Studies on Expression of Tyrosine Phenol-Lyase Gene in *Erwinia herbicola*

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Contents

List of Abbreviations

Amp	ampicillin
ATP	adenosine 5'-triphosphate
b	base(s)
bp	base pair(s)
Cam	chloramphenicol
cAMP	cyclic adenosine 3',5'-monophosphate
CRP	cAMP receptor protein
dCTP	deoxycytidine 5'-triphosphate
DNA	deoxyribonucleic acid
Glc	glucose
Gly	glycerol
Kan	kanamycin
mRNA	messenger RNA
PLP	pyridoxal 5'-phosphate
PTS	phosphoenolpyruvate-sugar phosphotransferase system
RNA	ribonucleic acid
TPL	tyrosine phenol-lyase
Tet	tetracycline
Tyr	tyrosine
U	unit(s)

Introduction

Some enteric bacteria, such as *Erwinia herbicola*, *Citrobacter freundii*, and *Escherichia intermedia*, can utilize L-tyrosine as a carbon source and a nitrogen source by degrading L-tyrosine to pyruvate, ammonia, and phenol. The enzyme which enables these bacteria to do that is tyrosine phenol-lyase (TPL; EC 4.1.99.2) (Kumagai *et al.* 1970a). TPL, formerly called β-tyrosinase, is a pyridoxal phosphate (PLP)-dependent multi-functional enzyme (Kumagai *et al.* 1970c, 1970d and 1975; Yamada and Kumagai 1975). It catalyzes the α, β-elimination and β-replacement reactions of L-amino acids retaining functional groups, such as -OH, -SH and phenolyl, at their β-carbons (Kumagai *et al.* 1969 and 1970c). It also catalyzes alanine-racemization (Kumagai *et al.* 1970b) and the reverse reaction of α, β-elimination (Yamada *et al.* 1972). Through the reverse reaction of α, β-elimination, TPL can synthesize L-tyrosine or 3, 4-dihydroxyphenylalanine (L-dopa) from pyruvate, ammonia, and phenol or pyrocatechol, respectively (Yamada *et al.* 1972). Its ability to synthesize L-dopa, which is used as a specific medicine for Parkinson's disease, is preferably utilized for industrial production today with *E. herbicola*, a TPL high-expression strain.

The expression of TPL is induced by L-tyrosine and is subject to catabolite repression (Enei *et al.* 1973). While the enzymatic properties and catalytic

mechanism of TPL is well studied, the regulation mechanism of its expression has not been examined so far. To elucidate it in more detail will be valuable for an effective production of L-dopa. In this study, the author described the transcriptional regulation of *tpl* in *E. herbicola*, the mode of expression of *tpl'-'lac* fusion in *Escherichia coli*, and the cloning of a positive regulator of *tpl* using *lac* reporter system in *E. coli*. In addition, the author proposed a hypothetical model for the activation mechanism of *tpl* by assessing the β-galactosidase activities of *tpl'-'lac* fusion carrying mutations in its regulatory region.

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Chapter 1.

Transcriptional Regulation of *tpl* in *Erwinia herbicola*

As mentioned above, the expression of tyrosine phenol-lyase (TPL) is induced by tyrosine and is subject to catabolite repression (Enei *et al.* 1973). Catabolite repression, also known as the glucose effect, had been intensively studied on the regulations of the expressions of enzymes involved in the carbon metabolism in bacteria. Those studies revealed that, in most cases, those phenomena were the consequences of the transcriptional regulations of those genes (Saier *et al.* 1996 and the references therein). Several transcriptional regulators which bring about catabolite repressions on their target genes have been identified so far and their machineries on them have also been discussed by many researchers.

cAMP receptor protein (CRP) is the most famous and examined regulator, which cause cAMP-dependent catabolite repression. CRP can bind to its target site when its ligand, cAMP, is bound to it and thereby can affect the gene expression positively or negatively with its binding location (upstream or downstream from the target promoter). cAMP is synthesized from ATP by adenylate cyclase, the activity of which is regulated via the phosphoenolpyruvate-sugar phosphotransferase system (PTS). Thus, the fluctuation of the concentration of cAMP in the cell results in cAMP-dependent catabolite

repression (Botsford and Harman 1992, Saier *et al.* 1996). On the other hand, catabolite activator and repressor protein (Cra), formerly called FruR, is known to cause cAMP-independent catabolite repression in *E. coli*. Cra, which also regulates the gene expression positively or negatively, binds to its recognition site without effectors and is released when fructose-1-phophate or fructose-1,6-bisphosphate binds to it (Saier *et al.* 1996 and the references therein). In addition, other factors those which cause cAMP-independent catabolite repression were also reported by them (Saier *et al.* 1996).

