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Cloning and Random Mutagenesis of the *Erwinia herbicola tyrR* Gene for High-Level Expression of Tyrosine Phenol-Lyase

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Tyrosine phenol-lyase (Tpl), which can synthesize 3,4-dihydroxyphenylalanine from pyruvate, ammonia, and catechol, is a tyrosine-inducible enzyme. Previous studies demonstrated that the *tpl* promoter of *Erwinia herbicola* is activated by the TyrR protein of *Escherichia coli*. In an attempt to create a high-Tpl-expressing strain, we cloned the *tyrR* gene of *E. herbicola* and then randomly mutagenized it. Mutant TyrR proteins with enhanced ability to activate *tpl* were screened for by use of the *lac* reporter system in *E. coli*. The most increased transcription of *tpl* was observed for the strain with the mutant *tyrR* allele involving amino acid substitutions of alanine, cysteine, and glycine for valine-67, tyrosine-72, and glutamate-201, respectively. A *tyrR*-deficient derivative of *E. herbicola* was constructed and transformed with a plasmid carrying the mutant *tyrR* allele (V67A Y72C E201G substitutions). The resultant strain expressed Tpl without the addition of tyrosine to the medium and produced as much of it as was produced by the wild-type strain grown under tyrosine-induced conditions. The regulatory properties of the mutant TyrR^{V67A}, TyrR^{Y72C}, TyrR^{E201G}, and TyrR$^{V67A\ Y72C\ E201G}$ proteins were examined in vivo. Interestingly, as opposed to the wild-type TyrR protein, the mutant TyrR^{V67A} protein had a repressive effect on the *tyrP* promoter in the presence of phenylalanine as the coeffector.

Tyrosine phenol-lyase (Tpl) (EC 4.1.99.2) normally catalyzes the degradation of tyrosine into pyruvate, ammonia, and phenol (26–28, 56). However, this reaction is reversible, and if catechol is substituted for phenol, L-dihydroxyphenylalanine (L-DOPA) is produced (24, 57). L-DOPA is used in the treatment of Parkinson's disease, which afflicts 1 out of every 1,700 individuals. About 250 tons of L-DOPA is now supplied per year, and more than half of it is produced by an enzymatic method involving Tpl (24, 57).

On an industrial scale, *Erwinia herbicola* cells with extremely high Tpl activity are prepared by cultivation in a medium containing L-tyrosine as an inducer of Tpl. The intact cells are then harvested by centrifugation and transferred to the reactor, as the catalyst, together with the substrate. This microbiological method is efficient; however, it actually has one serious drawback. Since Tpl is only synthesized under L-tyrosine-induced conditions (16, 49), the cells must be grown in medium supplemented with L-tyrosine. The extremely low solubility of L-tyrosine results in considerable carryover of it into the reactor, which severely complicates the separation of the final product, L-DOPA (hydroxyl derivative of L-tyrosine), from the remaining L-tyrosine. To avoid this drawback, the *tpl* genes of *E. herbicola* (17, 20, 50) and *Citrobacter freundii* (21) were cloned and expressed in *Escherichia coli* under the control of the *tac* promoter, respectively. In either case, Tpl was highly induced upon the addition of isopropyl-β-D-thiogalactopyranoside (IPTG); however, the L-DOPA productivity of the cells was inferior to that of *E. herbicola* cells. Some factors other than the level of Tpl expression should be considered in order to explain this observation, for example, the transmittance of substrates and L-DOPA through the cell membrane (Tpl is located in the cytoplasmic space) (50) and the tolerance of cells to catechol. It is noteworthy that *E. herbicola* possesses one copy of the *tpl* gene on its chromosome; nevertheless, it is the best source for L-DOPA production.

The regulatory mechanism underlying expression of *tpl* was investigated by means of the *lac* reporter system, and it was demonstrated that, at least in *E. coli*, both the TyrR protein and cyclic AMP receptor protein (CRP) participate in it (23, 47). The TyrR protein plays a major role in the regulation of genes that are essential for the biosynthesis, transport, and degradation of aromatic amino acids (1, 5, 8, 23, 34, 42, 47). TyrR contains a helix-turn-helix DNA-binding motif near its carboxyl end (60) and binds to DNA with a palindromic consensus sequence (TGTAAAN$_6$TTTACA) (19, 42). The central domain of the TyrR protein exhibits significant similarity to those of other regulators such as NtrC (40) and NifA (4), although TyrR completely differs from them in the respect that it regulates transcription from σ^{70}-dependent promoters, not σ^{54}-dependent promoters (11, 30, 33, 42). The N-terminal domain is considered to be involved in the interaction with the α subunit of RNA polymerase as a class I transcriptional activator (33). Using tyrosine, phenylalanine, and tryptophan as coeffectors (2, 42, 54), TyrR regulates transcription from target promoters positively and/or negatively in various manners, which depends on the locations of its binding sites (designated as TyrR boxes) (42). In vitro studies have shown that the TyrR protein ordinarily exists as a dimer in solution (12, 54, 55); however, in the presence of ATP and tyrosine (or a high concentration of phenylalanine), it undergoes a reversible conformational change to a hexameric form (54, 55).

The regulatory region of the *tpl* gene contains three TyrR boxes that are separated from each other by 11 helical turns and two CRP-binding sites that are juxtaposed between the two upstream TyrR boxes (3, 23). Evidence has been obtained that the tyrosine induction of *tpl* is caused by tyrosine-mediated hexamerization of the TyrR protein bound to three distant boxes (3, 23). DNA bending of the intervening region triggered

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TABLE 1. Strains, plasmids, and oligonucleotides used in this work

