (pMU1065, pMU1631) which could not grow on minimal medium containing 25 μg of chloramphenicol per ml if any of the aromatic amino acids were present. This observation enabled us to develop a strategy for the isolation of operator-constitutive *aroP* mutants by using plasmid pMU1631 in strains with pMU1065.

To facilitate the isolation of operator mutants, plasmids pMU1631 and pkk232.8 were linearized with *Hin*dIII and *Pvu*II, respectively, denatured, mixed, and reannealed to form heteroduplexes and mutagenized with sodium bisulfite

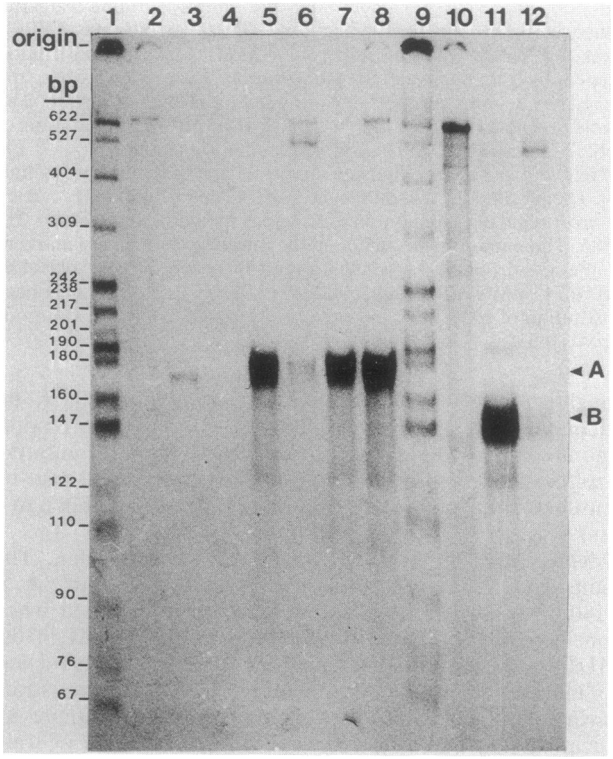

FIG. 5. Autoradigraph of ^{32}P-labeled S1-resistant fragments of 180 bp (band A) and 152 bp (band B) using probes 1 and 2, respectively, for hybridization with specific RNA preparations. Lanes: 1 and 9, standard marker fragments of pBR322 digested with *Hpa*II; 2, probe 1 (unhybridized); 3 through 8, probe 1 hybridized with RNA preparations from (i) JP3561(pMU400, pMU530) grown in minimal medium (MM) (lane 3) and in the same medium supplemented with repressing concentrations of the aromatic amino acids (MMA) (lane 4), (ii) JP3561(pMU400, pMU1409) grown in MM (lane 5) and in MMA (lane 6), (iii) JP3561 (pMU1409) *tyrR* grown in MM (lane 7) and in MMA (lane 8); 10, probe 1 (unhybridized); 11 and 12, probe 2 hybridized with RNA preparations from JP3561(pMU400, pMU1409) grown in MM (lane 11) and in MMA (lane 12).

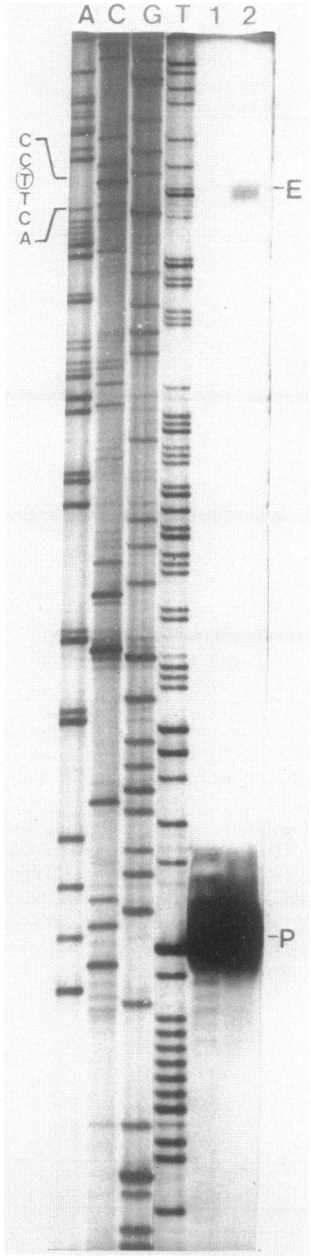

FIG. 6. Determination of the start site of *AroP* transcription by primer extension. A 120-bp *Sfa*NI ^{35}S-labeled probe was used as primer (band P), and the product of extension (band E) with this primer corresponded to the encircled T nucleotide on the sequencing ladder of the sense strand of the *aroP* regulatory region. A, C, G, and T are the various residues from the dideoxynucleotide sequencing reactions obtained when the identical mp8 clone used to generate the primer was sequenced. Lanes: 1, Unextended primer (P); 2, product of extension (E).