In this chapter, the author examined the induction of *tpl* in the presence of tyrosine using northern hybridization, together with catabolite repression whether it is cAMP-dependent or not. And then, the transcription start point of *tpl* was determined by primer extension mapping. Based on the results obtained, the nucleotide sequence of the *tpl* upstream region was scrutinized.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used in this chapter were *Erwinia herbicola* AJ2985 and derivatives of *E. coli* K-12. The relevant characteristics and sources of the strains and plasmids used are listed in Table 1-1.

Media and chemicals. *E. herbicola* was grown at 30 ℃ in basal medium which consisted of 5 g of peptone, 5 g of yeast extract, 5 g of meat

Table 1-1. Bacterial strains and plasmids used in this chapter

Strain or plasmid	Relevant characteristic	Source or reference
Strains		
Erwinia herbicola AJ2985		Ajinomoto, Co., Inc.
Escherichia coli K-12		
JM109	F'[*traD36 proA⁺B⁺ lacI*^{q} Δ(*lacZ*)*M15*] / *endA1 gyrA96 thi hsdR17 supE44 relA1* Δ(*lac-proAB*) *recA1*	Laboratory stock (Yanisch-Perron *et al.* 1985)
SH768	pSH768 / JM109	Laboratory stock (Suzuki *et al.* 1993)
Plasmid		
pSH768	*tpl*$^{+}_{E.herbicola}$ Amp^{r} ColE1*ori*	Laboratory stock (Suzuki *et al.* 1993)

extract, and 2 g of KH_2PO_4 per liter. L-Tyrosine as an inducer of TPL, D-glucose, and cAMP were added to the medium at final concentrations of 0.2 %, 0.2 %, and 5 mM, respectively, when indicated. *E. coli* strains were routinely grown at 37 ℃ in LB-medium (Sambrook *et al.* 1989). Ampicillin was used, if needed, at a final concentration of 100 μg/ml. [α-^{32}P]dCTP and [γ-^{32}P]ATP were purchased from ICN Biochemicals Inc. Restriction endonucleases were obatained from New England Biolabs, Takara Shuzo Co., Ltd. or Toyobo Co., Inc. The chemicals were all obtained commercially and not purified further.

Genetic techniques. Standard genetic procedures were performed essentially as described by Sambrook *et al.* (Sambrook *et al.* 1989) unless

otherwise stated. Total RNA was extracted from *E. herbicola* by the method of Aiba *et al.* (Aiba *et al.* 1981). Preparing a *tpl* specific probe for northern hybridization, the *Bgl*II-*Nae*I (0.4 kb) fragment digested from pSH768 (Suzuki *et al.* 1993) was labeled with [α-^{32}P]dCTP using Random Primer DNA Labeling Kit from Takara Shuzo Co., Ltd. For mapping the 5' end of *tpl* mRNA using primer extension reaction, two complementary primers (No. 172; 5'-AATGCGCGTCGAATAAGTCTGGTGC-3' and No.174; 5'-TGCGGAA AGGCTCGGCAGGATAGTT-3' see Fig. 1-3) were synthesized at Biologica, Co., Japan and labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase from Toyobo Co., Inc. SuperScript RNase H$^-$ reverse transcriptase (Gibco BRL) and RNase inhibitor (PROMEGA Co., Inc.) were used for extension reactions. DNA sequencing for analyzing the extension products were carried out by the method of Sanger *et al.* (Sanger *et al.* 1977) using the same primers and pSH768 (Suzuki *et al.* 1993) as a template with [α-^{32}P]dCTP and BcaBEST dideoxy Sequencing Kit from Takara Shuzo Co., Ltd. Autoradiographs were established by exposing X-ray films (Fuji Photo Film Co., Ltd.) with intensifying screens.

