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Regulatory Mutants of the *aroF-tyrA* Operon of *Escherichia coli* K-12

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The regulatory region of the *aroF-tyrA* operon was fused to the chloramphenicol acetyltransferase (*cat*) gene on a plasmid vector. Expression of the *cat* gene was subject to repression by *tyrR*$^+$. This fusion was used to isolate regulatory mutants with increased expression of the *cat* gene in which repression by *tyrR*$^+$ was affected. Nucleotide sequencing of these mutants has led to the identification of three sites involved in the repression of *aroF* by *tyrR*$^+$. The existence of a functional promoter divergently transcribing from the *aroF* regulatory region was also demonstrated by using the *cat* fusion vector. The expression of this promoter is also regulated by *tyrR*$^+$.

The *aroF-tyrA* operon is one of a number of genes or operons under the control of the regulator gene *tyrR*$^+$ (4, 6). Nucleotide sequencing of the *aroF* regulatory region has identified two sequences (referred to as TYR R boxes), upstream of the *aroF* promoter, which are closely homologous to sites of regulation by *tyrR*$^+$ in other genes (13, 15). A comparison of the sequences preceding *aroF* and *aroL*, another gene repressed by *tyrR*$^+$, led to the identification of a third possible TYR R box which overlaps the −35 region of the *aroF* promoter (10). By using a multicopy plasmid carrying the *aroF-tyrA* operon, Garner and Herrmann (13) isolated regulatory mutants in which expression of the operon was derepressed. The mutations in these strains were shown to occur in either of the two putative TYR R boxes upstream of the *aroF* promoter. However, it was not shown that these mutations specifically affected regulation of the operon by *tyrR*$^+$. A difficulty in working with multicopy plasmids carrying either the *aroF-tyrA* operon or *aroF-lac* gene fusions is that *tyrR* strains carrying these plasmids are unable to grow or grow poorly (20; unpublished data). This prevents the expression of regulatory mutants of such plasmids from being assayed in a *tyrR* strain.

This paper reports the construction of a gene fusion between the *aroF* regulatory region and the chloramphenicol acetyltransferase (*cat*) gene. Expression of this fusion can be assayed in both *tyrR*$^+$ and *tyrR* strains. By using this fusion, mutants no longer regulated by *tyrR*$^+$ have been generated both in vivo and in vitro. Nucleotide sequencing of these mutants has confirmed the observations of Garner and Herrmann (13) and has shown that the third TYR R box is also necessary for repression of *aroF* by *tyrR*$^+$.

Nucleotide sequencing of the region upstream of *aroF* has also identified an open reading frame (ORF) of at least 88 codons, which, if expressed, would be transcribed divergently from the *aroF* regulatory region (15). Hudson and Davidson (15) were unable to detect transcription of this ORF by using S1 nuclease mapping and suggested either that the gene is not preceded by a functional promoter or that it is transcribed at only a very low level. If there is a functional promoter, the position of the ORF (see Fig. 2) makes it likely that such a promoter would overlap one or more of the TYR R boxes involved in the regulation of *aroF* and may itself be regulated by *tyrR*$^+$.

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We also demonstrate, by constructing ORF-*cat* gene fusions, that the ORF is preceded by a functional promoter and that transcription of the ORF is regulated by *tyrR*$^+$. Furthermore, at least two of the three TYR R boxes involved in the regulation of *aroF* are also required for complete repression of the ORF promoter.

MATERIALS AND METHODS

Bacterial strains and plasmids. All bacterial strains are derivatives of *Escherichia coli* K-12. Descriptions and origins of bacterial strains and plasmids are given in Table 1.

Media. Cells were grown in Luria broth (LB) (19). Solid medium was Oxoid nutrient broth no. 2 plus 1% Oxoid agar with ampicillin (50 μg/ml), kanamycin (20 μg/ml), and chloramphenicol at various concentrations added when appropriate.

Chloramphenicol resistance levels. The level of resistance to chloramphenicol was determined semiquantitatively by growing a saturated culture of the cells to be tested in LB plus ampicillin, diluting the culture to 10^{-6}, and spotting 10-μl volumes on nutrient agar plates containing increasing concentrations of chloramphenicol. Growth was compared with that on a control plate without chloramphenicol and scored as being resistant (approximately equal to the control), partially resistant (less resistant than the control), or sensitive (no growth).

DNA manipulations. The general procedures used for restriction enzyme cleavage, exonuclease *Bal* 31 digestion, ligation, plasmid DNA isolation, gel purification of DNA fragments, and transformation have been described previously (18).