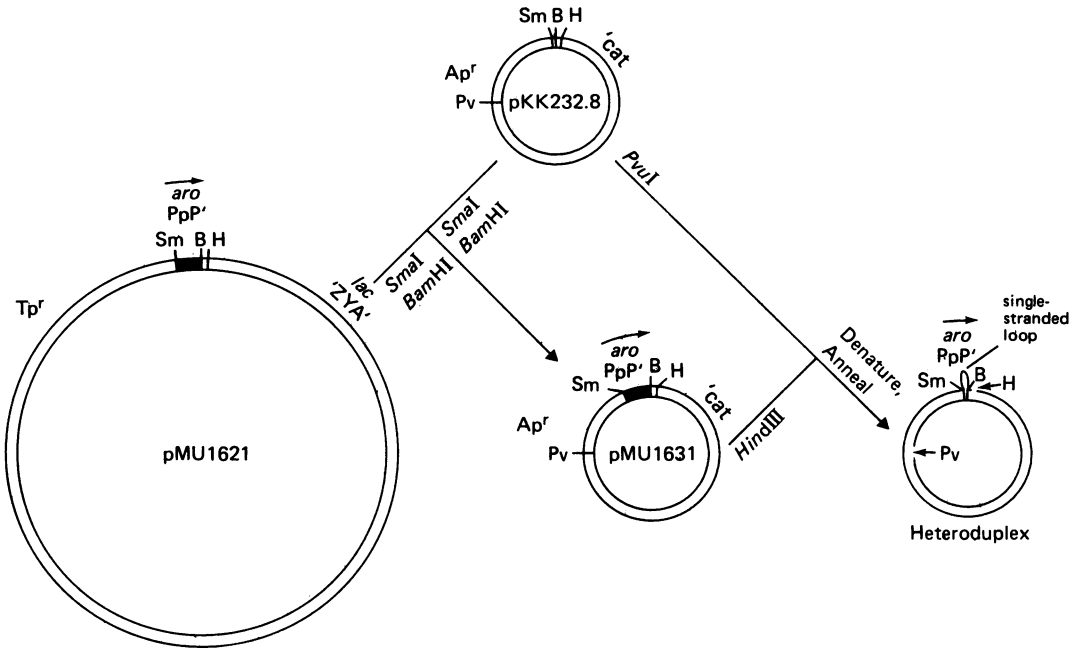

FIG. 7. Construction of the *AroP-cat* plasmid pMU1631 and formation of a heteroduplex structure from pMU1631 and pkk232.8. Plasmid pMU1631 was constructed by cloning the 0.3-kb *Sma*I-*Bam*HI *aroP* promoter fragment from pMU1621 into the *Sma*I and *Bam*HI sites of pkk232.8. A heteroduplex structure was formed when linear molecules of pMU1631 (cleaved with *Hin*dIII) and pkk232.8 (cleaved with *Pvu*I) were mixed and reannealed. Abbreviations: B, *Bam*HI; H, *Hin*dIII; Pv, *Pvu*I; Sm, *Sma*I; Ap^r, ampicillin resistance; Tp^r, trimethoprim resistance.

(Fig. 7) (30). After mutagenesis the DNA was used to transform the *ung* mutant strain BW310(pMU1065) selecting on glucose minimal medium containing chloramphenicol (25 μg/ml), ampicillin, and kanamycin and repressing concentrations of one or all of phenylalanine, tyrosine, and tryptophan. A number of derepressed clones were obtained. That the phenotypic changes were due to lesions occurring within the *aroP* regulatory region was confirmed by recloning the 0.3-kb *Sma*I-*Bam*HI fragment from each clone into pkk232.8 and checking the phenotype. The same fragments were also cloned into M13mp8 and M13mp9 for DNA sequencing.

