JOURNAL OF BACTERIOLOGY, Sept. 1997, p. 5914–5921 Vol. 179, No. 18
0021-9193/97/$04.00+0
Copyright © 1997, American Society for Microbiology

The *tpl* Promoter of *Citrobacter freundii* Is Activated by the TyrR Protein†

HONG QIU SMITH AND RONALD L. SOMERVILLE*

Department of Biochemistry, Purdue University, West Lafayette, Indiana 47907

Received 5 May 1997/Accepted 15 July 1997

The ability of microorganisms to degrade L-tyrosine to phenol, pyruvate, and ammonia is catalyzed by the inducible enzyme L-tyrosine phenol lyase (EC 4.1.99.2). To investigate possible mechanisms for how the synthesis of this enzyme is regulated, a variety of biochemical and genetic procedures was used to analyze transcription from the *tpl* promoter of *Citrobacter freundii* ATCC 29063 (*C. braakii*). By computer analysis of the region upstream of the *tpl* structural gene, two segments of DNA bearing strong homology to the known operator targets of the TyrR protein of *Escherichia coli* were detected. A DNA fragment of 509 bp carrying these operator targets plus the presumptive *tpl* promoter was synthesized by PCR and used to construct a single-copy *tpl-lacZ* reporter system. The formation of β-galactosidase in strains carrying this reporter system, which was measured in *E. coli* strains of various genotypes, was strongly dependent on the presence of a functional TyrR protein. In strains bearing deletions of the *tyrR* gene, the formation of β-galactosidase was reduced by a factor of 10. Several mutationally altered forms of TyrR were deficient in their abilities to activate the *tpl* promoter. The pattern of loss of activation function was exactly parallel to the effects of the same *tyrR* mutations on the *mtr* promoter, which is known to be activated by the TyrR protein. When cells carrying the *tpl-lacZ* reporter system were grown on glycerol, the levels of β-galactosidase were 10- to 20-fold higher than those observed in glucose-grown cells. The effect was the same whether or not TyrR-mediated stimulation of the *tpl* promoter was in effect. By deleting the *cya* gene, it was shown that the glycerol effect was attributable to stimulation of the *tpl* promoter by the cyclic AMP (cAMP)-cAMP reporter protein system. A presumptive binding site for this transcription factor was detected just upstream of the −35 recognition hexamer of the *tpl* promoter. The transcriptional start point of the *tpl* promoter was determined by chemical procedures. The precise locations of the TyrR binding sites, which were established by DNase I footprinting, agreed with the computer-predicted positions of these regulatory sites. The two TyrR operators, which were centered at coordinates −272.5 and −158.5 with respect to the transcriptional start point, were independently disabled by site-directed mutagenesis. When the upstream operator was altered, activation was completely abolished. When the downstream operator was altered, there was a fourfold reduction in reporter enzyme levels. The *tpl* system presents a number of intriguing features not previously encountered in TyrR-activated promoters. First among these is the question of how the TyrR protein, bound to widely separated operators, activates the *tpl* promoter which is also widely separated from the operators.

The ability of microorganisms to degrade L-tyrosine to phenol, pyruvate, and ammonia in a reaction catalyzed by the inducible enzyme L-tyrosine phenol lyase (EC 4.1.99.2) is restricted to certain genera within the *Enterobacteriaceae* family (23). Detailed studies, including the cloning and characterization of the structural gene for tyrosine phenol lyase, have been carried out for *Citrobacter freundii* (3, 22), *Erwinia herbicola* (15, 39), and *Escherichia intermedia* (25).

In *E. intermedia* and *E. herbicola*, the induction of tyrosine phenol lyase is stimulated when cells are grown in the presence of L-tyrosine (14, 23) and reduced in catabolite-repressed cells (14). Our current understanding (31) of the role of the TyrR protein in controlling rates of gene expression in response to exogenous L-tyrosine in *Escherichia coli* made it seem likely that the tyrosine phenol lyase (*tpl*) gene would belong to the TyrR regulon.

To investigate possible mechanisms for the regulation of tyrosine phenol lyase, we employed a variety of genetic and biochemical procedures to analyze transcription from the *tpl* promoter of *C. freundii*. The results suggest that the *tpl* promoter is subject to activation by the TyrR protein. In a parallel study (5), the *tyrR* gene of *C. freundii* has been characterized and found to bear a high degree of sequence identity to the *tyrR* gene of *E. coli*. By several criteria, the *tpl* gene thus qualifies for membership in the TyrR regulon.

* Corresponding author. Phone: (765) 494-1614. Fax: (765) 494-7897.

† Journal Paper no. 15465 of the Purdue University Agricultural Research Station.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and plasmids. The biological materials used are listed in Table 1. The P1 transduction method used to construct strains SP1625 and SP1626 was similar to that described by Miller (30).

Media. Basal medium was salts mix E of Vogel and Bonner (41). The following compounds were included when appropriate: L-tyrosine, 50 μg/ml; 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal), 40 μg/ml; glucose, 0.2%; glycerol, 0.2%; and ampicillin, 50 μg/ml. All minimal media contained thiamine hydrochloride (1 μg/ml) and biotin (0.1 μg/ml). Either L broth (26) or nutrient agar (31 g/liter; Difco) was used as complete medium for the propagation of bacterial strains. Bacteriophage λ derivatives were propagated and titrated on tryptone agar (16).

DNA preparations. Plasmid DNA and M13 replicative-form DNA were purified from cultures grown to saturation in L broth with Wizard Plus Miniprep resins purchased from Promega. Single-stranded M13 DNA was prepared by the procedure of Sanger et al. (33). The preparation of competent cells and their transformation were carried out by the procedure of Mandel and Higa (27). When thermosensitive lysogens were being transformed, the heat step was omitted.

Chemicals and reagents. Restriction endonucleases, T4 DNA ligase, and DNA polymerase I large (Klenow) fragment were purchased from New England Biolabs. *E. coli* RNA polymerase holoenzyme was purchased from Epicentre. TyrR protein was purified as previously described (11). TyrR protein concentra-