DNA sequencing. DNA sequence analysis was done by the method of Sanger et al. (21) with the M13mp10 sequencing vector. To sequence the endpoints of the deletions generated by using *Bal* 31 and the spontaneous mutations isolated by using pMU1743, the *Hin*dIII fragment carrying the *aroF* regulatory region from each plasmid was cloned into M13mp10. Of more than 40 mp10 clones carrying the *Hin*dIII fragments derived from these plasmids, all had the fragment inserted in the orientation in which the *aroF* promoter was transcribing through the *lacZ* gene. Since the *aroF* promoter is very strong (13), it may be that transcription from the promoter, when inserted in the opposite orientation, interfered with the adjacent replicative functions of the mp10 bacteriophage (23), thereby preventing the isolation of such

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant genotype[a]	Origin
Strain		
JP4098	*tyrR*$^+$ *zci-2*::Tn*10* Δ*lac*	J. Pittard
JP4099	*tyrR366 zci-2*::Tn*10* Δ*lac*	J. Pittard
CC146	JP4098(pMU1065)	This work
MC1061	Δ(*ara leu*)*X7697* Δ*lacX74*	M. Hynes
CC196	MC1061(pMU1065)	This work
CC418	CC146(pMU1742)	This work
CC527	CC146(pMU1743)	This work
Plasmids		
pKK232.8	*bla*$^+$ *cat*	2
pACYC177	*neo*$^+$ *bla*$^+$	8
pMU1741	*neo*$^+$ Φ(*aroFp-lacZ*α)	From pMC489 (7)
pMU1742	*bla*$^+$ Φ(*aroFp-cat*)	From pKK232.8
pMU1743	*bla*$^+$ Φ(*aroFp-cat*)	From pMU1742
pMU1754	*bla*$^+$ Φ(ORFp-*cat*)	From pMU1743
pMU1065	*neo*$^+$ *tyrR*$^+$	From pACYC177

[a] The nomenclature of bacterial genotypes follows that described by Bachmann (1). *zci-2*::Tn*10* describes the position of a transpositional insertion by using the nomenclature of Kleckner et al. (16).

clones. The consequence of this was that only one strand of the *aroF* regulatory region of each plasmid was sequenced. Nevertheless, comparison of the sequence of each clone with the sequence of the wild-type fragment derived from pMU1742 on the same gel allowed the deletion endpoints and point mutations to be unambiguously identified.

To determine the sequence of pMU1742 and its mutant derivatives, the *Pst*I fragment extending from the *bla* gene to the site adjacent to the *aroF* regulatory region (Fig. 1) was cloned into mp10, again in only the one orientation.

Construction of the *aroFp-cat* fusion plasmid. To construct an *aroF-cat* gene fusion, we used a previously constructed plasmid, pMU1741 (unpublished data), as a source of the *aroF* regulatory region for cloning purposes. pMU1741 consists of the two contiguous *Sau*3A restriction fragments (231 and 29 base pairs [bp]) which span the *aroF* regulatory region (Fig. 1) inserted into the *Bam*HI site of the promoter cloning vector pMC489 (7). A 300-bp *Bst*EII-*Bam*HI restriction fragment containing these two *Sau*3A fragments was purified, partially digested with *Sau*3A, and ligated with the promoter-cloning vector, pKK232.8 (2), which had been cleaved with *Bam*HI. Ligated plasmids were transformed into the high-frequency transforming strain MC1061, and transformants resistant to ampicillin and chloramphenicol (20 μg/ml) were selected. Plasmids from these transformants were shown by restriction enzyme cleavage patterns to contain either the 231-bp fragment or the two contiguous *Sau*3A fragments in the orientation in which the *cat* gene was transcribed from the *aroF* promoter. The presence of the two *Sau*3A fragments in one of these plasmids, pMU1742, was confirmed by nucleotide sequencing. Since the promoter-cloning vector, pKK232.8, was constructed with translational termination codons in all three reading frames between the cloning sites and the *cat* structural gene (2), fusions constructed with this vector are transcriptional and not translational fusions. The fusion plasmid, pMU1742, was used in the studies described below.

Chloramphenicol acetyltransferase (CAT) assays. Cells were grown in LB containing 10^{-4} M tyrosine, phenylalanine, and tryptophan plus appropriate antibiotics to mid-log phase. We determined CAT activities in duplicate by using sonicated cell extracts as done in the spectrophotometric method described by Shaw (22). The protein content of the extracts was determined by using the Bio-Rad Protein Assay reagent with known concentrations of immunoglobulins as standards. The CAT activity of each strain was determined two or three times on separate days. Individual activities differed from the mean by not greater than 30%.