Strain, plasmid, or oligonucleotide	Characteristic(s)	Source or reference
Strains		
E. herbicola		
AJ2985	Wild type	Laboratory stock
YG17	Δ*tyrR*::*kan*	This study
E. coli K-12		
CJ236	pCJ105 [F′ cat^+]/*dut-1 ung-1 thi-1 relA1*	22
CSH26	F^- *ara* Δ(*lac-pro*) *thi*	39
JM107	F′[*traD36* $proA^+B^+$ $lacI^q$ Δ(*lacZ*)*M15*]/*endA1 gyrA96 thi hsdR17 supE44 relA1* Δ(*lac-proAB*)	61
JP2144	λ^- *tsx-84 trpA9605 tyrR366 his-85 ilv-632*	5
MG1655	λ^- *rph*	Laboratory stock
MV1184	F′[*traD36* $proA^+B^+$ $lacI^q$ Δ(*lacZ*)*M15*]/*ara* Δ(*lac-proAB*) *rpsL thi* φ*80*Δ(*lac*)*M15* Δ(*srl-recA*)*306*::Tn*10*	52
TE2680	F^- λ^- IN(*rrnD-rrnE*)*1* Δ(*lac*)*X74 rpsL galK2 recD1903*::Tn*10d-tet*$^+$ *trpDC700*::*putPA1303*::[*kan* cat^+ ′*lac*]	T. Elliott (14)
TK314	TE2680 *trpDC700*::*putPA1303*::[*kan*$^+$ Φ(*tpl′*-′*lac*)]	23
TK453	JM107 Δ(*srl-recA*)*306*::Tn*10 tyrR366 trpDC700*::*putPA1303*::[*kan*$^+$ Φ(*tpl′*-′*lac*)]	This study
TK481	TK453 *trpDC700*::*putPA1303*::[*kan*$^+$ Φ(*tpl′*-′*lac*) (containing only *tplp*)]	This study
TK596	CSH26 Δ*tyrR*::*kan*$^+$ Δ(*srl-recA*)*306*::Tn*10*	This study
TK747	CSH26 *trpDC700*::*putPA1303*::[*kan*$^+$ Φ(*tpl′*-′*lac*)] Δ*tyrR*::*cat*$^+$ Δ(*srl-recA*)*306*::Tn*10*	This study
TK809	CSH26 Δ*tyrR*::*cat*$^+$ Δ(*srl-recA*)*306*::Tn*10*	This study
Plasmids		
pACYC177	p15A replicon bla^+ kan^+	7
pBR322	ColE1 replicon bla^+ tet^+	48
pMBO131	Mini-F replicon cat^+	41
pMU400	ColE1 replicon bla^+ $tyrR^+_{E.\ coli}$	J. Pittard (10)
pMW118	pSC101 replicon *lacZ*α$^+$ bla^+	Nippon Gene
pMW219	pSC101 replicon *lacZ*α$^+$ kan^+	Nippon Gene
pRS552	ColE1 replicon bla^+ kan^+ *rrnBT1* ′*lac*	R. W. Simons (46)
pTK#-13	ColE1 replicon bla^+ *tet*::$tyrR^+_{E.\ herbicola}$	This study
pTK#-20	ColE1 replicon bla^+ *tet*::$tyrR^+_{E.\ herbicola}$	This study
pTK479	p15A replicon bla^+; the 0.2-kb *Sma*I-*Nru*I fragment was removed from pACYC177	This study
pTK588	pSC101 replicon kan^+ *rrnBT1* Φ(*aroF′*-′*lac*)	This study
pTK589	pSC101 replicon kan^+ *rrnBT1* Φ(*tyrP′*-′*lac*)	This study
pTK631	pSC101 replicon *bla*::*tet*$^+$; the *tet* gene of pBR322 was inserted into the *Sca*I site in the *bla* gene of pMW118 and the 0.3-kb *Pvu*II fragment was removed to delete the *lacZ*α gene	This study
pTK723	p15A replicon bla^+ $tyrR^+_{E.\ coli}$; the 2.4-kb *Nde*I(end-filled)-*Hin*dIII fragment of pMU400 was ligated with the 3.5-kb *Hin*dIII-*Nru*I fragment of pACYC177	This study
pTK766	ColE1 replicon bla^+ *tet*::Δ$tyrR_{E.\ herbicola}$::*kan*$^+$	This study
pTK774	p15A replicon bla^+ Δ*kan*::*tyrRp*	This study
pTK775	p15A replicon bla^+ $tyrR^+_{E.\ herbicola}$; the 2.4-kb *Sal*I-*Ssp*I fragment of pTK#-20 was ligated with the 3.7-kb *Xho*I-*Sma*I fragment of pACYC177	This study
pTK871	Mini-F replicon cat^+ *rrnBT1* Φ(*tpl′*-′*lac*)	This study
pTK919	pSC101 replicon *bla*::*tet*$^+$ $tyrR^+_{E.\ herbicola}$	This study
pTK922	pSC101 replicon *bla*::*tet*$^+$ $tyrR5_{E.\ herbicola}$ (V67A Y72C E201G substitutions)	This study
pTZ19R	ColE1 replicon *lacZ*α$^+$ bla^+ f1*ori*	Pharmacia
pUC4K	ColE1 replicon bla^+ *lacZ*α::*kan*$^+$	Pharmacia
Oligonucleotides		
63	5′-CCGAATTCCAGACTGGCATGCGTATATTGC-3′, for cloning the *tyrP* regulatory region (upstream end)	
64	5′-CCGGATCCTTCACGCTTTCTTCTGTCCTGACGA-3′, for cloning the *tyrP* regulatory region (downstream end)	
65	5′-CCGAATTCGCTAAATGCATCGTCATCTTTTATG-3′, for cloning the *aroF* regulatory region (upstream end)	
66	5′-CCGGATCCTTTTGCATGATGGCGATCCTGTTTA-3′, for cloning the *aroF* regulatory region (downstream end)	
75	5′-GATTAAGGCCCACCATATGCGTTTAGAAG-3′, for random mutagenesis of the $tyrR_{E.\ herbicola}$ gene	
76	5′-TGAGCATGACAAAAAGCTTTACAGCCAG-3′, for random mutagenesis of the $tyrR_{E.\ herbicola}$ gene	
91	5′-TCAGACGGCATACAGGGGACCGTGC-3′, for site-directed mutagenesis of the $tyrR_{E.\ herbicola}$ gene (Y72C substitution)	

by the binding of CRP (13, 36, 43) facilitates the self-association of three TyrR dimers (3, 23).

To create a more efficient and available strain for L-DOPA production, we cloned the *tyrR* gene from an *E. herbicola* genomic library and randomly mutagenized it. Mutant forms of the TyrR protein resulting in high expression of *tpl* were screened for with the *lac* reporter system. The mutant *tyrR* allele obtained was then introduced into an *E. herbicola tyrR*-deficient strain, and the ability of its product to activate Tpl expression was evaluated.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used in this study were derivatives of *E. herbicola* or *E. coli* K-12. The strains and plasmids are listed in Table 1 with their characteristics. All *lac* fusions were created by use of pRS552 to produce translational fusions (46). Construction of the Φ(*tpl′*-′*lac*) gene was

described elsewhere (23). The DNA fragment containing the *rrnB* terminator (*rrnBT1*) and Φ(*tpl′*-*′lac*) gene in that order was cut off by *Hin*dIII and *Sal*I digestion and then integrated into the *E. coli* chromosome as described previously (14, 23), or the fragment was blunt-ended and then subcloned into a mini-F plasmid, pMBO131 (41), at the *Sal*I (end-filled) site. The Φ(*aroF′*-*′lac*) and Φ(*tyrP′*-*′lac*) genes were constructed as follows. DNA fragments containing the respective regions required for TyrR-mediated regulation and parts of the N-terminal region (1, 8, 19, 42) were amplified by PCR using the DNA polymerase from *Pyrococcus kodakaraensis* (KOD polymerase; Toyobo, Osaka, Japan) with the genomic DNA of *E. coli* strain MG1655 as a template and a pair of primers (primers 63 and 64 for *tyrP*, and primers 65 and 66 for *aroF* [Table 1]). The primers were designed to produce an *Eco*RI site at the upstream end and a *Bam*HI site at the downstream end in order to facilitate the connection with pRS552 (46). After being confirmed by sequencing (45), these fragments were subcloned into pRS552. The *Sal*I-*Hin*dIII 8-kb fragment was cut off as described above and then inserted into a low-copy-number plasmid, pMW118 (Nippon Gene, Tokyo, Japan). The construction of the other plasmids is described when they are first mentioned in the text.