TABLE 1. Bacterial strains, plasmids, viruses, and oligonucleotides

Strain, plasmid, phage, and oligonucleotide	Description or sequence	Source or reference
E. coli strains		
DH5α	F$^-$ ϕ 80 d*lac*ΔM15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 phoA hsdR17*(rK$^-$ mK$^+$) *supE44* λ$^-$ *thi-1 gyrA96 relA1*	18
TG-1	*supE thi-1* Δ(*lac-proAB*) Δ(*mcrB-hsdSM*)*5* (rK$^-$ mK$^-$) [F′ *traD36 proAB lacI*^{q} ZΔM15]	Stratagene
NK5031	Δ(*lacIZY*) MS265 *gyrA supF*	29
SP1312	F$^-$ *zah-735*::Tn*10* Δ(*argF-lac*)*U169*	19
SP1313	F$^-$ *zah-735*::Tn*10* Δ(*argF-lac*)*U169* Δ*tyrR*	19
CSH26(λRZ11)	*ara* Δ(*lac-pro*) *thi* (λ *plac5* cI$_{857}$ Sam7)	45
7200	λ$^-$ e14$^-$ *relA1 spoT1* Δ(*cya-1400*)::*kan*	35
SP1625	SP1312(λHQS10) *cya*::*kan*	This work
SP1626	SP1312(λHQS10-mut1) *cya*::*kan*	This work
Other bacteria		
C. braakii	(*C. freundii*)	ATCC 29063
Plasmids		
pRVT	*tpl* gene cloned in pBluescript	3
pMLB1034	Promoterless *lacZ*; Amp^r	6
pJC100	*tyrR*$^+$ gene cloned in pET3a	37
pJC131	*tyrR* (Δ4) in pJC100	10
pJC132	*tyrR* (Δ8) in pJC100	10
pJC134	*tyrR* (L3A) in pJC100	10
pJC135	*tyrR* (E4A) in pJC100	10
pJC137	*tyrR* (R2K) in pJC100	10
pJC138	*tyrR* (R2E) in pJC100	10
pJC142	*tyrR* (L3K) in pJC100	10
pJC144	*tyrR* (L3P) in pJC100	10
pJC145	*tyrR* (L3W) in pJC100	10
pJC146	*tyrR* (L3I) in pJC100	10
pUC19	Cloning vector	45
pUC19-tpl	Wild-type *tpl* promoter in pUC19	This work
pUC19-tpl Δ45	Deletion derivative of pUC19-*tpl*	This work
pUC19-tpl mut2Δ	G→A change in Box B of the *tpl* promoter	This work
pUC19-tpl mut2Δ45	G→A change in Box B of the *tpl* promoter	This work
pUC19-tpl mut3	Changes to −35 hexamer of *tpl* promoter	This work
pHQS10	Wild-type *tpl* promoter in pMLB1034	This work
pHQS10-mut1	*tpl* promoter in pMLB1034; G→A change in Box A	This work
pHQS10-mut2	*tpl* promoter in pMLB1034; G→A change in Box B	This work
pHQS10-mut3	*tpl* promoter in pMLB1034; altered −35 hexamer	This work
pHQS10-Δ65	Deletion derivative of *tpl* in pMLB1034 (Fig. 1)	This work
pHQS10-Δ130	Deletion derivative of *tpl* in pMLB1034 (Fig. 1)	This work
pHQS10-Δ179	Deletion derivative of *tpl* in pMLB1034 (Fig. 1)	This work
Phages		
m13mp18/p	Promoter cloning vector	36
m13mp18/p-tpl	Mutagenesis substrate	This work
m13mp18/p-tpl-mut1	G→A mutation in Box A (see Fig. 1)	This work
λHQS10	*tpl-lacZ* reporter	This work
λHQS10-mut1	*tpl-lacZ* reporter with G→A mutation in Box A	This work
λHQS10-mut2	*tpl-lacZ* reporter with G→A mutation in Box B	This work
λHQS10-mut3	*tpl-lacZ* reporter with altered −35 hexamer	This work
λHQS10-Δ64	*tpl-lacZ* reporter with 64-nucleotide deletion	This work
λHQS10-Δ130	*tpl-lacZ* reporter with 130-nucleotide deletion	This work
Oligonucleotide		
1355F	CGGAATTCTAACTCACTGAAGCAAATAGCA	
1356F	AGGGATCCGCCGGATAATTCATTTTGTTTC	
161H	GTGTAAAGCAAAGTGTATAAAACGGCGTTG	
896H	CTGAAAACTTAACTTTCTAGTCTGTGCTCA	
256J-II	TGAGCACAGACTAGAAAGTTAAGTTTTCAG	
293J	GCATCACCTACAACAGGCGCTTTTTAAATTATTGCCAG	
292J	CTGGCAATAATTTAAAAAGCGCCTGTTGTAGGTGATGC	
1412I	CGGAATTCTTTTAGTGATGT	
1413I	CGGAATTCAGCAATACAGTA	
1275I	CGGAATTCCCCTCTCCACG	
688I	ATGTGGAGGCACTATGCAAGATTTAATTGA	

tions were determined spectrophotometrically at 280 nm, with an extinction coefficient of 34,470 M^{-1} cm^{-1} (42). α-^{35}S-dATP and [α-^{32}P]dATP were purchased from Amersham. Special-purpose oligonucleotides for use in PCR and site-directed mutagenesis were synthesized in the Laboratory for Macromolecular Structure, Purdue University. All other chemicals were of the highest quality that was commercially available.

DNA sequence analysis. DNA sequences were determined by the method described by Sanger et al. (33), modified for the use of α-^{35}S-dATP as the labeling nucleotide (7). The reagents for sequencing were purchased from U.S. Biochemicals in kit form (Sequenase version 2.0) and used according to the directions of the supplier.

Construction of λ *tpl-lacZ* lysogens. *tpl* promoter fragments were inserted as *Eco*RI-*Bam*HI fragments into pMLB1034. The resulting pMLB1034 derivatives were transformed into CSH26(λRZ11). Lac$^+$ λ recombinants were isolated as described by Yu and Reznikoff (45) with NK5031 as the plating indicator. High-titer lysates of Lac$^+$ λ recombinants were used to lysogenize strains SP1312 (*tyrR*$^+$) and SP1313 (Δ*tyrR*). Two independently isolated lysogens of each promoter construction were used in β-galactosidase assays.

Enzyme assays. β-Galactosidase was measured as described elsewhere (30). Cells were grown at 30°C to early log phase in Vogel-Bonner medium in the presence of L-tyrosine (50 μg/ml) before being sampled. Assays were carried out in triplicate and have standard errors of ≤10%. The enzyme assay values are reported in Miller units (20).

DNase I footprinting. A DNA fragment of 527 bp carrying the *tpl* promoter was released from pUC19-*tpl* by digestion with *Eco*RI plus *Pst*I. The digest was subjected to electrophoresis on 0.9% agarose. The promoter-bearing fragment was purified by adsorption to DEAE paper (Schleicher and Schuell) followed by elution with 20 mM Tris (pH 8.0) containing 0.1 mM EDTA and 1 M NaCl. The DNA-containing solution was then phenol-chloroform treated and ethanol precipitated. For use, the *tpl* promoter DNA was dissolved in water at a concentration of 0.1 mg/ml. A sample of this fragment (0.3 μg) was selectively labeled at the *Eco*RI site by treatment with DNA polymerase I (Klenow fragment) in the presence of [α-^{32}P]dATP according to the method of Brenowitz et al. (9). The unincorporated radiolabel was removed with a G-25 spin column (Boehringer Mannheim), and residual protein was removed with a QIA spin column (Qiagen), according to the recommendations of the supplier. Radiolabeled DNA (15,000 cpm per tube) was allowed to interact at 37°C with TyrR protein and/or RNA polymerase holoenzyme (for a typical set of conditions, see Fig. 3) in 10 mM Tris (pH 8.0)–5 mM $MgCl_2$–1 mM $CaCl_2$–100 mM KCl–2 mM dithiothreitol in the presence of bovine serum albumin (50 μg/ml) and sonicated calf thymus DNA (2 μg/ml). The final volume of each binding reaction mixture was 180 μl. After 30 min, each tube was treated with 5 μl of DNase I (0.5 μg/ml) for 2 min. Digestion by DNase I was halted by the addition of 40 μl of 50 mM EDTA (pH 8.0). Each sample was then treated twice with 200 μl of phenol-chloroform-isoamyl alcohol (24:25:1). The DNA was precipitated with ethanol and resuspended in 5 μl of loading dye, and the suspension was heated for 10 min at 85 to 90°C and loaded onto an 8% acrylamide–6 M urea DNA sequencing gel. After electrophoresis, the gel was exposed overnight at −70°C to Kodak XAR-5 film.