RESULTS

Regulation of the *aroFp-cat* fusion. To assay the effect of *tyrR*$^+$ on the expression of the *aroF-cat* fusion, pMU1742 was transformed into the following strains: JP4098 (*tyrR*$^+$); JP4099 (*tyrR*); and CC146, which is JP4098 containing pMU1065, a plasmid derived from the cloning vector pACYC177 (8) into which the *tyrR*$^+$ gene had been cloned (E. Cornish, unpublished data). In addition, pACYC177 was subsequently transformed into strain JP4099(pMU1742). The CAT activity in each of these strains containing pMU1742 was assayed (Table 2). These results indicate that there was an 18-fold repression of CAT activity in a haploid *tyrR*$^+$ strain compared with a *tyrR* strain, while the presence of the multicopy plasmid carrying *tyrR*$^+$, pMU1065, caused a 154-fold decrease in CAT activity. The high level of expression of the gene fusion observed in strain JP4099(pMU1742, pACYC177) demonstrates that repression of CAT activity in strain JP4098(pMU1742, pMU1065) was due to repression by *tyrR*$^+$ and not to some effect of the second plasmid such as a change in plasmid copy number.

It is not clear why the *aroF-cat* fusion plasmid is stable in a *tyrR* strain, whereas previously isolated *aroF-lac* fusion plasmids (unpublished data) and the *aroF-tyrA* operon itself on a multicopy plasmid (20) are not. This may be due to a relatively low copy number for the *aroF-cat* plasmid; however, the copy numbers of these different plasmids have not been determined. Alternatively, transcription from the derepressed *aroF* promoter may proceed into the plasmid vector, interfering with its replication. This would not occur with the *aroF-cat* fusion, since the *cat* gene on the fusion vector, pKK232.8, is flanked by strong transcriptional terminators (2), which would prevent transcription from the *aroF* promoter entering the plasmid vector. Otherwise, it may simply be that overproduction of β-galactosidase and the gene products of *aroF* or *tyrA* or both inhibit cell growth, whereas overproduction of CAT does not.

The resistance of the four strains described above to chloramphenicol was also tested. Strains JP4098(pMU1742), JP4099(pMU1742), and JP4099(pMU1742, pACYC177) were all resistant to 200 μg of chloramphenicol per ml and

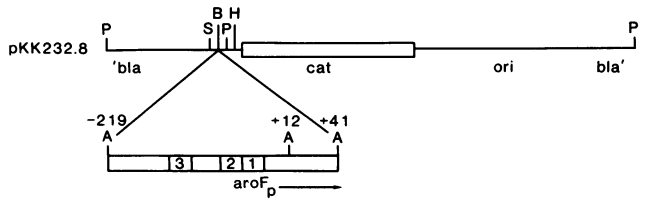

FIG. 1. Construction of the *aroF-cat* fusion plasmid, pMU1742. Two contiguous *Sau*3A (A) restriction fragments which span the *aroF* regulatory region were ligated into the *Bam*HI (B) site of pKK232.8. For convenience, pKK232.8 has been linearized at the *Pst*I (P) site within the *bla*$^+$ gene. The positions of the *Sau*3A sites are given with respect to +1, the start point of transcription of *aroF*. The approximate positions of the TYR R boxes numbered 1, 2, and 3 are indicated. The arrow indicates the direction of transcription from the *aroF* promoter. Other restriction enzyme cleavage sites are *Sma*I (S) and *Hin*dIII (H).

TABLE 2. Regulation of wild-type and mutant *aroF-cat* fusions

Plasmid	Mutation[a] (TYR R box mutated)	*tyrR* genotype[b]	CAT activity (U/mg of protein) (% of *tyrR*$^-$)
pMU1742	Wild type	−	7.4 (100)
		+	0.41 (5.5)
		p$^+$	0.048 (0.65)
		p$^-$	8.0 (108)
Deletion plasmids			
pMU1743	Deletion to −180 (none)	−	8.2 (100)
		p$^+$	0.052 (0.64)
pMU1744	Deletion to −120 (none)	−	7.5 (100)
		p$^+$	0.019 (0.25)
pMU1745	Deletion to −79 (box 3)	−	9.0 (100)
		p$^+$	0.38 (4.2)
pMU1747	Deletion to −43 (box 3, box 2)	−	6.1 (100)
		p$^+$	6.0 (98)
Spontaneous mutants of pMU1742			
pMU1748	A insertion at −30 (box 1)	−	7.8 (100)
		p$^+$	2.5 (32)
pMU1749	A insertion at −52 (box 2)	−	8.3 (100)
		p$^+$	4.6 (55)
pMU1750	G-to-A transition at −60 (box 2)	−	8.3 (100)
		p$^+$	4.9 (59)
Spontaneous mutants of pMU1743			
pMU1751	A-to-G transition at −35 (box 1)	−	14.9 (100)
		p$^+$	0.24 (1.6)
pMU1752	G-to-A transition at −37 (box 1)	−	13.2 (100)
		p$^+$	1.4 (11)
pMU1753	A deletion at −52 (box 2)	−	6.2 (100)
		p$^+$	2.4 (39)