Media and chemicals. Bacto MacConkey agar base was purchased from Difco Laboratories (Detroit, USA) and D-lactose was added at a final concentration of 1% as a fermentable carbon source. For the cultivation of *E. herbicola*, basal medium consisting of 0.5% peptone, 0.5% yeast extract, 0.5% meat extract, and 0.2% KH_2PO_4 (pH 8.0) was used. L-Tyrosine was added as an inducer of *tpl* at a final concentration of 0.1%. M63-glucose (39) was used as the minimal medium (MM) for *E. coli*, and L-proline and thiamine-HCl were added as growth requirements at final concentrations of 30 and 1 μg/ml, respectively. Ampicillin, kanamycin, chloramphenicol, and tetracycline were used at final concentrations of 50, 30, 15, and 15 μg/ml, respectively. The chemicals were all obtained commercially and not purified further.

Genetic techniques. Standard recombinant DNA procedures were used essentially as described by Sambrook et al. (44). The method for generalized transduction involving the P1 phage was that described by Miller (39). The *tyrR* transductant was selected based on resistance to 0.2 mM L-3-fluorotyrosine (5). To prevent gene conversion, strains were made *recA* with Tn*10* as a marker (52). The transductants were examined for sensitivity to nitrofurantoin (1.5 μg/ml) (37).

Determination of DNA sequences. DNA sequences were determined by the method of Sanger et al. (45) using a Thermo sequenase fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham) and a DSQ-2000L sequencer (Shimadzu, Kyoto, Japan).

Construction of an *E. herbicola* genomic library. Genomic DNA was extracted from *E. herbicola* AJ2985 (53), partially digested with *Sau*3AI, and then fractionated by low-melting-point agarose gel electrophoresis to obtain 4- to 8-kb fragments. A recovered DNA fragment was inserted into a compatible *Bam*HI site of pBR322 (48). A ligation mixture was used to transform TK453 (a derivative of *E. coli* strain [see text and Table 1]), with about 20,000 transformants being obtained.

Random mutagenesis of the *tyrR* gene using error-prone PCR. Localized random mutagenesis was carried out by the error-prone PCR amplification method (35) using pTK#-20 containing the *tyrR* gene of *E. herbicola* as a template, and synthetic oligonucleotides 75 and 76 (Table 1) as a pair of primers. Primer 75 was designed to introduce an *Nde*I site in the initiation codon and primer 76 was designed to introduce a *Hin*dIII site downstream of the putative transcription terminator of the *tyrR* gene. The amplified 1.6-kb DNA fragment was treated with *Nde*I and *Hin*dIII and then ligated with pTK774 (Table 1) that had been predigested similarly. pTK774 carries the wild-type promoter and 5′ untranslated region of the *tyrR* gene except that GCAATG of the translation initiation site was changed to CATATG (*Nde*I) so that the amplified fragment is placed downstream of the *tyrR* wild-type promoter.

Site-directed mutagenesis. Site-directed mutagenesis was carried out by the method of Kunkel et al. (29). To replace the tyrosine residue at amino acid position 72 with cysteine, the oligonucleotide 91 (Table 1) and single-stranded pTK852, which was generated by inserting the 0.4-kb *Eco*RI-*Pst*I fragment of pTK#-20 into pTZ19R, were used. The entire fragment used for later manipulation was sequenced to ensure that no base change other than those planned had occurred.

β-Galactosidase assay. To study TyrR-mediated regulation, M63-glucose (39) was used as the minimal medium. Either tyrosine or phenylalanine was added as an effector of the TyrR protein (2, 42, 54) at a final concentration of 1 mM. Cultures were grown at 37°C to the mid-exponential phase and then subjected to β-galactosidase assaying according to the method of Miller (39). Assays were performed in duplicate for three separate cultures, and the values obtained showed less than 10% error.

Preparation of anti-Tpl antibodies and immunoblotting. Tpl was purified from an *E. herbicola* cell extract as described previously (27, 50). One milligram of the protein emulsified in Freund's complete adjuvant was used to immunize a female New Zealand White rabbit. Booster immunizations with 1 mg of the protein in Freund's incomplete adjuvant were administered twice with an interval of 2 weeks. A small amount of blood was taken to test for anti-Tpl activity after each booster immunization. Whole blood was collected and kept at 37°C for 1 h, and then the clot was removed by centrifugation to obtain crude antiserum. The immunoglobulin G fraction was purified from the crude antiserum by protein-A Sepharose CL-6B column chromatography as recommended by the supplier (Amersham).

Immunoblotting was performed as described previously (18) with slight modifications. In brief, an overnight liquid culture was diluted with the same medium to give an optical density of 1.0 at 600 nm. Cells were collected from 1 ml of dilution by centrifugation and suspended in 100 μl of cracking buffer (60 mM Tris-HCl [pH 6.8], 1% 2-mercaptoethanol, 1% sodium dodecyl sulfate [SDS], 10% glycerol, 0.01% bromophenol blue), and then boiled for 5 min. The whole-cell extract was separated on an SDS–12.5% polyacrylamide gel (32) and then electroblotted onto a polyvinylidene difluoride membrane (Millipore). Anti-Tpl antibodies and anti-rabbit immunoglobulin, horseradish peroxidase-linked whole antibodies (from donkey) (Amersham), were used in 4,000-fold dilution and 3,000-fold dilution, respectively. Specific cross-reactions were visualized with ECL chemiluminescent detection agent (Amersham). The image on X-ray film was analyzed with a Fuji Film ImageGauge program, and the values were estimated within the linear range.