Preparation of RNA. RNA was prepared from 250 ml of mid-log-phase cells, according to the procedure of Salser et al. (32). Two sets of cellular RNA were prepared, either from *E. coli* DH5α(pHQS10) or *C. freundii* ATCC 29063. The *E. coli* strain was grown at 37°C in L broth plus ampicillin (50 μg/ml); the *C. freundii* strain was grown at 30°C in tryptic soy broth (Difco) supplemented with 0.2% L-tyrosine. The RNA was stored at −20°C in the form of an ethanol slurry. A third preparation of RNA was obtained by in vitro transcription with pUC19-*tpl* as the template. The reaction mixture contained, in a final volume of 50 μl, 2 μg of template, bovine serum albumin (10 μl of 250 μg/ml), RNA polymerase holoenzyme (10 U), 2 μg of TyrR protein, 1 μl of RNase inhibitor, and ribonucleoside triphosphates (15 μl of 5 mM stock solution). After 30 min at 37°C the reaction was terminated with 50 μl of 40 mM EDTA containing 300-μg/ml tRNA. The RNA was purified by two successive precipitations with ethanol and stored at −20°C.

Oligonucleotide-directed mutagenesis. The introduction of the G→A change in Box A (see Fig. 1) was conducted with M13mp18/p-*tpl* as template by using the Sculptor in vitro mutagenesis kit from Amersham. The mutagenic oligonucleotide was 161 H (Table 1), and the procedure was according to the directions of the supplier. Changes to Box B and to the −35 hexamer of the *tpl* promoter were made with the oligonucleotide pairs 896H–256J-2 and 292J-293J, respectively, by using the Quick-Change Site-Directed Mutagenesis kit from Stratagene, according to the directions of the supplier. Each mutational change was verified by DNA sequencing and the removal or addition of a diagnostic restriction endonuclease cleavage site.

Primer extension analysis of transcriptional start point. A 10-ng sample of a 30-residue oligodeoxynucleotide (688I [Table 1]), complementary to *tpl* mRNA (Fig. 1), was hybridized with 10 to 50 μg of RNA in 0.15 M KCl–0.01 M Tris (pH 8.3)–1 mM EDTA by heating at 100°C for 2 min followed by quick cooling on ice. The extension reaction was carried out as described by Curtis (12) in a final volume of 15 μl for 40 min at 42°C. At that time, excess nonradioactive dATP was added, and incubation was continued for an addition 40 min. The reaction was stopped by adding an equal volume of sequencing stop solution and heating to 90°C for 2 min. A 5-μl sample was loaded onto an 8% acrylamide–6 M urea sequencing gel. The extension products were run next to a sequencing ladder generated with the same oligodeoxynucleotide and pUC19-*tpl* as template.

***Rsa*I protection assay for binding of TyrR protein to the *tpl* promoter.** To simplify the interpretation of the protection assay, a derivative of pUC19-*tpl* with one less *Rsa*I site was constructed. A sample of pUC19-*tpl* was digested with *Sna*BI and *Bam*HI and then treated with the Klenow fragment of DNA polymerase in the presence of an equimolar mixture of deoxyribonucleoside triphosphates. The product of the latter reaction was circularized with T4 DNA ligase. This procedure eliminated the *Rsa*I site at coordinate +137 (see Fig. 1), generating pUC19-*tpl*Δ45. Reaction mixtures contained, in a final volume of 20 μl, 1.5 to 2 μg of target DNA (pUC19 *tpl* Δ45), 1 mM Tris-Cl (pH 7.5), 1 mM $MgCl_2$, 5 mM NaCl, and 0.1 mM dithioerythritol. Digestion with *Rsa*I (5 U/tube) was carried out at 37°C for 2 h. The digestion products were analyzed by electrophoresis on 0.9% agarose gels. When appropriate, TyrR protein, ATP, and L-tyrosine were also added. For specific conditions, see Fig. 5.

RESULTS

Analysis of DNA sequences upstream of *tpl* genes. The DNA sequences adjacent to the known *tpl* genes (3, 15, 22, 25, 39) were scrutinized for the presence of target sites for the TyrR protein. A consensus table based on authentic TyrR boxes was used as the reference system, as previously described (21). Several potential operator targets were detected in the region upstream of the *tpl* gene of *C. freundii*, the sequence of which was determined by Antson et al. (3). With the first A of the starting Met codon of the *tpl* structural gene designated coordinate +1, possible TyrR boxes were detected with centers at coordinates −434.5, −321.5, −205.5, −116.5, and −47.5.

Construction of *lacZ* reporter systems specific for the *tpl* promoter. The *tpl* promoter was synthesized by PCR with pRVT (3) as the template and oligonucleotides 1355F and 1356F (Table 1) as primers. Oligonucleotide 1355F hybridized with DNA sequences upstream of the putative TyrR box at coordinate −434.5. It was designed to introduce a cleavage site for *Eco*RI. Oligonucleotide 1356F was designed to permit an in-frame connection (via a *Bam*HI site) between the first five codons of the *tpl* gene and the *'lacZ* gene of pMLB1034 (6). The PCR product was digested with *Eco*RI and *Bam*HI, yielding a DNA fragment of 509 bp that was inserted into similarly cleaved pMLB1034. The resulting plasmid was named pHQS10. The *tpl-lacZ* transcription-translation fusion of pHQS10 was then transferred by standard techniques (4) into an integration-proficient derivative of bacteriophage λ. The resulting phage was named λHQS10. Single-copy lysogens of the Δ(*lac*) host strains SP1312 (*tyrR*$^+$) and SP1313 (Δ*tyrR*) were prepared. Double lysogens were identifiable on the basis of colony color on X-Gal medium or through standard β-galactosidase assays. The enzyme data from these isolates were rejected. Essentially identical procedures were followed in the construction of LacZ reporter systems carrying mutationally altered *tpl* promoters (see below), except that mutagenesis was carried out prior to insertion into pMLB1034. The mutagenesis substrates were either M13-*tpl* subclones or pUC19-*tpl* subclones. The sequence of the *tpl* promoter is shown in Fig. 1.

Regulation by TyrR of the *tpl* promoter. Strains of *E. coli* carrying LacZ reporter systems specific for the *tpl* promoter were grown and assayed for β-galactosidase activity (Table 2). In *tyrR*$^+$ strains, there was substantial production of β-galactosidase. In strains bearing a deletion of the *tyrR* gene, there was a reduction of approximately 10-fold in reporter enzyme levels. This effect was reversed in the *tyrR* deletion strain when a plasmid (pJC100 [37]) that mediated the expression of a functional TyrR protein was introduced. These results suggested that the *tpl* promoter, like the *mtr* and *tyrP* promoters, was subject to positive control by the TyrR protein.