RESULTS AND DISCUSSION

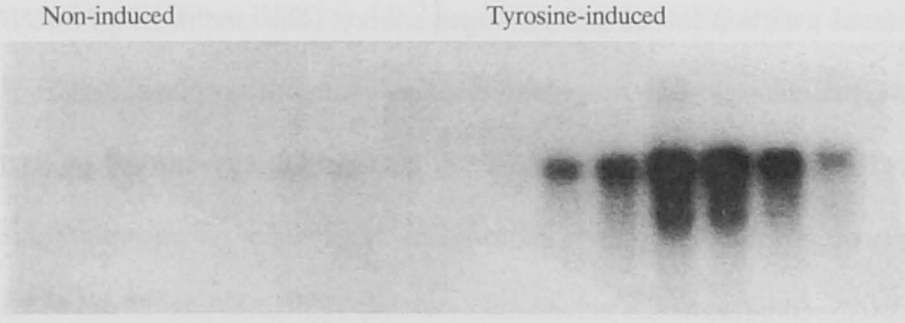
Increased amount of *tpl* mRNA in the presence of tyrosine. As mentioned earlier, the expressions of enzymes being subject to catabolite

repression are usually regulated transcriptionally (Saier *et al.* 1996). To test the possibility of the transcriptional regulation of *tpl*, northern hybridization was performed. Total RNAs were prepared from *E. herbicola* AJ2985 cells grown in the medium with and without tyrosine at the time of 0, 2, 3.5, 5, 6.5, 8.5, and 11.5 hours after the cell density (OD_{600}) reached to 0.5. As shown in Fig. 1-1, much *tpl* mRNA was detected only in the cells grown in the medium containing tyrosine. The amount of *tpl* mRNA was maximum in the late exponential phase, which suggested that the cells started to grow using preferred nutrients in the medium and then, with the depletion of preferred nutrients, they began to induce TPL to utilize tyrosine in the medium as a carbon source and a nitrogen source. The amount of *tpl* mRNA gradually decreased as the amount of tyrosine getting fewer in the medium.

The result indicated that the presence of tyrosine either greatly enhanced the stability of *tpl* mRNA or greatly activated the synthesis of *tpl* mRNA. That was discussed later.

cAMP-dependent catabolite repression of *tpl*. To examine whether catabolite repression of TPL is cAMP-dependent or cAMP-independent, northern hybridization was performed. *E. herbicola* was grown in the presence of tyrosine to the late-exponential phase, and a portion of culture was collected for the RNA preparation (lane 1 in Fig. 1-2). The remainder of culture medium was divided in two and one half was

continuously cultivated in the same condition. Another half was cultivated after adding glucose to the medium at a final concentration of 0.2 %. Thirty minutes later, a portion of each culture was collected for the RNA preparation (lane 2 and 3 in Fig. 1-2) and then the latter culture containing 0.2 % glucose was divided equally again. One was continuously cultivated and another was cultivated after the addition of cAMP to the medium at a final concentration of 5 mM. Half an hour later, total RNA was extracted from each cell (lane 4 and 5 in Fig. 1-2). Prepared total RNAs were similarly analyzed by northern



Time (hour)

	0.0	2.0	3.5	5.0	6.5	8.5	11.5
Non-induced							
Cell density (OD_{600})	0.5	2.4	4.0	5.1	5.7	5.5	5.3
Tyrosine-induced							
Cell density (OD_{600})	0.5	2.3	3.6	5.2	5.9	5.6	5.4

Fig. 1-1. Increased amount of *tpl* mRNA in the presence of tyrosine. Northern hybridization was performed as described in the text. Briefly, total RNAs were prepared from cells grown in the medium with and without tyrosine at the indicated time (0 time correspondigs OD_{600} = 0.5). The membrane blotted was hybridized with a *tpl* specific probe labeled with [α-^{32}P]dCTP and then autoradiographed. Cell density (OD_{600}) at each time is also indicated.

hybridization and the result was shown in Fig. 1-2. The addition of glucose to the medium severely diminished the amount of *tpl* mRNA which had existed much before the addition of glucose and was exsiting much in the absence of glucose (compare lane 1, 2, and 3 in Fig. 1-2). Decreased amount of *tpl* mRNA was slightly releaved by the addition of cAMP to the medium (compare lane 3, 4, and 5). These results indicated that the expression of *tpl* was subject to cAMP-dependent catabolite repression, and proposed that the transcription of *tpl* was regulated through cAMP receptor protein (CRP).