[a] The positions of the mutations are shown in Fig. 2.
[b] The *tyrR* genotypes indicate the following strains: −, JP4099 (*tyrR*); +, JP4098 (*tyrR*$^+$); p$^+$, CC146(pMU1065) (multicopy *tyrR*$^+$); and p$^-$, JP4099(pACYC177) (*tyrR*$^-$).

sensitive to 400 μg/ml, while strain CC146(pMU1742) was resistant to 20 μg/ml and sensitive to 50 μg/ml. These resistance levels do not strictly mirror the CAT activities of these strains, suggesting that, particularly at higher levels of CAT activity, increases in activity are not reflected by a proportional increase in chloramphenicol resistance. Unpublished results with other fusions suggest that for low CAT levels, corresponding to the range of resistance from 0 to about 60 μg of chloramphenicol per ml, small changes in resistance levels are reflected by changes in CAT levels. However, as CAT enzyme levels increase further, they are not accompanied by proportional changes in Cm^r. The reason for this observation is not understood. It was clear from these results that *aroF* regulatory mutants with increased resistance to chloramphenicol could be selected only in the presence of pMU1065. Two other strains, MC1061 (*tyrR*$^+$) and CC196 [MC1061(pMU1065)], when transformed with pMU1742, showed similar resistance levels to those of the corresponding strains above. The latter of these strains was also used to select or screen for regulatory mutants in which Cm^r was increased.

Isolation of *aroF* regulatory mutants; deletion mutants constructed in vitro. Garner and Herrmann (13) have identified two regulatory sites upstream of the *aroF* promoter (Fig. 2). To determine whether these sites are involved in the regulation of *aroF* by *tyrR*$^+$, a number of deletions entering this region were constructed. pMU1742 was cleaved with *Sma*I and digested with exonuclease *Bal* 31 to generate the deletions. The rate of digestion by *Bal* 31 is known to be sequence dependent (17), and so to assess the extent of digestion, samples were taken at various time intervals, digested with *Pst*I, and run on agarose gels. It was apparent that the *Bal* 31 digestion had not proceeded at a uniform rate, since discrete bands were seen which were present at a number of successive time intervals. Ultimately, the predominance of deletions of particular sizes prevented the isolation of a wide range of different-sized deletions. DNA ends which had 5′ single-stranded extensions were made double stranded by using DNA polymerase 1 (Klenow fragment) and were then ligated with *Hin*dIII linkers. After digestion with *Hin*dIII, the mixture was ligated with an excess of pKK232.8 which had also been cleaved with

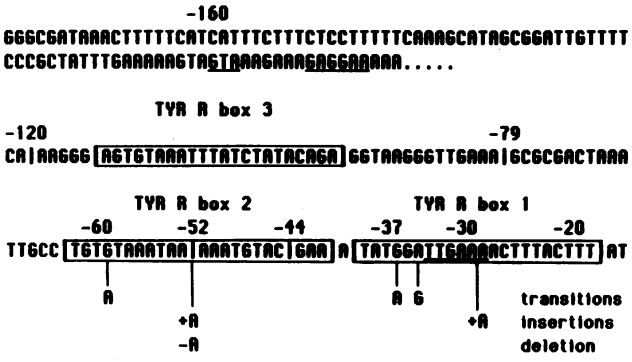

FIG. 2. Nucleotide sequence changes in *aroF* regulatory mutants. The sequence is numbered backwards from +1, the start point of transcription of *aroF*. The last digit of each number is aligned with the nucleotide to which the number refers. Deletion endpoints are indicated by vertical bars, and point mutations are shown below the sequence. The TYR R boxes identified by sequence comparisons (13, 15) are boxed, and the −35 promoter sequence contained within TYR R box 1 is underlined. The initiation codon and putative ribosome-binding site (both underlined) of the divergently transcribed ORF are indicated on a segment of the complementary strand.

*Hin*dIII. This ligation mixture was then transformed into strain CC196, and transformants resistant to ampicillin and chloramphenicol (10 μg/ml) were selected. These transformants were then screened for those resistant to 50, 100, and 200 μg of chloramphenicol per ml.

Crude plasmid preparations from these transformants were digested with *Sma*I and *Hin*dIII in separate digestions, and the DNA fragments were separated on agarose and polyacrylamide gels, respectively. The presence of a *Sma*I site demonstrates that the plasmid was derived from pKK232.8 and not from the original pMU1742 plasmid which had been digested at the *Sma*I site with *Bal* 31. The *Hin*dIII digests demonstrated the presence of a fragment in each plasmid, ranging in size from approximately 100 to 300 bp, inserted in the *Hin*dIII site of pKK232.8. The endpoints of four deletions were determined by nucleotide sequencing (see Materials and Methods), and the results are shown in Fig. 2.