The single-base changes in the nucleotide sequence of each of the mutant plasmids are shown in Fig. 4. These base changes were either C→T or G→A transitions, as would be expected with bisulfite mutagenesis. The base changes occurring in each of these plasmids were found to be located in one or the other of the two palindromic arms of the first TYR R box (Fig. 4). Three of the mutant plasmids, pMU1674, pMU1676, and pMU1677, involved a $\frac{G}{C}\rightarrow\frac{A}{T}$ transition occurring in the left arm of the first TYR R box, and a fourth, pMU1678, involved a $\frac{C}{G}\rightarrow\frac{T}{A}$ transition in the right arm of the same box.

Chloramphenicol acetyltransferase assays carried out on two of the plasmid-bearing strains, BW310(pMU1065, pMU1678) and BW310(pMU1065, pMU1676), showed that partial constitutivity of *aroP* expression occurred in the presence of the three aromatic amino acids (Table 4). In these mutants the ability of tryptophan or phenylalanine to affect repression was greatly diminished. Tyrosine on the other hand continued to be a fairly effective co-repressor.

Also shown in Fig. 4 are the mutations present on a plasmid, pMU1663, which was isolated after longer treatment with bisulfite. There are mutations in this plasmid in both TYR R boxes, as well as a number outside the TYR R box region. Repression in strains carrying this plasmid by

TABLE 4. Specific activities of chloramphenicol acetyltransferase of BW310(pMU1065) strains bearing pMU1631 and its derivatives containing mutations in the *aroP* operator

Plasmid	Mutation	Sp act of chloramphenicol acetyltransferase (nmol/min per mg of protein) in[a]:				
		MM	MMP	MMY	MMT	MMA
pMU1631	Wild-type	1,197 (1.0)	100 (0.1)	145 (0.1)	526 (0.4)	97 (0.1)
pMU1678	G→A at 456[b]	1,594 (1.0)	1,257 (0.8)	526 (0.3)	1,541 (1.0)	882 (0.6)
pMU1676	C→T at 471	1,468 (1.0)	887 (0.6)	381 (0.3)	999 (0.7)	708 (0.5)
pMU1663	G→A at 316, 324, 456, 479, 567, 580, and 583	986 (1.0)	906 (0.9)	1,103 (1.1)	1,017 (1.0)	1,053 (1.0)

[a] For definitions of abbreviations, see Table 3, footnote *a*. The ratios of the specific activities in various media with respect to that in minimal medium are in brackets.

[b] Nucleotide number corresponds to numbering in nucleotide sequence of Fig. 4.

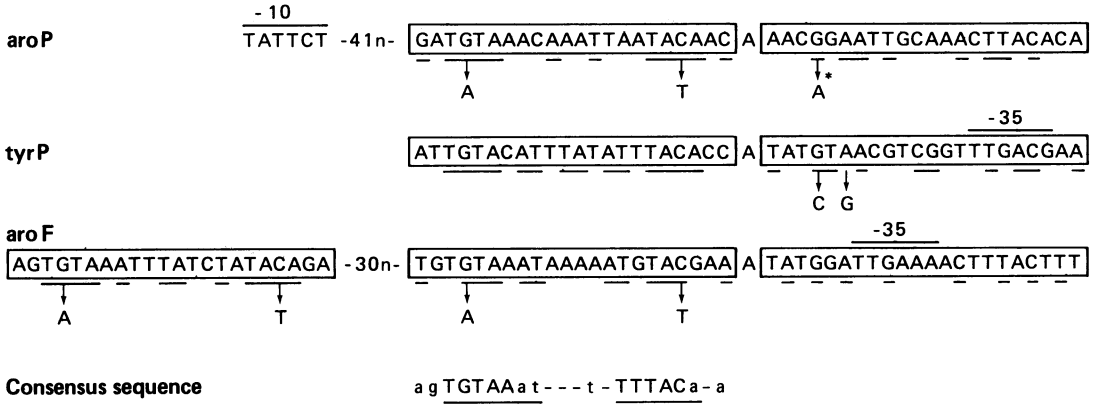

FIG. 8. Location of single-base substitution mutations in TYR R boxes of *aroP*, *tyrP* (22), and *aroF* (18). The consensus for the TYR R box is given; capital letters indicate the common bases of at least 9 of 12 boxes of genes within the *tyrR* regulon while lower-case letters denote those common to at least 6 boxes. Nucleotide changes in various operator constitutive mutants are marked by arrows. The arrow accompanied with an asterisk (*) marks the G→A substitution in the second TYR R box which was only found on pMU1663. The −35 and −10 regions are overlined. Symmetrical sequences are underlined.

any or all of the three amino acids was completely abolished (Table 4).