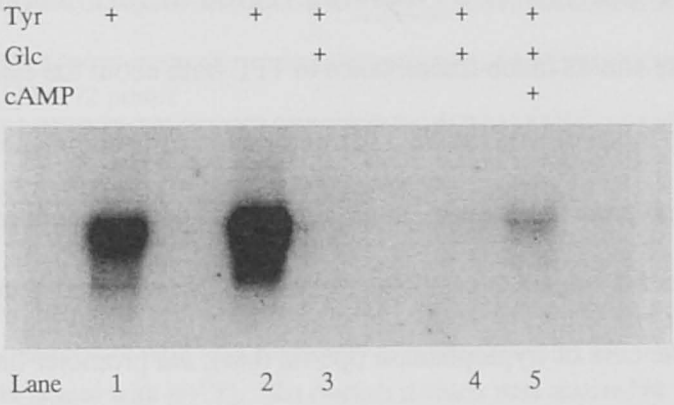
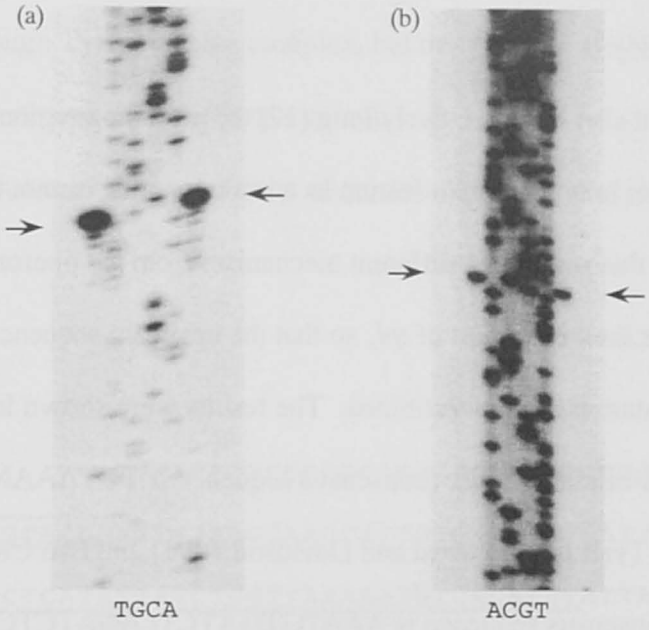


Fig. 1-2. cAMP-dependent catabolite repression of *tpl*. Northern hybridization was performed as described in the text. Briefly, total RNAs were prepared from cells grown in; lane 1, the medium containing tyrosine (Tyr); lane 2, further 30 minutes cultivation of lane 1; lane 3, further 30 minutes cultivation of lane 1 in the presence of glucose (Glc); lane 4, further 30 minutes cultivation of lane 3; lane 5, further 30 minutes cultivation of lane 3 in the presence of cAMP. The signs '+' above the lanes indicate that the cells were grown in the medium containig tyrosine, glucose, and cAMP, respectively. The membrane blotted was hybridized with a *tpl* specific probe labeled with [α-^{32}P]dCTP and autoradiographed.

Determination of the transcription start site of *tpl*. For further analysis of the expression mechanism of *tpl*, the transcription start point was determined by primer extension mapping. Two oligonucleotides (No. 172 and 174, see Fig. 1-3) spanning different regions of *tpl* and total RNA extracted from cells grown in the presence of tyrosine were used for extension reactions as specific primers and template, respectively. The results were shown in Fig. 1-3. In either case, the transcription start site was determined as a nucleotide G which located at -121 b from A of initiation codon of *tpl*. Just upstream region of the transcription start site, presumptive σ^{70}-dependent promoter was found and underlined in Fig. 1-3.