Construction of the *E. herbicola* Δ*tyrR*::*kan* strain. The chromosomal region in *E. herbicola* corresponding to the *tyrR* gene was deleted and replaced with the kanamycin resistance gene (*kan*) through a homologous recombination event. A plasmid carrying the $\Delta tyrR_{E.\ herbicola}$::*kan* gene was constructed as follows. pTK#-13, which contains long flanking regions on both the upstream and downstream sides of the *E. herbicola tyrR* gene, was digested with *Eco*RI, resulting in the production of 5.5-, 6.5-, 1.6-, and 0.3-kb DNA fragments. The *kan* gene was isolated from pUC4K (Amersham) by *Eco*RI digestion and then ligated, in the proper orientation, with the 5.5- and 6.5-kb fragments of pTK#-13 obtained above. As a result, a plasmid in which almost all the *E. herbicola tyrR* gene (1.6 kb) and the proximal downstream 0.3-kb fragment were replaced with the *kan* gene was constructed (pTK766). An 8.0-kb *Fsp*I fragment containing 4.0- and 2.2-kb DNA regions corresponding to either side of the chromosomal *tyrR* locus was recovered from pTK766, and then introduced into *E. herbicola* by electroporation. Transformants in which the correct replacement event had occurred were screened for by genomic Southern hybridization analysis with the *kan* and *tyrR* genes as specific probes.

Nucleotide sequence accession number. The GenBank accession number of the *E. herbicola tyrR* gene is AF035010.

RESULTS AND DISCUSSION

Cloning of the *tyrR* gene from *E. herbicola*. In *E. herbicola*, expression of Tpl is induced by tyrosine and is subject to cyclic AMP-dependent catabolite repression (49). In a previous study, the *lac* reporter system of *E. coli* was employed to elucidate the regulatory mechanism of *tpl* (23). Although the *tpl* gene is not normally found in *E. coli* (15, 25), both induction and repression of this gene were observed in the same manner as observed in *E. herbicola* (23). Consequently, the TyrR protein and CRP of *E. coli* were identified as regulators of *tpl* that are responsible for tyrosine induction and carbon catabolite repression, respectively (23). Somerville and his colleagues have also shown that the expression of *C. freundii tpl* is regulated by the TyrR protein, integration host factor, and CRP in *E. coli* (3, 47). They carried out precise in vitro experiments; however, their studies were demonstrated with the noncognate (*E. coli*) TyrR protein. In this study, therefore, as part of an effort to elucidate the regulatory mechanism of *tpl* and to find a suitable means of constructing a Tpl high expression strain, we attempted to clone the *tyrR* gene of *E. herbicola*.

A derivative of *E. coli* strain JM107 (61), TK453, carrying the Φ(*tpl′*-*′lac*) gene, *tyrR* mutation, and *recA* mutation was constructed by P1 transduction using TK314 (23), JP2144 (5), and MV1184 (52) as donors, respectively. An *E. herbicola* genomic library was constructed using TK453 as a host, spread on MacConkey agar-lactose plates containing 0.1% tyrosine as an inducer, and then screened for red color formation. At this time, three possible reasons were considered for this phenotype change; (i) the gene for a positive regulator of *tpl* was cloned, the product of which triggered expression of the fusion; (ii) the gene for β-galactosidase was cloned, the activity of which was expressed; and (iii) an unknown factor(s) was involved, such as one causing a pH decrease. In order to exclude the second and third possibilities, a plasmid extracted from a red color-forming colony was subsequently introduced into another *E. coli* strain (TK481). In strain TK481, the upstream