To test whether the *aroF-cat* fusion on these plasmids was repressed by *tyrR*$^+$, the deletion plasmids were transformed into strains JP4099 and CC146 and assayed for CAT activity (Table 2). These results demonstrate that the region upstream of the *aroF* promoter is essential for the regulation of *aroF* by *tyrR*$^+$. A deletion which extended up to 120 bp from the start point of transcription of *aroF* (Fig. 2) (pMU1744) had little effect on repression of the gene fusion. If anything, this deletion appeared to enhance the repression of the fusion by *tyrR*$^+$. In contrast, a deletion which extended through TYR R box 3, leaving box 2 intact, caused partial derepression of the fusion in the presence of pMU1065. In the latter deletion mutant, pMU1745, CAT activity was repressed only 24-fold by pMU1065 with respect to the *tyrR* strain compared with the 154-fold repression seen with pMU1742. Deletions which extended further, into TYR R box 2 (pMU1747), caused complete derepression. Although these deletions caused derepression of the *aroF* promoter in strain CC146, they had no effect on *aroF* expression in strain JP4099. This demonstrates that the deletions affect regulation of *aroF* by *tyrR*$^+$. No deletions extending beyond TYR R box 2 were isolated, presumably because such deletions would have extended into the −35 sequence of the *aroF* promoter.

Isolation of *aroF* regulatory mutants; spontaneous mutants isolated in vivo. Strain CC418 which carries both pMU1742 and pMU1065 and is sensitive to 50 μg of chloramphenicol per ml was used to select for spontaneous mutants with increased Cm^r. Cultures derived from individual colonies were subcultured to medium containing ampicillin, kanamycin, and chloramphenicol (100 μg/ml) and allowed to grow to saturation, diluted 1:100 in the same medium, and again grown to saturation. Kanamycin was included in the medium to ensure the maintenance of pMU1065. To isolate plasmids which carried mutations conferring increased resistance to chloramphenicol, crude plasmid DNA was prepared from each of these cultures and transformed into CC196. Transformants resistant to ampicillin, kanamycin, and chloramphenicol (100 μg/ml) were selected. One transformant derived from each of seven original independent cultures was selected for further analysis.

A second set of mutants was isolated by using strain CC527 which carries pMU1065 and pMU1743, a deletion derivative of pMU1742. The deletion in pMU1743 has no effect on the regulation of the *aroF-cat* fusion by pMU1065 (Table 2). The advantage of using pMU1743 is that the entire *aroF* regulatory region lies within a 246-bp *Hin*dIII restriction fragment, which facilitates subsequent manipulations. In an attempt to isolate spontaneous mutants which were only partially derepressed, resistance to 40 μg of chloramphenicol per ml was selected in essentially the same manner as described above. However, in this case, five cycles of growth in the presence of 40 μg of chloramphenicol per ml were necessary to obtain resistant cultures. In addition, when plasmid DNA obtained from these resistant cultures was transformed into CC196, transformants were grown in the absence of chloramphenicol for 6 h before being selected on plates containing chloramphenicol (40 μg/ml).

The nucleotide sequence of the *aroF* regulatory region of 10 mutants was determined. Each of the plasmids had a single mutation in the *aroF* regulatory region. Six different mutations were detected. Three of these mutations altered the nucleotide sequence of TYR R box 2 (Fig. 2) and were identical to mutations previously isolated by Garner and Herrmann (13). The other three mutations were within the putative TYR R box overlapping the *aroF* promoter (TYR R box 1). Mutant plasmids were transformed into strains JP4099 and CC146 and assayed for CAT activity and Cm^r (Table 2). Again, while these mutations caused derepression of the gene fusion in strain CC146, they had no great effect on expression in the *tyrR* strain, JP4099, demonstrating that the mutations affected the regulation of the fusion by *tyrR*$^+$. For pMU1748, the CAT activity assayed in strain JP4099 shows that although the mutation in this plasmid increases the distance between the −35 and −10 regions of the *aroF* promoter by one nucleotide pair, it has no appreciable effect on the level of expression from this promoter in the *tyrR* strain. The other two point mutations in TYR R box 1 (pMU1751 and pMU1752) are immediately upstream of the −35 promoter sequence and appeared to increase the expression of the fusion in the *tyrR* strain by about 60 to 80%. Since the day-to-day variation in the CAT assays of any one strain was only 20 to 30%, it is likely that this increase is due to a small increase in promoter efficiency.