Sequence analysis of 5' flanking region of *tpl*. It is known that tryptophanase shows much resemblance to TPL both about the catalyzing reactions and mode of expression. The expression of tryptophanase, which degrades tryptophan to pyruvate, ammmonia, and indole, is induced by tryptophan and is subject to catabolite repression (Stewart and Yanofsky 1985). In the case of tryptophanase operon (*tna*), *tna* promoter lies in an approximately 300 b upstream region from its structural gene (*tnaA*) and the resultant long transcribed region contains a leader peptide consisting of 24 amino acids (*tnaC*). Stewart and Yanofsky showed that a leader peptide synthesis was important for the elongation of transcription of the downstream

(A)



(B)

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                                          -35                    -10
......AAAGGCGCGCATAGTGACGCGGCTATTTTCATCGCATGATAAATCCCGCATGATG
      +1
GTGTCGTATTATTTCCACCTCAATTCTGAGGTTATTGTTATATCTTCCTGTGCATTTCATC
          No.172 primer
TATGCACCAGACTTATTCGACGCGCATTTTTCTGCGTATGAAAATGGATAACTGGAGAAAT
                  No.174 primer
AAACATGAACTATCCTGCCGAGCCTTTCCGCATTAAAA....
      Met Asn Tyr Pro Ala Glu Pro Phe Arg Ile Lys
```

Fig. 1-3. (A) Mappings of 5'-end of *tpl* mRNA. Primer extensions were performed using reverse transcriptase with total RNA from *E. herbicola* and (a), No. 172 primer or (b), No. 174 primer labeled with [γ-^{32}P]. The reaction products were analyzed by gel electrophoreses next to the sequence ladders generated by the method of Sanger *et al.* (Sanger *et al.* 1977) using the same primers and pSH768 (Suziki *et al.* 1993) as a template. Arrows indicate the extension products. Both reaction products corresponded to the same nucleotide which was G positioned at -121 b from A of the initiation codon. (B) The nucleotide sequence of the upstream region of *tpl* and the amino acid sequence of N-terminus of TPL are shown. The complementary regions to No. 172 and 174 primers are indicated by underlines. The transcription start site determined above is boldfaced and shown as +1, and presumptive σ^{70}-dependent promoter is underlined with wavy lines.

gene encoding tyrptophanase and tryptophan-specific transporter (Stewart and Yanofsky 1986).

As for *tpl*, it also has a relatively long (121 b) preceding region before its structural gene, however, such feature as a leader peptide cannot be recognized in this region. A different mechanism from *tna* operon should be considered for the expression of *tpl*, so that the upstream sequence from the transcription start site was scrutinized. The results were shown in Fig. 1-4.

Three TyrR-binding motifs (consensus sequence is TGTAAAN$_6$TTTACA, referred to as TyrR box) (Pittard and Davidson 1991) and two CRP-binding motifs (the consensus sequence is AAATGTGATCT/ AGATCTCATTT) (Berg and von Hippel 1988; Botsford and Harman 1992) were found in the upstream region of *tpl*. Possible TyrR boxes was centered at -313.5 base (Box 1), -200.5 base (Box 2), and -85.5 base (Box 3) with respect to the transcription start point (Fig. 1-4). TyrR is well studied, especially in *E. coli*, to be the regulator for genes encoding the proteins responsible for aromatic amino acid biosyntheses and transports. TyrR binds to its recognition sites with aromatic amino acids as effectors and regulates the transcription of those genes positively or negatively (Camakaris and Pittard 1973; Cornish *et al.* 1982 and 1986; Cobbett 1988; Pittard and Davidson 1991; Yang *et al.* 1993; Argaet *et al.* 1994; Lawley and Pittard 1994; Kwok *et al.* 1995). These findings led the author to an assumption that the induction of TPL in the