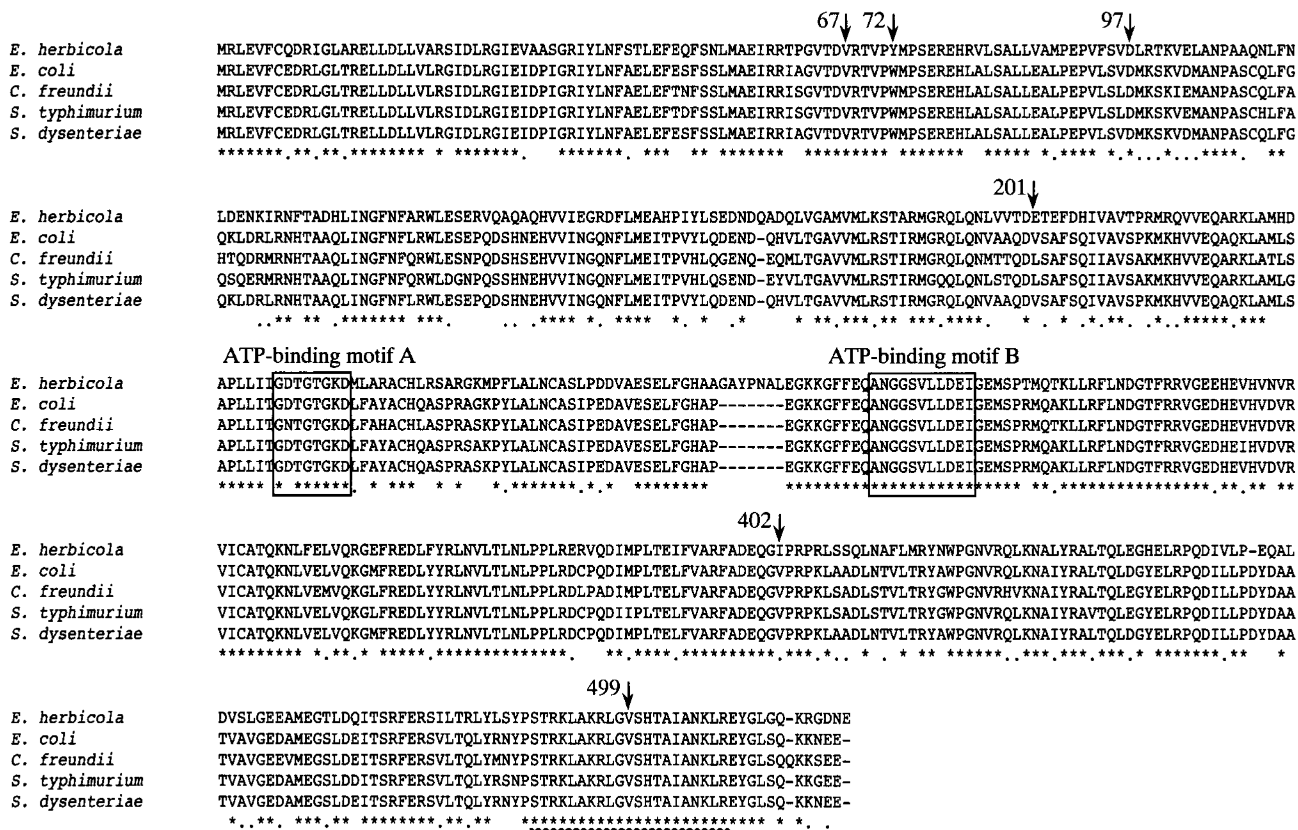


FIG. 1. Multiple amino acid sequence alignment of TyrR proteins. The deduced amino acid sequence of the *E. herbicola* TyrR protein was aligned with those of the TyrR proteins from *E. coli* (GenBank accession number M12114) (9), *S. enterica* serovar Typhimurium (*S. typhimurium*) (GenBank accession number U90141) and *C. freundii* (GenBank accession number U90140) (Bai and Somerville, unpublished data), and *S. dysenteriae* (GenBank accession number AF153317) (38) by use of the Clustal W 1.6 program (51). The asterisks indicate residues that are conserved in all five TyrR proteins, and the dots indicate positions at which only conservative changes have occurred. Only the *E. herbicola* TyrR protein possessed seven extra amino acid residues between the two ATP-binding motifs (A and B), which are enclosed by boxes. The sequences in the HTH motifs are identical in all five proteins and are underlined with a wavy line. Mutations mapped in this experiment are indicated by arrows labeled with their amino acid positions.

regulatory region of the fusion was deleted, leaving only the *tpl* promoter. Therefore, when a gene encoding an activator of *tpl* was introduced into TK481, expression of the fusion would remain basal (forming a white colony) since the activator did not have its target region. On the other hand, in the second and third cases, transformants would show red color again.

In this way, we obtained 20 positive clones. Every plasmid produced the same DNA fragment (1.6 kb) on *Eco*RI digestion and conferred the TyrR$^+$ phenotype (5) on the host strain. One of these plasmids, pTK#-20, with the shortest insert (6 kb), was studied further. By means of the TyrR phenotypic check (5), it was confirmed that a 3.5-kb *Sal*I fragment certainly contained the gene of interest. The nucleotide sequence was determined and deposited in GenBank (accession number AF035010). The sequence analysis proved that the cloned gene was *tyrR*.

Multiple amino acid sequence alignment of TyrR proteins. Analysis of the *E. herbicola tyrR* gene revealed two potential translation initiation codons separated by 46 frames. Since removal of the upstream ATG codon did not affect the ability of the protein to activate *tpl* (data not shown) and the amino acid sequence deduced from the downstream ATG codon showed good agreement with those of other TyrR proteins (Fig. 1), we concluded that the downstream ATG is the actual translation initiation codon.

The primary structure of the *E. herbicola* TyrR protein was aligned with and compared to those of other bacterial TyrR proteins by use of the Clustal W 1.6 program (51) (Fig. 1). The *E. coli tyrR* gene has been sequenced by Cornish et al. (9), and Bai and Somerville have cloned and sequenced the *tyrR* genes of *Salmonella enterica* serovar Typhimurium and *C. freundii* (GenBank accession numbers U90141 and U90140, respectively) (Q. Bai and R. L. Somerville, unpublished data). Recently, McDonough and Butterton determined the DNA sequence of the *tyrR* gene of *Shigella dysenteriae* (GenBank accession number AF153317) (38). While the TyrR proteins of *E. coli*, *S. enterica* serovar Typhimurium, *C. freundii*, and *S. dysenteriae* exhibited marked resemblance (more than 90% identity) to each other, the TyrR protein of *E. herbicola* showed relatively low (less than 72% identity) similarity to the others. As shown in Fig. 1, all five TyrR proteins had identical helix-turn-helix (HTH) motifs in their C-terminal domains (60); therefore, they could recognize the common DNA sequence (TGTAAAN$_6$TTTACA) (19, 42). Only the *E. herbicola* TyrR protein possessed seven extra amino acid residues within the central domain; however, deletion of this region did not alter its regulatory properties at least in *E. coli* (data not shown).

Screening for mutant TyrR proteins with enhanced ability to activate *tpl*. As mentioned above, in the case of L-DOPA production with *E. herbicola* cells, the presence of tyrosine in the medium is absolutely required but is troublesome. To date, various mutant forms of the TyrR protein have been isolated and analyzed in vivo and in vitro (19, 31, 58–60); however,

TABLE 2. Mutant TyrR proteins leading to high expression of the Φ(*tpl′*-*′lac*) gene in *E. coli* grown in basal medium[a]

tyrR allele present on pACYC177[b]	Sp. act. of β-galactosidase (Miller units)	Mutation(s) (putative amino acid replacement[s])
None	27.4	
tyrR$^+$	100	
tyrR2[c]	389	GTC for V-67→GCC for A (V67A), GTT for V-499→ATT for I (V499I)
tyrR3	393	GTC for V-67→GCC for A (V67A)
tyrR4	231	GAT for D-97→GGT for G (D97G), ATT for I-402→GTT for V (I402V)
tyrR5[d]	863	GTC for V-67→GCC for A (V67A), TAT for Y-72→TGT for C (Y72C), GAA for E-201→GGA for G (E201G)
tyrR6	850	GTC for V-67→GCC for A (V67A), TAT for Y-72→TGT for C (Y72C), GAA for E-201→GGA for G (E201G)

[a] Basal medium consists of 0.5% peptone, 0.5% meat extract, 0.5% yeast extract, and 0.2% KH_2PO_4.

[b] Four *tyrR* alleles (*tyrR5* and *tyrR6* were identical) were obtained through two different cycles of error-prone PCR. The randomly mutagenized *tyrR* gene was expressed under the control of its wild-type promoter on the pACYC-derived plasmid (pTK774 [see text]).

[c] 25 PCR cycles.

[d] 30 PCR cycles.

these studies were mainly focused on proteins with impaired capacity to activate or repress the gene expression. In order to obtain a constitutive activator form of TyrR, localized random mutagenesis was carried out. The DNA region containing the open reading frame and putative transcription terminator of the $tyrR_{E.\ herbicola}$ gene was amplified by the error-prone PCR method (35). The amplified fragments were placed under the control of the *tyrR* wild-type promoter (6). A derivative of *E. coli* strain CSH26, TK747, carrying the Φ(*tpl′*-*′lac*) gene and Δ*tyrR*::*cat*$^+$ gene was transformed with two independently derived plasmid libraries and then spread on basal medium plates containing 2 mM X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), and about 50,000 transformants were obtained. The mutagenized *tyrR* genes were then screened for the ability of their products to activate the *tpl* promoter without additional tyrosine in the medium. Colonies were visually screened for enhanced blue color formation. One hundred highly blue-colored colonies were selected from the bulk of the population and then streaked on the same plate again. Finally, five colonies that exhibited the deepest blue were selected as candidates. The β-galactosidase activities of these strains grown in the basal medium are shown in Table 2. The *tyrR2*, *tyrR3*, and *tyrR4* alleles were obtained with 25 cycles of error-prone PCR, and *tyrR5* and *tyrR6* were obtained with 30 cycles. The highest activity was attained by the strain carrying the *tyrR5* allele, and it was eight times as high as that of the strain carrying the wild-type *tyrR* gene.