Mutationally altered forms of TyrR unable to activate transcription from the *tpl* promoter. In previous studies, it was found that a number of in-frame deletions and amino acid

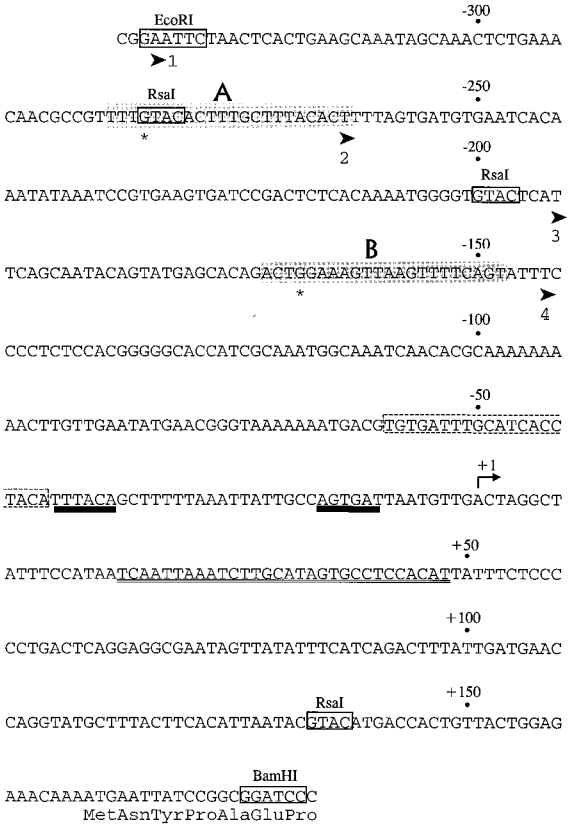

FIG. 1. The *tpl* promoter and associated regulatory elements. Only the nucleotide sequence of the messenger-equivalent strand is shown. The coordinate system has been changed from the original publication (3) by assigning +1 to the start point of transcription. DNA fragments used in the functional analysis of the *tpl* promoter were synthesized by PCR, with pRVT1 (3) as the template. Cleavage sites for restriction endonucleases *Eco*RI and *Bam*HI were installed at the indicated locations (−329 and +180) in order to facilitate the construction of single-copy reporter systems. Cleavage sites for restriction endonuclease *Rsa*I (−278 and −198) that were used in studies of the binding of the TyrR protein to Box A operator targets (Fig. 3) are indicated. Residues marked by an asterisk (−280 and −166) are the locations of operator mutations that were generated during the course of this study. Note that the change of G (−280) to A leads to the elimination of an *Rsa*I cleavage site and that the change of G (−166) to A creates a cleavage site for *Bfa*I. The 5′ end points of three truncated derivatives of the *tpl* promoter (Table 1) are shown as broad arrowheads at coordinates −262, −193, and −144. The presumptive −10 and −35 recognition elements are underlined with heavy lines. The presumptive target site for cAMP-CRP (coordinates −58 through −29) is outlined with dashed lines. The hybridization site for the oligodeoxynucleotide primer used in the determination of the transcriptional start point is indicated by double underlining.

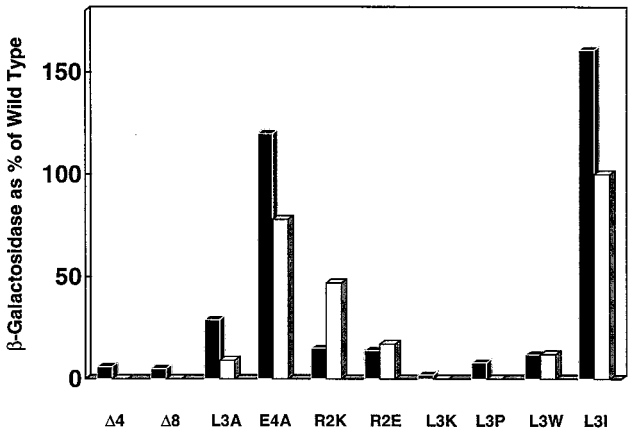

FIG. 2. Effects of single amino acid switches and deletion mutations within the TyrR protein on the ability to activate the *tpl* promoter. Into strain SP1313(λHQS10) were introduced a series of plasmids carrying different *tyrR* alleles (Table 1). The plasmid-bearing strains were grown in minimal salts-glucose medium and assayed as described in Materials and Methods. The 100% values used as the basis of comparison were 515 Miller U for assays involving the *tpl* reporter system and 5,090 Miller U for assays involving the *mtr* reporter system. Dark bars, *tpl* reporter system of λHQS10; open bars, *mtr* reporter system as described by Cui and Somerville (10).

switches near the N terminus of TyrR reduced or abolished the positive control function of this protein. These structural changes did not diminish the ability of TyrR to negatively regulate repressible promoters (10, 43). To compare the TyrR-mediated activation of *tpl* with the *mtr* system, a set of plasmids specifying mutationally altered forms of TyrR were introduced into a Δ(*tyrR*) host strain carrying a *tpl-lacZ* reporter system. When the β-galactosidase levels of the plasmid-bearing strains were measured (Fig. 2), there were variations in reporter enzyme levels that closely paralleled the results that had been obtained (10) with the activated *mtr* promoter. Mutational alterations that abolished TyrR activation of *mtr* (Δ4, Δ8, L3K, L3P, and L3W) also disallowed activation of the *tpl* promoter. Lesions that partly reduced the activation of *mtr* (L3A, R2K, R2E, and L3W) had similar effects on the operation of the *tpl* promoter. Finally, certain changes in TyrR that did not alter the activation of *mtr* (E4A and L3I) led to proteins that were fully functional in the activation of *tpl*. This set of results is fully consistent with a role for TyrR as an activator of the *tpl* promoter.

TABLE 2. β-Galactosidase levels in strains harboring *tpl-lacZ* reporter systems

Strain	Relevant genotype	Carbon source	β-Galactosidase (Miller U)
SP1312(λHQS10)	*tyrR*$^+$	Glucose	140
SP1313(λHQS10)	Δ(*tyrR*)	Glucose	13.5
SP1313(λHQS10)(pJC100)	Δ(*tyrR*) *tyrR*$^+$	Glucose	515
SP1312(λHQS10 mut1)	G→A in Box A	Glucose	17
SP1313(λHQS10 mut1)	G→A in Box A	Glucose	16
SP1312(λHQS10 mut2)	G→A in Box B	Glucose	39
SP1312(λHQS10)	*tyrR*$^+$	Glycerol	3,340
SP1312(λHQS10 mut1)	G→A in Box A	Glycerol	375
SP1625(λHQS10)	*tyrR*$^+$ Δ(*cya*)	Glycerol	55.7
SP1626(λHQS10 mut1)	G→A in Box A	Glycerol	9.25
SP1625(λHQS10)	*tyrR*$^+$ Δ(*cya*)	Glycerol + cAMP	219
SP1626(λHQS10 mut1)	G→A in Box A	Glycerol + cAMP	12
SP1312(λHQS10 mut3)	Altered −35 region	Glycerol	84

Identification of TyrR binding sites by DNase I footprinting. A fragment of DNA identical to the segment that had been used to construct the *tpl* reporter systems was preparatively isolated from pUC19-*tpl* as described in Materials and Methods. The fragment was selectively radiolabeled with ^{32}P at the *Eco*RI end. The promoter-bearing fragment was mixed with TyrR protein and/or RNA polymerase holoenzyme under a variety of conditions and then treated briefly with pancreatic DNase. The resulting digest was analyzed by gel electrophoresis (Fig. 3). Two regions of the *tpl* promoter were protected by bound TyrR protein. The first (designated Box A in Fig. 1) was centered 434.5 nucleotides upstream of the *tpl* gene. The second (designated Box B in Fig. 1) was centered 321.5 nucleotides upstream of the structural gene. Both TyrR binding sites, identified earlier by computer analysis, were close matches to the canonical TyrR target sequence ($TGTAAAN_6TTTACA$). Protection of Boxes A and B was negligible in the absence of RNA polymerase (data not shown), a feature of target recognition by TyrR that has not been previously encountered.