Bidirectional regulation from the *aroF* operator. To demonstrate that the ORF proceeding divergently from the *aroF* regulatory region is preceded by a functional promoter, we took advantage of the fact that one deletion plasmid,

pMU1743, contained a 246-bp *Hin*dIII fragment containing the entire *aroF* regulatory region fused to the *cat* gene of pKK232.8. This *Hin*dIII fragment extended from a position within the 5′ region of the ORF coding sequence to a position within the 5′ untranslated region of *aroF* (Fig. 2 and 3). To demonstrate that the ORF was transcribed, the *Hin*dIII fragment in pMU1743 was inverted to form an ORF-*cat* gene fusion. To achieve this, pMU1743 was cleaved with *Hin*dIII and then religated and transformed into the *tyrR* strain JP4099. Transformants resistant to ampicillin were selected and then screened for resistance to 5 μg of chloramphenicol per ml. Most of the transformants were Cm^s and were found to contain plasmids which lacked the *Hin*dIII insert. About 6% of the transformants screened were Cm^r. These fell into two classes on the basis of their level of Cm^r. One class was resistant to 200 μg of chloramphenicol per ml and was identical in phenotype to JP4099(pMU1743). The second class was resistant to only 20 μg of chloramphenicol per ml. To determine the orientation of the *Hin*dIII fragment within each plasmid, crude plasmid preparations were digested with *Bam*HI, and the resulting fragments were separated on agarose gels. Cleavage with *Bam*HI can distinguish between the two orientations (Fig. 3). This demonstrates that the *Hin*dIII fragment had been inverted in the plasmids in the second class of transformants, and since the *cat* gene is being expressed, this result indicates that the ORF is preceded by a functional promoter.

To determine whether the ORF promoter was regulated by $tyrR^+$, one plasmid carrying an ORF-*cat* fusion, pMU1754, was transformed into JP4098 and CC146. In addition, pACYC177 was transformed into strain JP4099(pMU1754). The CAT enzyme activity of each of these strains was determined. These results (Table 3) demonstrate that expression of the ORF is regulated by $tyrR^+$. Expression of the ORF-*cat* fusion was repressed twofold in a haploid $tyrR^+$ strain, while CAT activity in the strain carrying the multicopy $tyrR^+$ plasmid, CC146, was not detected by the assay used, although the cells were resistant to 2 μg of chloramphenicol per ml. This represents at least a 16-fold repression in the presence of pMU1065. In contrast, the introduction of pACYC177 into strain JP4099(pMU1754) caused only a twofold decrease in CAT activity. This decrease may have been due to some effect on plasmid copy number but demonstrates that the repression of the ORF-*cat* fusion occurring in the presence of pMU1065 was largely due to the $tyrR^+$ gene. These results can be compared with the CAT activities conferred on the same strains by the *aroF-cat* fusion plasmid, pMU1743 (Table 3).

The TyrR protein is known to act in concert with all three aromatic amino acids, singly or in combination, to regulate the various members of its regulon (4). *aroF* is regulated by

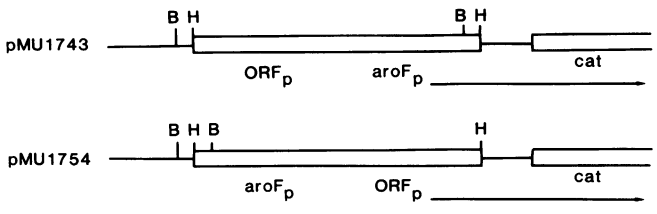

FIG. 3. Formation of the ORF-*cat* fusion plasmid, pMU1754. Details of the *aroF-cat* plasmid, pMU1743, and ORF-*cat* plasmid are shown. Digestion of pMU1743 with *Hin*dIII (H) and inversion of the 246-bp *Hin*dIII fragment formed pMU1754. Digestion with *Bam*HI (B) was used to distinguish between the two plasmids.

TABLE 3. Regulation of wild-type and mutant ORF-*cat* and *aroF-cat* fusions by $tyrR^+$

Mutation (TYR R box)[a]	*tyrR* genotype[b]	CAT activity (U/mg of protein) (repression ratio)[c]	
		ORF-*cat*	*aroF-cat*
None	−	0.032	8.2
	+	0.014 (2.3)	ND[d]
	p^+	<0.002 (>16)	0.052 (158)
	p^-	0.015 (2.1)	ND
G-to-A transition at −37 (box 1)	−	0.026	13.2
	p^+	0.013 (2.0)	1.4 (9.4)
A deletion at −52 (box 2)	−	0.039	6.2
	p^+	0.013 (3.0)	2.4 (2.6)

[a] The positions of the TYR R box mutations are shown in Fig. 2.
[b] The *tyrR* genotypes are described in Table 2, footnote *b*.
[c] The repression ratio is the ratio of the CAT activity conferred on JP4099 by a given plasmid to the activity conferred by the same plasmid on the second strain. The CAT activities for the *aroF-cat* fusion (pMU1743) and its mutant derivatives (pMU1752 and pMU1753) are from Table 2.
[d] ND, Not done.