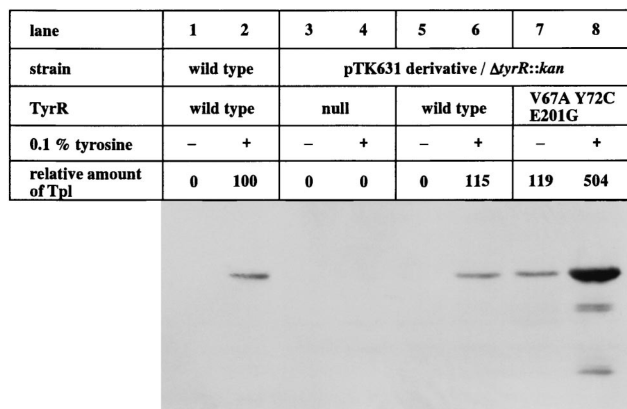
Mapping of mutations by DNA sequencing. The DNA sequences of the above five *tyrR* alleles were determined. The *tyrR5* and *tyrR6* alleles were found to be identical. Although these *tyrR* alleles were isolated in two independent experiments, the substitution of alanine for valine at position 67 (V67A) was seen in both cases (*tyrR2*, *tyrR3*, and *tyrR5*), suggesting a significant effect of this mutation on the ability of the TyrR protein to activate *tpl*. The *tyrR2* allele contained mutations leading to substitutions of alanine and isoleucine for valine-67 (V67A) and valine-499 (V499I), respectively. Valine-499 of the *E. herbicola* TyrR protein corresponds to valine-492 of the *E. coli* TyrR protein (Fig. 1) (9). Replacement of valine-499 with isoleucine (V499I) caused discordance within the conserved HTH motif of the TyrR protein; however, the effect of this substitution was thought to be negligible, at least as to the activation of *tpl*, because the β-galactosidase level of the strain carrying the *tyrR2* allele was almost equal to that of the strain carrying the *tyrR3* allele. Mutations in the *tyrR4* allele resulted in amino acid substitutions of glycine and valine for aspartate-97 (D97G) and isoleucine-402 (I402V), respectively. It seems likely that the replacement of isoleucine at position 402 with valine (I402V) has no or a little, if any, effect on the function of the TyrR protein because, as can be seen in Fig. 1, all the other TyrR proteins have valine residues at the corresponding position. The change of aspartate-97 to glycine (D97G) seemed to have a moderate effect on the ability of the protein to activate the *tpl* promoter. This substitution (D97G) has already been demonstrated in a study on the *E. coli* TyrR protein to cause a twofold increase in transcription from the *mtr* and *tyrP+4* promoters (59). Our results exactly agree with the case of the *E. coli* TyrR protein, provided that the I402V substitution has no effect on the function of the *E. herbicola* TyrR protein.

As mentioned above, the highest expression of *tpl* was exhibited by the strain carrying the *tyrR5* allele ($TyrR^{V67A\ Y72C\ E201G}$). Therefore, to create a practical strain for L-DOPA production, we then tried to introduce the *tyrR5* allele into *E. herbicola*.

Expression of Tpl in *E. herbicola* carrying a mutant *tyrR* allele. Before introducing the *tyrR5* allele into *E. herbicola*, the chromosomal locus corresponding to the *tyrR* gene was replaced with the kanamycin resistance gene, as described under Materials and Methods. Although the DNA fragment used for this recombination event contained a small N-terminal part of the *tyrR* gene, this Δ*tyrR*::*kan* allele did not exhibit negative dominance (data not shown). Following confirmation of the genetic cross by Southern hybridization analysis, the *tyrR* allele was introduced into the *E. herbicola* Δ*tyrR*::*kan* strain by use of the pSC101-derived vector (pMW118; Nippon Gene), which was shown to be stably maintained for more than 100 generations in the absence of selective pressure (data not shown). Since *E. herbicola* showed resistance to ampicillin for an unknown reason, the tetracycline resistance gene (*tet*) was substituted for the *bla* gene on pMW118. Also, in order to prevent read-through transcription into a subcloned gene (*tyrR*) from the *lac* promoter present in pMW118, the *lacZ*α gene was removed by *Pvu*II digestion, followed by self-ligation of the remaining large fragment. The wild-type *E. herbicola tyrR* gene was cloned into the *Pvu*II site to give pTK919, and the 1.5-kb *Sac*II-*Msc*I internal region was replaced with the corresponding region of the *tyrR5* allele to give pTK922.

The wild-type *E. herbicola* strain and the *E. herbicola* Δ*tyrR*::*kan* strain transformed with one of the following three plasmids—pTK631 (pSC101 replicon *bla*::*tet*$^+$), pTK919 (pSC101 replicon *bla*::*tet*$^+$ *tyrR*$^+$), or pTK922 [pSC101 replicon *bla*::*tet*$^+$ *tyrR5* ($TyrR^{V67A\ Y72C\ E201G}$)]—were cultured in the basal medium with and without additional tyrosine, and then expression of Tpl in these strains was assessed. Since Tpl easily loses its activity once cells are broken, we monitored the expression by immunoblotting instead of measuring the catalytic activity. Whole-cell extracts were obtained by disrupting cells and then subjected to SDS-polyacrylamide gel electrophoresis. The result of immunoblotting with anti-Tpl antibodies is presented in Fig. 2. The level of Tpl expression was expressed as a percentage relative to the amount of Tpl in the wild-type *E. herbicola* cells grown under tyrosine-induced conditions. Some smaller cross-reactants that appeared in lane 8 of Fig. 2 might result from degradation of Tpl.