Site-directed mutagenesis of TyrR targets. The highly conserved first G residues of each TyrR target were changed to A residues, as described in Materials and Methods. The initial screening for the desired changes was facilitated by the fact that the G-to-A change within Box A eliminates a *Rsa*I site, while the G-to-A change in Box B creates a *Bfa*I site. The complete nucleotide sequence of each mutant promoter was verified by DNA sequencing. Each mutant promoter was then incorporated via pMLB1034 subclones into integration-proficient Lac^+ derivatives of bacteriophage λ for evaluation of promoter function in vivo. When the *tpl* promoter was altered via a G-to-A mutation in Box A, there was no detectable TyrR-mediated activation (Table 2). The reporter enzyme levels in $TyrR^+$ cells carrying *tpl* promoters with Box A mutations were essentially identical to those observed in Δ(*tyrR*) hosts, i.e., there was a 10-fold reduction in reporter enzyme levels compared to that for the *tpl* promoter with an intact Box A. The effect of the G-to-A mutation in Box B was not as severe (Table 2). As measured in terms of differential rate of β-galactosidase synthesis, the Box B mutation reduced the TyrR-mediated activation of the *tpl* promoter by only 3- to 4-fold.

Deletion of TyrR target sites abolishes *tpl* promoter function. To investigate whether the *tpl* promoter could function without the participation of the TyrR protein, three altered versions of the *tpl* promoter having progressively larger deletions of DNA from the upstream region were constructed. This was accomplished by PCR with oligonucleotides 1412I, 1413I, and 1275I (Table 1) to shorten the *tpl* promoter by 65, 130, and 179 nucleotide pairs, respectively. The endpoints of these deletion derivatives are indicated in Fig. 1. The three truncated *tpl* promoters were installed within *lacZ* reporter constructs in the usual fashion. In no case was there significant β-galactosidase activity (data not shown).

Quantitative aspects of the binding of TyrR to Box A of the *tpl* promoter. To investigate TyrR-operator interaction in more detail, a series of in vitro studies using a well-defined system was carried out. The protection of the cleavage site for *Rsa*I within Box A (Fig. 1) formed the basis of a convenient assay for the binding of TyrR. The binding of TyrR to Box A was associated with the appearance of a DNA fragment of 359 bp. Lack of operator protection was indicated by *Rsa*I fragments of 279 and 80 bp.

The *Rsa*I protection assay (Fig. 4) confirmed the DNase I footprinting results and the site-directed mutagenesis studies. In the absence of ligands, the TyrR protein was able to form stable complexes with the Box A segment of pUC19-*tpl*Δ45. Half-maximal protection was observed at a TyrR concentration of 15 pM. The affinity of the TyrR protein for Box A was greatly enhanced by ATP and tyrosine. In the presence of these ligands, half-maximal protection occurred at a protein concentration of 0.09 to 0.125 pM. Surprisingly, the protection by TyrR of the *Rsa*I site within Box A of pUC19-*tpl* Δ45 did not require RNA polymerase. By DNase I footprinting TyrR-operator interaction had been undetectable in the absence of RNA polymerase. The most likely reason for this difference is that the two methods for observing TyrR-operator interaction

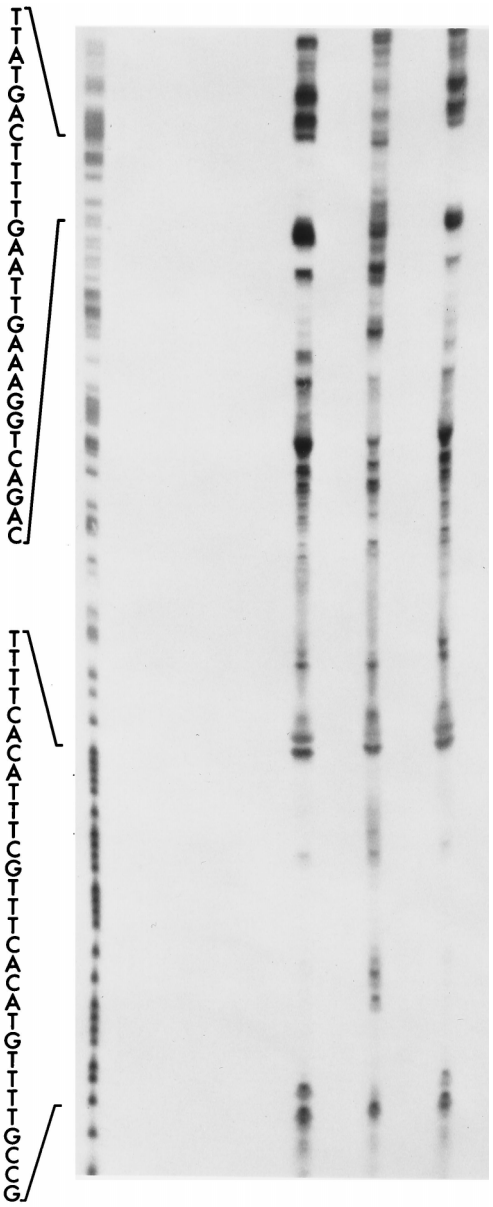

FIG. 3. DNase I footprinting analysis of TyrR binding sites within the *tpl* promoter. Incubations (final volume, 200 μl) were carried out in siliconized microcentrifuge tubes according to the procedure of Brenowitz et al. (9) as described in Materials and Methods. Left lane, (G+A) standards, prepared by treatment with formic acid (28). A DNA fragment, labeled at one end, carrying the *tpl* promoter was allowed to interact with proteins under several conditions. Lanes: 1, RNA polymerase holoenzyme (500 nM) plus TyrR (12 nM); 2, RNA polymerase holoenzyme (1,000 nM); 3, RNA polymerase holoenzyme (1,000 nM) plus TyrR (12 nM). ATP (200 μM) and L-tyrosine (200 μM) were present in each tube. The sequence shown reads from the 5′ end (bottom of figure) toward the 3′ end (top) as shown in Fig. 1.

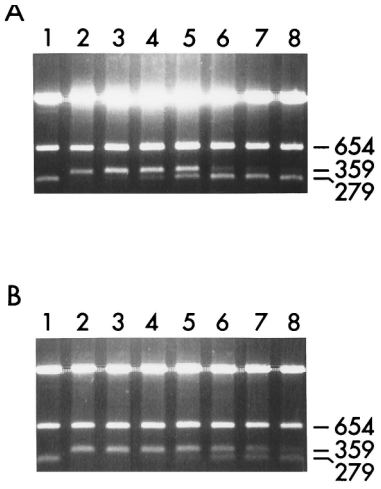

FIG. 4. Binding of the TyrR protein to Box A in vitro. The interaction of TyrR protein with pUC19-*tpl*Δ45 and subsequent treatment with *Rsa*I were carried out as described in Materials and Methods. (A) No ATP or L-tyrosine. Lanes: 1, no TyrR protein; 2 to 8, TyrR protein present at final concentrations of 300, 150, 60, 30, 15, 10, and 7.5 pM, respectively. (B) ATP (2 mM) and L-tyrosine (0.5 mM) present. Lanes: 1, no TyrR protein; 2 to 8, TyrR protein present at final concentrations of 30, 15, 7.5, 3.75, 2.5, 1.875, and 1.2 pM, respectively. The sizes (in base pairs) of fragments are indicated on the right. A DNA fragment of 80 bp which was released by *Rsa*I cleavage within Box A (Fig. 1) was not visible in these experiments.

employed DNA fragments that were topologically different. When pUC19-*tpl*Δ45 was treated with *Xmn*I (a single site in pUC19 far removed from the *tpl* insert), the resulting linear DNA was unable to interact with unliganded TyrR protein, as judged by *Rsa*I protection assays. In the presence of ATP, TyrR protein was able to interact with Box A in linear DNA, but four to five times more protein was required for *Rsa*I protection compared to the situation when the target was circular (data not shown). Evidently the circularity and/or state of supercoiling of pUC19-*tpl* enables TyrR to firmly associate with its target in the absence of RNA polymerase. With linear DNA, TyrR-operator binding is either weak, or the complex, if it does form, readily undergoes dissociation. When the Box A-specific *Rsa*I protection assay was used to study the binding of TyrR to DNA molecules bearing lesions in Box B, there were no observable differences from DNA molecules having a wild-type Box B (data not shown).