$tyrR^+$ in concert with tyrosine as an effector molecule (or with phenylalanine at high concentrations) (3, 4). To determine which of the aromatic amino acids acts as the effector molecule(s) in regulating the expression of the ORF-*cat* fusion, strain JP4098(pMU1754) was assayed for CAT activity after growth in minimal medium in the presence of the three aromatic amino acids together and separately and in their absence. No significant differences were observed (data not shown). This may be because in a haploid $tyrR^+$ strain, the multicopy ORF-*cat* fusion is almost fully derepressed owing to titration of the repressor and relatively small effects exerted by the putative effector(s) may not be detected. Strain CC146(pMU1754) was also assayed for CAT activity after growth in the presence and absence of the aromatic amino acids; however, under neither growth condition was CAT activity detected. From this we can draw no conclusions regarding the effector molecule which modulates the repression of the ORF by $tyrR^+$.

Three regulatory mutants of the *aroF-cat* fusion plasmid pMU1743 have been characterized (Table 2). The mutations in these plasmids lie within either TYR R box 1 or box 2. To determine whether these mutations also affected the repression of the ORF promoter by $tyrR^+$, the *Hin*dIII fragment carrying the *aroF* and ORF promoters in each of these plasmids was inverted as described above. The resulting ORF-*cat* fusion plasmids carrying the TYR R box mutations were transformed into JP4099 and CC146. In each case, the level of chloramphenicol resistance conferred on CC146 by the plasmid was increased relative to that conferred by the wild-type ORF-*cat* fusion plasmid, pMU1754 (data not shown). The CAT activities conferred by two of these plasmids with mutations in TYR R boxes 1 and 2, respectively, are shown in Table 3. These results demonstrate that the mutant ORF-*cat* plasmids are repressed only 2- to 3-fold in the presence of pMU1065 compared with at least 16-fold repression of the wild-type fusion.

DISCUSSION

We have described the construction of an *aroF-cat* gene fusion which has been used to select for *aroF* regulatory mutants. The particular advantage of this fusion is that it is stable in a *tyrR* strain. This has made possible the formal

Consensus	agTGTAAAT--|-t-TtTACA-a	
TYR R box 3	AGTGTAAATtt|aTcTaTACAgA	16/17
TYR R box 2	tGTGTAAATaa|aagTgTACgaA	13/17
TYR R box 1	taTGgAttgaa|aacTTTACttt	8/17

FIG. 4. Nucleotide sequence of TYR R boxes in *aroF*. The consensus sequence is that determined by DeFeyter et al. (10). Nucleotides in the individual TYR R boxes which correspond to the consensus sequence are in capital letters, and those which differ are in lowercase letters. The numbers of positions which correspond to the consensus sequence are shown on the right. The vertical bar indicates the center of dyad symmetry of each box, and symmetrical nucleotides are underlined.

mutants. The particular advantage of this fusion is that it is stable in a *tyrR* strain. This has made possible the formal demonstration that the mutants isolated are no longer fully repressed by $tyrR^+$ and permits the residual level of repression to be determined. These studies have demonstrated that three sequences (or TYR R boxes) are necessary for the full repression of the *aroF* promoter by $tyrR^+$. Mutations in any one of these TYR R boxes result in at least partially constitutive expression of *aroF*.

In a previous study, Garner and Herrmann isolated *aroF* regulatory mutants by using the intact *aroF-tyrA* operon on a multicopy plasmid (13). The mutations they described were in either TYR R box 2 or box 3 (called $aroF_o1$ and $aroF_o2$, respectively, in their paper). In contrast, in this study, all the mutants isolated in vivo have mutations in TYR R box 1 or box 2 but none in box 3. It is not clear whether this difference is due simply to chance or to the different selection procedures used to isolate the mutants. In this work, spontaneous mutations in box 3 may not have been isolated because of the relatively small increase in Cm^r expected for box 3 mutants compared with box 1 or box 2 mutants. It was apparent that even when the selection for mutants was only 40 μg of chloramphenicol per ml, most mutants obtained were actually resistant to 200 μg/ml. It may be possible that box 3 mutants were at a selective disadvantage relative to other mutants during some stage of the isolation procedure.

It is possible to compare the effects of mutations in the corresponding positions of TYR R boxes 1 and 2. For example, the mutant with a single base-pair insertion in the center of box 1 retains a threefold repression by $tyrR^+$, while the corresponding mutation in box 2 allows only twofold repression. More striking is the comparison of the mutants with GC-to-AT transitions in the left arms of boxes 1 and 2. The mutation in box 2 allows only 2-fold residual repression, while the corresponding box 1 mutant is repressed 10-fold. This suggests that a greater degree of repression can be achieved by boxes 2 and 3 together than by boxes 1 and 3 together. One model to explain these observations is that binding of repressor to box 1 requires or is enhanced by binding to box 2, and therefore mutations in box 1 have a lesser effect than corresponding mutations in box 2.