While the *tyrR* null mutant did not express detectable amounts of Tpl, the strain carrying the wild-type *tyrR* gene on the plasmid induced Tpl, as with the wild-type *E. herbicola*



lane	1	2	3	4	5	6	7	8
strain	wild type		pTK631 derivative / Δ*tyrR*::*kan*					
TyrR	wild type		null		wild type		V67A Y72C E201G	
0.1 % tyrosine	–	+	–	+	–	+	–	+
relative amount of Tpl	0	100	0	0	0	115	119	504

FIG. 2. Immunoblot analysis of Tpl expression in various *E. herbicola* strains. Wild-type cells (lanes 1 and 2) and Δ*tyrR*::*kan* cells transformed with pTK631 (pSC101replicon *bla*::*tet*$^+$, lanes 3 and 4) or pTK631 carrying the *tyrR* allele [*tyrR*$^+$, lanes 5 and 6; *tyrR5* ($\text{TyrR}^{V67A\ Y72C\ E201G}$), lanes 7 and 8] were grown in basal medium in the presence (+) or absence (−) of additional (0.1%) tyrosine for 13 h at 30°C. Whole-cell extracts were obtained by disrupting cells and separated by SDS–12.5% polyacrylamide gel electrophoresis (32). Following electroblotting onto a polyvinylidene difluoride membrane, immunodetection with anti-Tpl antibodies was carried out as described in the text. The level of Tpl expression was expressed as a percentage relative to the amount of Tpl in the wild-type *E. herbicola* cells grown under tyrosine-induced conditions.

strain, when tyrosine was added to the medium. In the case where the *tyrR5* allele was substituted for the wild-type *tyrR* gene, the cells expressed Tpl even in the absence of additional tyrosine in the medium and produced as much of it as was produced by wild-type cells grown under tyrosine-induced conditions. Furthermore, when tyrosine was added, expression of Tpl increased more than fivefold compared to that in the wild-type cells grown in the medium supplemented with tyrosine. These observations indicate that we had obtained a very powerful strain for L-DOPA production.

Effects of V67A, Y72C, and E201G substitutions on regulatory properties of TyrR protein. To obtain a better understanding of the $\text{TyrR}^{V67A\ Y72C\ E201G}$ protein, three amino acid substitutions were singly introduced into the protein by means of genetic arrangement or site-directed mutagenesis, and then the effects of these amino acid replacements on the regulatory properties of the TyrR protein were investigated in vivo. The *tyrR*- and *lac*-deficient derivative of *E. coli* (TK596 or TK809) was transformed with two compatible plasmids. One was a pACYC-derived plasmid (7) containing one of the *tyrR* alleles (encoding the mutant TyrR^{V67A}, TyrR^{Y72C}, TyrR^{E201G}, and $\text{TyrR}^{V67A\ Y72C\ E201G}$ proteins), and the other was a low-copy-number plasmid carrying the Φ(*aroF′*-*′lac*), Φ(*tyrP′*-*′lac*), or Φ(*tpl′*-*′lac*) gene, whose promoter represents a major type of TyrR regulon (23, 42, 47). A parallel set of strains in which the wild-type *tyrR* gene of either *E. coli* or *E. herbicola* was present instead of the above *tyrR* alleles was also constructed. The *aroF* and *tyrP* genes of *E. coli* encode tyrosine-repressible 3-deoxy-arabinoheptulosonate 7-phosphate synthase and tyrosine-specific permease, respectively. The expression of *aroF* is repressed by tyrosine or phenylalanine (1, 5, 8, 42), while the expression of *tyrP* is activated by phenylalanine and repressed by tyrosine (19, 33, 42). The regulatory region of *aroF* encompasses one weak and two strong TyrR boxes. The weak box lies inside the RNA polymerase binding region (−35 sequence), and the strong boxes lie upstream of the weak box. Ligand-induced self-association of the TyrR protein (54, 55) causes cooperative binding of TyrR molecules to the strong and weak boxes in the *aroF* regulatory region, which results in elimination of RNA polymerase from the promoter and consequently causes repression of transcription of the *aroF* gene (1, 5, 8, 42). In the case of *tyrP*, the strong and weak TyrR boxes are juxtaposed. The strong box lies just upstream of the RNA polymerase binding site, while the weak one overlaps the −35 promoter. Repression by tyrosine was also caused by the cooperative binding of the TyrR protein to two adjacent boxes, whereas phenylalanine-mediated activation was brought about by the single TyrR dimer, which binds to the strong box upstream of the promoter (19, 33, 42).

Strains were grown in MM or in MM containing either tyrosine or phenylalanine and then were subjected to β-galactosidase assay. The results are shown in Table 3. When the wild-type *E. herbicola tyrR* gene was introduced into a *tyrR*-deficient background, transcription from the *aroF* promoter remarkably decreased (2,500 to 530 Miller units). Expression of *aroF* was moderately repressed by phenylalanine (1.9-fold) and severely repressed by tyrosine (17-fold) in the presence of TyrR. These results indicate that the TyrR protein acts as a repressor on the *aroF* promoter. On the *tyrP* promoter, the TyrR protein also had a repressive effect (39 to 14 Miller units for MM, 38 to 19 Miller units for MM plus F, and 39 to 0.5 Miller units for MM plus Y). The TyrR protein slightly activated *tyrP* transcription in the presence of phenylalanine (1.4-fold) and severely repressed it in the presence of tyrosine (28-fold). The presence of TyrR hardly affected the basal transcription of *tpl* (85 against 98 Miller units). Expression of *tpl* was activated 2.6-fold and 30-fold upon the addition of phenylalanine and tyrosine, respectively. It is easily speculated that the ligand-mediated conformational change of the TyrR protein is necessary to activate *tpl*.

The β-galactosidase activities of the strains carrying the wild-type *E. herbicola tyrR* gene were, in any case, almost equal to those of the strains carrying the *E. coli tyrR* gene, indicative of equivalent properties of the two TyrR proteins. However, on close examination of the ligand-mediated regulation, a slight difference was recognized with respect to the magnitude of phenylalanine-mediated activation of the *tyrP* and *tpl* promoters. When the cells carrying the *E. coli tyrR* gene were grown in the medium supplemented with phenylalanine, transcription from the *tyrP* and *tpl* promoters increased threefold (10 to 30 Miller units) and fourfold (99 to 410 Miller units), respectively, compared to that in cells grown in MM. On the other hand, the *E. herbicola* TyrR protein activated these promoters 1.4-fold (14 to 19 Miller units) and 2.6-fold (98 to 260 Miller units), respectively, in the presence of phenylalanine as the coeffector. A minor disparity in the phenylalanine-mediated regulation was also observed in the *aroF* expression. In the presence of phenylalanine as a supplement, the *E. coli* TyrR protein repressed the *aroF* transcription more than the *E. herbicola* TyrR did (2.4- versus 1.9-fold). These results reveal a small but certain difference between the TyrR proteins of *E. coli* and *E. herbicola* concerning either the affinity to phenylalanine or the eventual structural change upon the binding of phenylalanine.