Transcriptional start point of the *tpl* promoter. Three different samples of RNA, prepared from *C. freundii* and from *E. coli*(pHQS10) and by in vitro transcription of pUC19-*tpl* were analyzed by primer extension with reverse transcriptase, as described in Materials and Methods. In each case (Fig. 5), a single transcriptional start point, corresponding to the A residue designated +1 in Fig. 1, was detected. This result shows that *tpl* mRNA contains an untranslated leader region of 165 nucleotides. Inspection of the region upstream of the transcriptional start point showed the presence of hexamers with substantial homology to the −10 and −35 recognition elements typical of σ^{70} promoters (Fig. 1). Experimental support for the correctness of the −35 hexamer was obtained by site-directed mutagenesis (see below). The −10 hexamer was chosen on the basis of its similarity to the consensus −10 hexamers reported in other systems.

Role of the cAMP-CRP system in *tpl* promoter function. Suzuki et al. (40) recently reported that the formation of *tpl* mRNA in *E. herbicola* AJ2985 was drastically curtailed when cells were cultivated in the presence of glucose. These workers also detected a possible cyclic AMP (cAMP) receptor protein (CRP) target site just upstream of the −35 recognition element of the *E. herbicola tpl* promoter. Inspection of the *tpl* promoter of *C. freundii* revealed a similarly situated CRP site (Fig. 1). Several experiments were, therefore, carried out to determine whether catabolite repression via the CRP system was a feature of the operation of the *C. freundii* promoter. Two $TyrR^+$ strains bearing *tpl-lacZ* reporter systems were cultivated in medium containing the noncatabolite-repressing carbon source glycerol. One of the two strains had a wild-type *tpl* promoter; the other bore a *tpl* promoter with a G→A change in Box A (Fig. 1) that prevented TyrR-mediated activation. The β-galactosidase levels in glycerol-grown cells (Table 2) were at least 20-fold higher than the enzyme levels seen in glucose-grown cells. The magnitude of the carbon source effect was the same whether or not TyrR-mediated stimulation was occurring (Table 2 [compare rows 1 and 6 and rows 5 to 7]). This suggests that cAMP-CRP-mediated stimulation of *tpl* promoter activity operates independently of TyrR-mediated activation. The overall amplitude of regulation of the *tpl* promoter via the two control mechanisms that have been demonstrated is thus about 200-fold.

Direct verification of a role for the cAMP-CRP system was obtained by deleting the *cya* gene from the pair of strains used in the previous experiment. This was accomplished by P1 transduction, with strain 7200 (35) as the donor. The resulting *cya*::*kan* derivatives (SP1625 and SP1626) were grown in glycerol-Casamino Acids medium either in the presence or the absence of cAMP. In the absence of cAMP, the *cya* mutant strains produced about 50 times less β-galactosidase than isogenic cya^+ strains (Table 2, rows 9 and 10). The production of β-galactosidase was stimulated by a factor of 4 to 5 when cAMP (0.5 mM) was present in the growth media (Table 2, rows 10 and 11). The failure of cAMP to restore β-galactosidase levels to control values undoubtedly reflects the hydrolysis of this compound by cAMP phosphodiesterase.

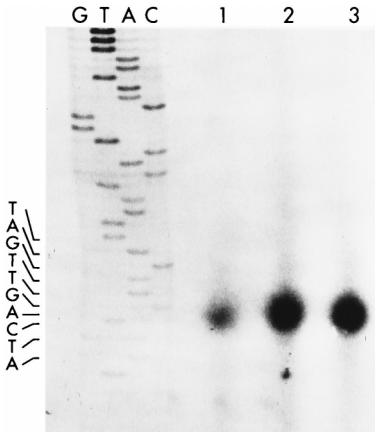

FIG. 5. Primer extension analysis of the *tpl* promoter. Lanes: 1, assay with an oligonucleotide primer corresponding to positions +19 through +48 of the *tpl* sequence (Fig. 1) and total RNA extracted from *C. freundii* grown at 30°C in trypticase soy broth (BBL) supplemented with L-tyrosine (500 μg/ml); 2, total RNA extracted from *E. coli* DH5α(pHQS10) grown at 37°C in L broth plus ampicillin (25 μg/ml); 3, RNA prepared by in vitro transcription of pUC19-*tpl* with the σ^{70} form of RNA polymerase. In all cases, annealing was accomplished by heating for 2 min at 100°C followed by cooling in an ice bath. The extension reactions were carried out at 42°C for 40 min with 120 U of reverse transcriptase. G, T, A, and C correspond to a parallel dideoxy sequencing ladder generated with the same primer. The sequence depicted on the left is the reverse complement of the sequence read from the autoradiogram and corresponds to the message-equivalent strand shown in Fig. 1.

Site-directed mutagenesis of the *tpl* promoter. To confirm the location of the *tpl* promoter, oligonucleotide-directed mutagenesis was used to change the −35 hexamer from TTTACA to ACAGGC. The resulting construct, named *tpl* mut 3, was incorporated in standard fashion into a single-copy *lacZ* reporter system. When cells harboring λ HQS10-mut3 were assayed in a $TyrR^+$ background, the β-galactosidase levels were reduced by more than 10-fold compared to control values (Table 2, row 12). This result suggests that the assignment of the location of the *tpl* promoter from the primer extension assays is correct.

DISCUSSION

In this report, we have presented several lines of evidence that transcription from the *tpl* promoter is subject to positive control by the TyrR protein. In tester strains bearing a deletion of the *tyrR* gene, there was at least a 10-fold reduction in the production of β-galactosidase from a single-copy *tpl-lacZ* reporter system. Promoter function was completely restored when a plasmid expressing the $tyrR^+$ gene was introduced into this *E. coli* strain. When several mutationally altered *tyrR* genes bearing lesions known to alter the activation of *mtr* or *tyrP* were separately introduced into such tester systems, there were reductions in *tpl* promoter activity that exactly paralleled those that had been observed in these two other TyrR-stimulated transcriptional units. By computer analysis, three DNA segments that might qualify as operator targets for TyrR were identified. Two TyrR boxes far upstream from the transcriptional start point (at −272.5 and −158.5) were shown to be valid targets for the binding of TyrR by DNase I footprinting, by a restriction endonuclease protection assay, and by the introduction of single nucleotide switches that either completely or partially disabled the in vivo activation of the *tpl* promoter. The third computer-predicted operator target failed to bind TyrR in vitro but happened to lie in a region of the *tpl* promoter that was almost certainly a binding site for CRP (see below).