DISCUSSION

The regulatory role of the TyrR protein acting in conjunction with any of the three aromatic amino acids to repress transport by the general aromatic system as had been observed in an earlier study (35) was confirmed in the present study and was shown to occur at the level of transcription of the gene *aroP*.

An examination of the nucleotide sequence of the fragment containing the wild-type *aroP* operator locus revealed the presence of two TYR R boxes. These boxes were later confirmed to be the repressor-binding site on the *aroP* regulatory region by the isolation and sequence analysis of *aroP* operator mutants. This observation supports the hypothesis that the TYR R box is indeed the operator site in each of the genes belonging to the *tyrR* regulon. Operator mutants have also been sequenced in *aroF* (18) and *tyrP* (22), and these mutations have been located within TYR R boxes.

The TYR R boxes in *aroP* are imperfect palindromes, and although they each exhibit an identity of only 11/17 with the TYR R box consensus sequence (16; Fig. 8), they retain the as yet invariant $GN_{14}C$ arrangement in each box. Either one or the other of these critical bases has been altered in all single-base substitution operator mutants so far isolated in *aroF* (18), one of two such mutants in *tyrP* (22), and four such mutants in *aroP* (Fig. 8). The operator mutants in *aroF* and *tyrP* were spontaneous mutants and were not isolated by using sodium bisulfite mutagenesis (which specifically affects C · G base pairs only), yet these G and C bases on the right and left arms of the TYR R box have been altered. These two particular bases are apparently important and are likely to play a role in establishing contact with the TyrR protein.

The single-base mutations in the consensus palindromic arms of the TYR R boxes of *aroP*, *tyrP* (22), and *aroF* (18) are shown in Fig. 8. It was observed that base substitutions at corresponding sites in either arm of the TYR R box result in operator constitutivity of *aroP* and *aroF*.

It has previously been observed that the genes of the *tyrR* regulon which are repressed by the TyrR protein in the presence of tyrosine (*aroF*, *aroL*, and *trP*) have two adjacent TYR R boxes separated by a single A residue (22). The analysis of *aroP* adds a further confirmation to this observation. The two unique mutant types obtained in the present study contained single-base substitutions in the first TYR R box which brought about major effects on tryptophan- and phenylalanine-mediated repression but only reduced tyrosine-mediated repression by 20%, suggesting that the first TYR R box in *aroP* is involved in tryptophan- and phenylalanine-mediated repression. The only strain in which tyrosine-mediated repression was completely abolished had multiple mutations, one in each of the two TYR R boxes and a number of others both upstream and downstream from the regulatory region. We are currently investigating whether a single mutation in the second box will abolish tyrosine repression or whether multiple mutations are required. In the case of each of two *tyrR* operator mutants, however, mutations in a single base located in the right-hand TYR R box (Fig. 8) resulted in complete loss of tyrosine repression (22).

The location of the *aroP* operator site some 31-bp downstream from the transcription start site was unexpected. However, the use of both S1 mapping and primer extension methods to determine the transcription start site confirms this observation. In the case of other genes of the *tyrR* regulon, the TYR R boxes in *tyrP*, *aroF*, *aroG*, and *tyrR* are located upstream from or around the −35 region. In *aroL*, a TYR R box lies around the −35 region, while two other such boxes are situated downstream from the −10 hexamer (16). The downstream location of the TYR R box in *aroP* may explain the observation that *aroP* is the last repressible of all the genes of the *tyrR* regulon. Herrin and Bennett (19) have studied the effects of inserting a 33-bp *lac* operator fragment at various positions upstream or downstream from the *trp* promoter located on a *trp* promoter-*galK* fusion plasmid. They observed that repression still occurred when the operator fragment was placed 27 and 58 bp downstream from the start site of transcription. However, the extent of repression was less than that seen with a similar plasmid in which the operator fragment was placed 2 bp downstream from the start site of transcription. These researchers suggested that repression occurring in the former cases is likely to be due to physical blockage of RNA polymerase elongation rather than that of RNA polymerase initiation. In the case of *aroP*, the nonoverlap of the *aroP* operator sequence with the −35 to −10 region may also argue for the occurrence of physical

blockage of RNA polymerase elongation, rather than the occurrence of competitive binding between RNA polymerase and the TyrR repressor. However, the possibility of steric hindrance caused by the large TyrR protein (subunit molecular weight of 53,099) (13) against binding by RNA polymerase cannot be ruled out. In vitro binding studies with RNA polymerase and the purified TyrR protein would be needed to elucidate their interactions at the *aroP* regulatory region.

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