This suggestion is supported by the observation that the deletion mutant retaining only box 1 is completely derepressed, while the deletion mutant retaining both boxes 1 and 2 can be repressed 24-fold (compared with approximately 150-fold in the wild type). This is perhaps reflected by the relative lack of dyad symmetry in box 1 and its more limited homology to the TYR R box consensus sequence (Fig. 4). It is noteworthy that the two members of the TyrR regulon which have only a single TYR R box, *aroG* and *tyrR* itself, show a high degree of symmetry within the TYR R box and a high level of homology to the consensus sequence. Furthermore, only a three- to eightfold repression of these genes is achieved by repressor binding at these single TYR R boxes (4, 5). It is perhaps not surprising, therefore, that repressor is unable to bind at TYR R box 1 alone in the *aroF* regulatory region.

All the mutants selected in vivo by Garner and Herrmann (13) and in this study retain some residual level of repression by $tyrR^+$. With such point mutations, it is not possible to distinguish between a situation in which a mutation only partially disrupts the ability of a given TYR R box to bind repressor and one in which the remaining two boxes can function, to some extent, independently of the third, completely inactivated, box. The deletion of box 3 in this study demonstrates that while box 3 is necessary for complete repression of *aroF*, boxes 1 and 2 together can achieve an intermediate level of repression.

The only other member of the TyrR regulon which is known to be controlled by three TYR R boxes is the *aroLM* operon. In this case, the positioning of the boxes with respect to each other is almost identical to *aroF*, except for an additional nucleotide pair between boxes 2 and 3 in *aroL* (10). A notable difference is that in *aroL*, it is box 3 which overlaps the −35 promoter sequence. In a wild-type strain, *aroL* can be repressed only 6-fold by $tyrR^+$ (12) compared with 40- to 50-fold for *aroF* (4). It is likely that the positioning of the TYR R boxes with respect to the promoters of *aroF* and *aroL* is an important factor in determining the levels of repression achieved. Also, in *aroL*, it is box 2 which bears the least identity with the TYR R box consensus sequence. Perhaps for *aroL*, repressor binding to box 1 assists repressor binding to box 2, and this, too, plays a role in limiting the level of repression of *aroL* which can be achieved.

Recent studies of the arabinose operon have shown that repression involves an interaction between two sites separated by some 300 bp and that the interaction between these sites is dependent on their being separated by an integral number of turns of the DNA helix (11). In vitro studies have also shown that cooperative binding of λ repressor to adjacent operator sites is similarly dependent on the number of helical turns between the sites (14). It is likely that TYR R box 3 in *aroF* interacts in a similar way with box 1 or box 2 or both. Experiments aimed at investigating this possibility are currently in progress.

The studies on the ORF proceeding divergently from the *aroF* regulatory region demonstrate that this ORF is preceded by a functional promoter, that this promoter is, like *aroF*, regulated by $tyrR^+$, and that at least two of the TYR R boxes involved in the repression of *aroF* are also required for the repression of this gene. A comparison of the CAT activities conferred on the *tyrR* strain, JP4099, by the wild-type ORF-*cat* and *aroF-cat* fusions suggests that the ORF is transcribed at very low efficiency: less than 1% of the level of transcription of *aroF*. It is not clear to what extent the divergent ORF promoter is repressed by $tyrR^+$ compared with repression of *aroF*, since in the presence of the multicopy $tyrR^+$ plasmid, expression of the ORF-*cat* fusion could not be detected. There are other examples of divergently transcribed genes that are regulated from a common site, for instance, in the ArgR regulon (9). This is the first example of bidirectional regulation involving genes in the *tyrR* regulon. It appears that this uncharacterized ORF is a new member of the *tyrR* regulon; however, it may be

fortuitous that it is regulated by *tyrR*$^+$ and is of no physiological consequence to the cell. In view of the apparent low level of transcription of the ORF, it is probably of some physiological importance to the cell that the ORF is regulated by *tyrR*$^+$ over a relatively wide range.

The function of this gene is yet to be determined. We have constructed a strain carrying a deletion of the proximal region of the ORF on the bacterial chromosome, and this mutation confers no apparent phenotype on the cell, indicating that the gene is not involved in any essential biosynthetic pathway (unpublished results). Hudson and Davidson (15) have suggested, from an analysis of the codon usage of the region of the gene sequenced, that this gene may itself code for a regulatory protein. This suggestion is in accord with the observed low level of transcription of the ORF. Experiments aimed at analyzing possible effects of the ORF deletion mutation on the expression of other genes involved in the biosynthesis of the aromatic amino acids and vitamins are in progress.

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