In contrast to the situation that prevails in the other TyrR-regulated promoters, the operator targets of the *tpl* promoter lie far upstream of the transcriptional start point and are separated from each other by a considerable amount of DNA (114 bp). In the case of the *mtr* promoter, there are two TyrR binding sites whose centers are separated by 30 bp, just upstream of the −35 recognition element for RNA polymerase (20, 34). The centers of the two TyrR boxes that are important for TyrR-mediated activation of *tyrP* are separated by 23 bp; one of the boxes overlaps the −35 region of the *tyrP* promoter (2). The importance to transcriptional activation alterations in spacing between TyrR boxes has been studied in the *tyrP* system by Andrews et al. (1). These workers found that TyrR-mediated activation became undetectable when the two TyrR boxes in the region upstream of the promoter were separated by more than 32 bp. In the present study, we found that even when pairs of TyrR binding sites were far upstream from the promoter and separated by 10 helical turns of DNA, they still contributed in critical fashion to the control of transcription initiation. When either or both TyrR boxes were eliminated from the system, the transcriptional activity of the *tpl* promoter either became virtually undetectable (Box A) or was sharply reduced (Box B).

If cooperative interaction between TyrR dimers is an important aspect of transcriptional activation, this process can evidently occur over distances much greater than those heretofore observed. Within the *tpl* promoter, the activation sites for TyrR lie far upstream of the RNA polymerase binding site, at coordinates −272.5 and −158.5. The use of such locations, which have been classified "remote" by Gralla and Collado-Vides (17), suggests that looping out of intervening DNA could occur in order for RNA polymerase to be touched by the TyrR protein. Whether such looping occurs, or whether accessory factors such as histonelike proteins play a role in the process, remains to be investigated.

Role of cAMP-CRP in transcription from the *tpl* promoter. The tyrosine phenol lyase system of *C. freundii* has a number of features in common with the tryptophanase system of *E. coli*. The two enzymes catalyze analogous pyridoxal phosphate-dependent α,β-elimination reactions. At the primary structure level, 43% of the amino acid residues are identical (3, 22). Both tryptophanase and tyrosine phenol lyase are subject to catabolite repression (8, 14). In the case of the tryptophanase promoter, catabolite repression is attributable to a perfect consensus cAMP-CRP binding site ($TGTGAN_6TCACA$) centered at coordinate −61.5 (13). A similar presumptive cAMP-CRP binding site ($TGTGA\ N_6\ TCACC$) is centered at coordinate −50.5 of the *tpl* promoter (Fig. 1). The rate of utilization of the *tpl* promoter increases about 10-fold when cells are grown on glycerol, a non-catabolite-repressible carbon source (Table 2). The introduction of a *cya* (adenylate cyclase) mutation essentially eliminated transcription from the *tpl* promoter in glycerol-grown cells. This effect was partially overcome by the addition of cAMP. To a first approximation, the activation of the *tpl* promoter by cAMP-CRP and by the TyrR protein appears to be independent and additive, rather than synergistic. As previously noted (3), there does not appear to be a sequence encoding a leader peptide between the *tpl* promoter and the *tpl* gene. This is a major point of difference from the tryptophanase system, in which transcriptional antitermination mechanistically connected to the translation of a leader peptide is a major determinant of gene expression (38).

Recently, the regulation of the *tpl* gene of *E. herbicola* AJ2985 was analyzed by Suzuki et al. (40). Using Northern blotting methods, these authors demonstrated that *tpl* mRNA was induced by tyrosine and repressed by glucose, in agreement with the general conclusions of the present study. By primer extension mapping, the start point of transcription in *E. herbicola* was found to lie 121 bp upstream of the initiation codon of the *tpl* gene. In contrast, the untranslated leader region of *C. freundii* was 165 nucleotides long (Fig. 1 and 5). Suzuki et al. postulated the existence of a TyrR box centered at coordinate −65.5, a CRP binding site at coordinate −40.5, and a target homologous to the *lac* operator centered at coordinate +26 (40). This distribution of control elements is quite different from that of *C. freundii* (Fig. 1). Our analysis of the known *tpl* promoters suggests that these punctuation elements are of two separate categories. The first is represented by *C. freundii* (3, 22) and *E. intermedia* (25), while the second is found within *E. herbicola* (15, 39). Two features distinguish the two promoter classes. First, the distances between the transcriptional start points and the starting ATG of the structural gene are different. Second, the locations of operator targets for the TyrR protein are dissimilar. Both classes of *tpl* promoter appear to be subject to catabolite repression. In our scrutiny of the region upstream of the *tpl* promoter of *E. herbicola*, we detected possible TyrR targets at approximately the same locations as those that were identified in *C. freundii*. It would be of interest to investigate whether such sites are capable of interacting with the TyrR protein.

ACKNOWLEDGMENTS

For his advice and assistance during the early phases of this work, we thank Ihor Skrypka. We are also grateful to Robert S. Phillips for

supplying pRVT DNA and to the reviewers of this paper for their helpful comments.

Financial support for this work came from grants from the U.S. Public Health Service (GM22131) and the U.S. Army Research Office (DAAH 04-95-1-01 38).