presence of tyrosine would result from the activation of transcription of *tpl* mediated through TyrR-tyrosine complex, but not from the stabilization of *tpl* mRNA as mentioned in an earlier paragraph.

The facts that catabolite repression of *tpl* was dependent on cAMP and the

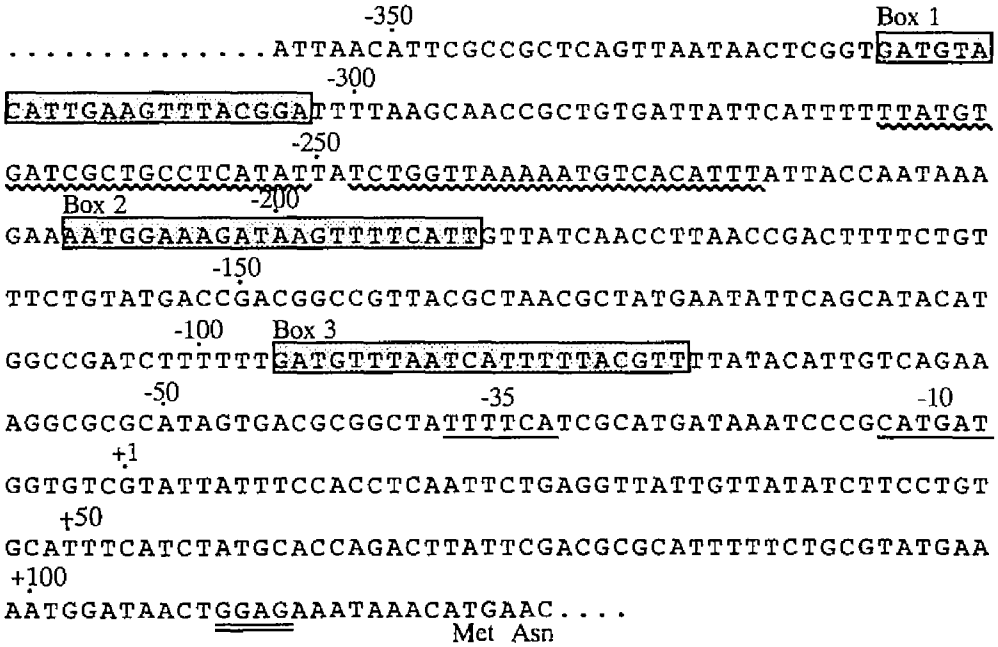

Fig. 1-4. Nucleotide sequence of the upstream region of *E. herbicola tpl* (Suzuki *et al.* 1993). Three possible TyrR binding sites (the consensus sequence is TGTAAAN$_6$TTTAC A) (Pittard and Davidson 1991) are enclosed with stippled boxes. Two possible CRP binding sites (the consensus sequence is AAATGTGATCT/ AGATCTCATTT) (Berg and von Hippel 1988; Botsford and Harman 1992) are underlined with wavy lines. The presumed -35 and -10 promoter regions (underlined), the SD sequence (double-underlined), and the initial methioninyl and second asparaginyl codons are indicated. The bases with dots above are positioned with respect to the transcription start site (+1) (Suzuki *et al.* 1995).

finding of possible CRP-binding sites in the upstream region of *tpl* strongly suggested that CRP-mediated transcriptional regulation would also occur.

Based on the above description, the author investigated the mode of expression of *tpl* in *E. coli* by means of constructing a single-copy *tpl'-'lac* fusion and assessing β-galactosidase activities of it in *E. coli*. That was described in the next chapter.

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SUMMARY

The expression of tyrosine phenol-lyase (TPL) is induced by tyrosine and is subject to catabolite repression. Using northern hybridization, the author proved that the variation of the expression of TPL was mainly due to the transcriptional regulation of its gene. The presence of tyrosine in the medium greatly increased the amount of *tpl* mRNA, and the increased amount of *tpl* mRNA by tyrosine was severely diminished by the addition of glucose to the medium. The glucose effect was slightly releaved by the addition of cAMP, that meant the cAMP-dependent catabolite repression occurred in the regulation of *tpl*. The 5'-end of *tpl* mRNA was determined using primer extension mapping and the upstream region was scrutinized. Three possible TyrR-binding sites and two possible cAMP receptor protein (CRP)-binding sites were found in the upstream region from its transcriptional start point.

Chapter 2.

Expression of *tpl'-'lac* Fusion in *Escherichia coli*

The expression of tyrosine phenol-lyase (TPL) is induced by tyrosine and is subject to catabolite repression (Enei *et al.* 1973). In the previous chapter, the author proved that the expression of TPL was regulated transcriptionally in *Erwinia herbicola* and suggested that it might be regulated through TyrR and CRP proteins (Suzuki *et al.* 1995). Recently, Smith and Somerville showed that the *tpl* promoter of *Citrobacter freundii* was activated by the *Escherichia coli* TyrR protein (Smith and Somerville 1997), although the mechanism of its regulation has not been clear yet.