As compared to the strains carrying the wild-type *tyrR* gene of *E. herbicola*, the strains carrying the mutant *tyrR* allele involving the V67A substitution or Y72C substitution exhibited increased levels of transcription from all promoters when the cells were grown in MM. One might explain the increased transcription from the *aroF* and *tyrP* promoters as the results of the instability or impaired capacity of the TyrR protein (the presence of the TyrR protein decreased the transcription from these promoters; compare β-galactosidase values of the *tyrR*-deficient strain with those of the *tyrR*$^+$ strain in Table 3); however, if so, how can one explain the activation of *tpl* [see line MM for the Φ(*tpl′*-*′lac*) gene in Table 3]? As mentioned previously, self-association of the TyrR dimers bound to three

TABLE 3. Regulation of expression of the Φ(*aroF′-′lac*), Φ(*tyrP′-′lac*), and Φ(*tpl′-′lac*) genes by mutant TyrR proteins

lac fusion[a]	Growth medium[b]	Sp. act. (Miller units) of β-galactosidase (mode of expression) of various *lac* fusions in the presence of mutant TyrR proteins[c]						
		E. coli (wild type)	*E. herbicola*					None
			Wild type	TyrR^{V67A}	TyrR^{Y72C}	TyrR^{E201G}	TyrR$^{V67A\ Y72C\ E201G}$	
Φ(*aroF′-′lac*)	MM	550	530	650	790	560	900	2,500
	MM+F	230 (R2.4)[d]	280 (R1.9)	280 (R2.3)	330 (R2.4)	360 (R1.6)	390 (R2.3)	2,500
	MM+Y	29 (R19)	31 (R17)	43 (R15)	42 (R19)	33 (R17)	62 (R15)	2,600
Φ(*tyrP′-′lac*)	MM	10	14	43	23	14	64	39
	MM+F	30 (A3.0)	19 (A1.4)	26 (A0.6)	26 (A1.1)	26 (A1.9)	42 (A0.7)	38
	MM+Y	0.3 (R33)	0.5 (R28)	1.7 (R25)	0.9 (R26)	0.5 (R28)	3.4 (R19)	39
Φ(*tpl′-′lac*)	MM	99	98	160	140	100	400	85
	MM+F	410 (A4.1)	260 (A2.6)	1,000 (A6.3)	340 (A2.4)	260 (A2.6)	1,200 (A3.0)	85
	MM+Y	3,200 (A32)	2,900 (A30)	9,300 (A58)	5,100 (A36)	3,200 (A32)	13,000 (A33)	84

[a] The β-galactosidase activities of the Φ(*aroF′-′lac*) and Φ(*tyrP′-′lac*) genes were assayed in *E. coli* TK809, and the β-galactosidase activity of the Φ(*tpl′-′lac*) gene was assayed in *E. coli* TK596. The Φ(*aroF′-′lac*) and Φ(*tyrP′-′lac*) genes were on the pSC101-derived plasmid, and the Φ(*tpl′-′lac*) gene was on the mini-F plasmid.

[b] *E. coli* strains were grown in M63-glucose MM or MM containing phenylalanine (MM+F) or tyrosine (MM+Y) as the coeffector of the TyrR protein at a final concentration of 1 mM.

[c] *E. coli* cells with an appropriate *lac* fusion were transformed with the pACYC-derived plasmid (pTK479) (None) or pTK479 containing the *tyrR* allele of either *E. coli* or *E. herbicola*.

[d] The values in parentheses represent the ratios of effector-mediated regulation. R and A indicate ratios of repression and activation, respectively. The ratio of repression was determined as the level of β-galactosidase in the cells grown in MM divided by that in the cells grown in the medium supplemented with a coeffector. The ratio of activation was determined as the level of β-galactosidase in the cells grown in the medium supplemented with a coeffector divided by that in the cells grown in MM.

distant TyrR boxes is required to activate the transcription of *tpl* (3, 23). Considering that the TyrR protein routinely acts as a repressor on the *aroF* promoter regardless of the presence or absence of a ligand (1, 5, 8, 42), it is likely that the V67A and Y72C substitutions changed the structure of the TyrR protein to an attractive form for RNA polymerase to interact with rather than altering the affinity of the protein to coeffectors. It is probable that atypical recruiting of RNA polymerase occurs on the *aroF* promoter.

Interestingly, as opposed to the wild-type TyrR protein, the mutant TyrR^{V67A} protein had a repressive effect on the *tyrP* promoter when phenylalanine was added as the coeffector (compare MM to MM plus F with regard to *tyrP*). Since repression of *tyrP* is caused by the cooperative binding of the TyrR protein to the promoter (19, 42), it was suggested that the V67A substitution stimulated the self-association of the TyrR protein in the presence of phenylalanine. The fact that the extents of phenylalanine- and tyrosine-mediated activation of *tpl* increased 2.3-fold (activation ratio [A], A2.6 to A6.3) and 1.9-fold (A30 to A58), respectively, upon the replacement of valine-67 with alanine also implies the efficient hexamerization of this mutant protein. At present, however, it is quite difficult to figure out the effect of the V67A substitution on the regulatory properties of the TyrR protein. Studies so far on the *E. coli* TyrR protein have distinguished the activation function of the protein from its ligand-mediated self-association function. But, if so, how does the mutant TyrR^{V67A} protein with the ability of facilitated self-association concomitantly activate transcription from the *aroF* and *tyrP* promoters in cells grown in MM? In vitro studies on the TyrR^{V67A} protein are necessary to clarify this problem.

As mentioned above, substituting cysteine for tyrosine-72 (Y72C) also increased transcription from the adopted three promoters; however, the mode of ligand-mediated regulation was not significantly different from that in the case of the wild-type TyrR protein of *E. herbicola*. Needless to say, the most-elevated level of transcription was seen in cells carrying the *tyrR5* allele (the mutant TyrR$^{V67A\ Y72C\ E201G}$ protein). As expected, repression of *tyrP* by phenylalanine was observed in this strain as much as in the strain carrying the *tyrR3* allele (TyrR^{V67A}).

In order to construct a Tpl high expression strain, we attempted to obtain a mutant TyrR protein with enhanced ability to form a hexamer with a lower amount of tyrosine. The error-prone PCR method was employed for this purpose, and as a result, the *tyrR5* allele (the mutant TyrR$^{V67A\ Y72C\ E201G}$ protein) was obtained. *E. herbicola* cells carrying this *tyrR5* allele expressed as much Tpl without the addition of tyrosine to the basal medium as that produced by the tyrosine-induced wild-type cells. It should be mentioned, however, that the hexameric form of the TyrR protein causes repression of the genes that are required for the biosynthesis and transport of aromatic amino acids. Therefore, there is a possibility that ligand-irresponsive hexamerization of TyrR may result in a growth defect of cells. The regulatory properties of the mutant TyrR$^{V67A\ Y72C\ E201G}$ protein were investigated in vivo, and it was shown that not only the *tpl* promoter but also the *aroF* (biosynthesis) and *tyrP* (transport) promoters were activated, which might alleviate the growth deficiency.

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