REFERENCES

1. **Andrews, A. E., B. Dickson, B. Lawley, C. Cobbett, and A. J. Pittard.** 1991. Importance of the position of TyrR boxes for repression and activation of the *tyrP* and *aroF* gene in *Escherichia coli*. J. Bacteriol. **173:**5079–5085.
2. **Andrews, A. E., B. Lawley, and A. J. Pittard.** 1991. Mutational analysis of repression and activation of the *tyrP* gene in *Escherichia coli*. J. Bacteriol. **173:**5068–5078.
3. **Antson, A. A., T. V. Demidkina, P. Gollnick, Z. Dauter, R. L. Von Tersch, J. Long, S. N. Berezhnoy, R. S. Phillips, E. H. Harutyunyan, and K. S. Wilson.** 1993. Three-dimensional structure of tyrosine phenol-lyase. Biochemistry **32:**4195–4206.
4. **Aronson, B. D., M. Levinthal, and R. L. Somerville.** 1989. Activation of a cryptic pathway for threonine metabolism via specific IS3-mediated alteration of promoter structure in *Escherichia coli*. J. Bacteriol. **171:**5503–5511.
5. **Bai, Q., and R. L. Somerville.** Cloning and characterization of the *tyrR* genes of *Citrobacter braakii* and *Salmonella typhimurium*. Unpublished data.
6. **Berman, M. L., and D. T. Jackson.** 1984. Selection of *lac* gene fusions in vivo: *ompR-lacZ* fusions that define a functional domain of the OmpR gene product. J. Bacteriol. **159:**750–756.
7. **Biggin, M. D., T. J. Gibson, and G. F. Hong.** 1983. Buffer gradient gels and ^{35}S label as an aid to rapid DNA sequence determination. Proc. Natl. Acad. Sci. USA **80:**3963–3965.
8. **Botsford, J. L., and R. D. DeMoss.** 1971. Catabolite repression of tryptophanase in *Escherichia coli*. J. Bacteriol. **105:**303–312.
9. **Brenowitz, M., D. Senear, E. Jamison, and D. Dalma-Weiszhausz.** 1993. Quantitative DNase I footprinting, p. 1–43. *In* A. Revzin (ed.), Footprinting of nucleic acid-protein complexes. Academic Press, San Diego, Calif.
10. **Cui, J., and R. L. Somerville.** 1993. A mutational analysis of the structural basis for transcriptional activation and monomer-monomer interaction in the TyrR system of *Escherichia coli* K-12. J. Bacteriol. **175:**1777–1784.
11. **Cui, J., and R. L. Somerville.** 1993. The TyrR protein of *Escherichia coli*. Analysis by limited proteolysis of domain structure and ligand-mediated conformational changes. J. Biol. Chem. **268:**5040–5047.
12. **Curtis, S. E.** 1987. Genes encoding the beta and epsilon subunits of the proton-translating ATPase from *Anabaena* sp. strain PCC 7120. J. Bacteriol. **169:**80–86.
13. **Deeley, M. C., and C. Yanofsky.** 1982. Transcription initiation at the tryptophanase promoter of *Escherichia coli* K-12. J. Bacteriol. **151:**942–951.
14. **Enei, H., H. Matsui, H. Nakazawa, S. Okumura, and H. Yamada.** 1973. Culture conditions for the preparation of cells containing high tyrosine phenol lyase activity. Agric. Biol. Chem. **37:**485–492.
15. **Foor, F., N. Morin, and K. A. Bostian.** 1993. Production of L-dihydroxyphenylalanine in *Escherichia coli* with the tyrosine phenol-lyase gene cloned from *Erwinia herbicola*. Appl. Environ. Microbiol. **59:**3070–3075.
16. **Gottesman, M. E., and M. B. Yarmolinsky.** 1968. Integration-negative mutants of bacteriophage λ. J. Mol. Biol. **31:**487–497.
17. **Gralla, J. D., and J. Collado-Vides.** 1996. Organization and function of transcription regulatory elements, p. 1232–1245. *In Escherichia coli* and *Salmonella*. Cellular and Molecular Biology ASM Press, Washington, D.C.
18. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580.
19. **Heatwole, V., and R. L. Somerville.** 1991. The tryptophan-specific permease gene, *mtr*, is differentially regulated by the tryptophan and tyrosine repressors in *Escherichia coli* K-12. J. Bacteriol. **173:**3601–3604.
20. **Heatwole, V. M., and R. L. Somerville.** 1991. Cloning, nucleotide sequence and characterization of *mtr*, the structural gene for a tryptophan-specific permease of *Escherichia coli* K-12. J. Bacteriol. **173:**108–115.
21. **Heatwole, V. M., and R. L. Somerville.** 1992. Synergism between the Trp repressor and Tyr repressor in repression of the *aroL* promoter of *Escherichia coli* K-12. J. Bacteriol. **174:**331–335.
22. **Iwamori, S., S. Yoshino, K.-I. Ishiwata and N. Makiguchi.** 1991. Structure of tyrosine phenol-lyase genes from *Citrobacter freundii* and structural comparison with tryptophanase from *Escherichia coli*. J. Ferment. Bioeng. **72:**147–151.
23. **Kumagai, H., H. Matsui, and H. Yamada.** 1970. Formation of tyrosine phenol-lyase by bacteria. Agric. Biol. Chem. **34:**1259–1261.
24. **Kumaji, H., H. Yamada, H. Matsui, H. Ohkishi, and K. Ogata.** 1970. Tyrosine phenol lyase. J. Biol. Chem. **245:**1767–1772.
25. **Kurusu, Y., M. Fukushima, K. Kohama, M. Kobayashi, M. Terasawa, H. Kumagai, and H. Yukawa.** 1991. Cloning and nucleotide sequencing of the tyrosine phenol lyase gene from *Escherichia intermedia*. Biotechnol. Lett. **13:**769–772.
26. **Lennox, E. S.** 1955. Transduction of linked genetic characters of the host by bacteriophage P1. Virology **1:**190–206.
27. **Mandel, M., and A. Higa.** 1970. Calcium-dependent bacteriophage DNA infection. J. Mol. Biol. **53:**154–159.
28. **Maxam, A., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65:**499–560.
29. **Meyer, B., R. Maurer, and M. Ptashne.** 1980. Gene regulation at the right operator (O_R) of bacteriophage λ. II. O_R1, O_R2, and O_R3: their roles in mediating the effects of repressor and cro. J. Mol. Biol. **139:**163–194.
30. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 352–355. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. **Pittard, A. J., and B. E. Davidson.** 1991. TyrR protein of *Escherichia coli* and its role as repressor and activator. Mol. Microbiol. **5:**1585–1592.
32. **Salser, W., R. F. Gesteland, and A. Bolle.** 1967. *In vitro* synthesis of bacteriophage lysozyme. Nature **215:**588–591.
33. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
34. **Sarsero, J. P., and A. J. Pittard.** 1991. Molecular analysis of the TyrR protein-mediated activation of *mtr* gene expression in *Escherichia coli* K-12. J. Bacteriol. **173:**7701–7704.
35. **Shah, S., and A. Peterkofsky.** 1991. Characterization and generation of *Escherichia coli* adenylate cyclase mutants. J. Bacteriol. **173:**3238–3242.
36. **Somerville, R. L., B. T. Hagewood, B. D. Aronson, and V. M. Tesmer.** 1992. Frameshifted derivatives and promoterless frameshifted derivations of M13mp18 and M13mp19 useful in the mutational analysis of cloned genes or punctuation elements. Methods Mol. Cell. Biol. **3:**103–107.
37. **Somerville, R. L., T.-L. N. Shieh, B. Hagewood, and J. Cui.** 1991. Gene expression from multicopy T7 promoter vectors proceeds at single copy rates in the absence of T7 RNA polymerase. Biochem. Biophys. Res. Commun. **181:**1056–1062.
38. **Stewart, V. J., and C. Yanofsky.** 1985. Evidence for transcription antitermination control of tryptophanase gene expression in *Escherichia coli* K-12. J. Bacteriol. **164:**731–740.
39. **Suzuki, H., K. Nishihara, N. Usui, H. Matsui, and H. Kamagai.** 1993. Cloning and nucleotide sequence for *Erwinia herbicola* AJ2982 tyrosine phenol-lyase gene. J. Ferment. Bioeng. **75:**145–148.
40. **Suzuki, H., T. Katayama, K. Yamamoto, and H. Kumagai.** 1995. Transcriptional regulation of tyrosine phenol-lyase gene of *Erwinia herbicola* AJ2985. Biosci. Biotechnol. Biochem. **59:**2339–2341.
41. **Vogel, H. J., and D. M. Bonner.** 1956. Acetylornithinase of *Escherichia coli*: partial purification and some properties. J. Biol. Chem. **218:**97–106.
42. **Wilson, T. J., P. Maroudis, G. J. Howlett, and B. E. Davidson.** 1994. Ligand-induced self-association of the *Escherichia coli* regulatory protein TyrR. J. Mol. Biol. **238:**309–318.
43. **Yang, J., H. Camakaris, and A. J. Pittard.** 1996. Further genetic analysis of the activation function of the TyrR regulatory protein of *Escherichia coli*. J. Bacteriol. **178:**1120–1125.
44. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.
45. **Yu, X. M., and W. S. Reznikoff.** 1984. Deletion analysis of the CAP-cAMP binding site of the *Escherichia coli* lactose promoter. Nucleic Acids Res. **12:**5449–5464.