To elucidate the regulation mechanism of *tpl*, the author investigated the mode of expression of *tpl* using *lac* reporter system in *E. coli*. To minimize experimental errors on assessing the β-galactosidase activities and to facilitate later genetic experiments, all were constructed as *tpl'-'lac* translational fusions and introduced into *E. coli* chromosome as single-copy fusions. In this chapter, the author constructed the *tpl'-'lac* fusions carrying a series of deletions in the upstream regions and examined the effect of the upstream length on its expression by assessing β-galactosidase activities of those strains. In addition, the modes of expression of *tpl'-'lac* fusion in *E. coli* *tyrR* and/or *crp* mutants were examined.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used in this chapter were derivatives of *E. coli* K-12. The relevant characteristics and sources of the strains and plasmids used are listed in Table 2-1.

Media and chemicals. *E. coli* strains were routinely grown at 37 ℃ in LB-medium (Sambrook *et al.* 1989). To study the regulation of *tpl'-'lac* fusions in *E. coli*, M63-glycerol and M63-glucose (Miller 1992) were used as the minimal media. L-Tyrosine was added as an inducer at a final concentration of 2 mM. L-Tryptophan and thiamine-HCl were added as growth requirements, if needed, at final concentrations of 20 μg/ml and 1 μg/ml, respectively. Ampicillin, kanamycin, chloramphenicol, and tetracycline were used at final concentrations of 100 μg/ml, 30 μg/ml, 30 μg/ml, and 15 μg/ml, respectively. The chemicals were all obtained commercially and not purified further.

Genetic techniques. Standard recombinant DNA procedures were performed essentially as described by Sambrook *et al.* (Sambrook *et al.* 1989) unless otherwise stated. The method of generalized transduction using P1*vir* phage was that described by Miller (Miller 1992). The *tyrR*$^-$ transductant was selected as to its resistance to 0.2 mM L-3-fluorotyrosine as described by Camakaris and Pittard (Camakaris and Pittard 1973). The *crp*$^-$ transductant was first selected as to its resistance to tetracycline derived from

Table 2-1. Bacterial strains and plasmids used in this chapter

Strain or plasmid	Relevant characteristic	Source or reference
Strains		
BD18	*rpsL* Δ(*cya*)*851* Δ(*crp*)*96 thi zhd-732*::Tn*10*(Tet^r)	NIG[a] (Benner *et al.* 1985)
CJ236	pCJ105 [F' Cam^r] / *dut1 ung1 thi-1 relA1*	Laboratory stock (Joyce and Grindley 1984)
JM109	F'[*traD36 proA*$^+$*B*$^+$ *lacI*^{q} Δ(*lacZ*)*M15*] / *endA1 gyrA96 thi hsdR17 supE44 relA1* Δ(*lac-proAB*) *recA1*	Laboratory stock (Yanisch-Perron *et al.* 1985)
JP2144	λ$^-$ *tsx-84 trpA9605 tyrR366 his-85 ilv-632*	CGSC[b] (Camakaris and Pittard 1973)
SH768	pSH768 / JM109	Laboratory stock (Suzuki *et al.* 1993)
TE2680	F$^-$ λ$^-$ IN(*rrnD-rrnE*)*1* Δ(*lac*)*X74 rpsL galK2 recD1903*::Tn*10d*-Tet^r *trpDC700*::*putPA1303*::[Kan^r-Cam^r-*lac*$^+$]	Elliott T (Elliott 1992)
TK314	TE2680 but *trpDC700*::*putPA1303*::[Kan^r *tpl'-lac*$^+$(carrying -350 element)]	This chapter
TK319	TK314 but Tet^s mutant	This chapter
TK334	TE2680 but *trpDC700*::*putPA1303*::[Kan^r *tpl'-lac*$^+$(carrying -300 element)]	This chapter
TK337	TE2680 but *trpDC700*::*putPA1303*::[Kan^r *tpl'-lac*$^+$(carrying -630 element)]; *Sma*I-*Bam*HI 750 b fragment from pTK306 was fused with pRS552.	This chapter
TK339	TK319 but Δ(*crp*)*96 zhd-732*::Tn*10*(Tet^r)	This chapter
TK348	TE2680 but *trpDC700*::*putPA1303*::[Kan^r *tpl'-lac*$^+$(carrying -150 element)]	This chapter
TK357	TK319 but *tyrR366*	This chapter
TK358	TK339 but *tyrR366* Δ(*crp*)*96 zhd-732*::Tn*10*(Tet^r)	This chapter
Plasmids		
pMU400	*tyrR*$^+_{E.coli}$ Amp^r ColE1*ori*	Pittard J (Cornish *et al.* 1982)
pRS552	Amp^r Kan^r *'lac*$^+$ ColE1*ori*	Simons RW (Simons *et al.*1987)

to be continued

Table 2-1. *continued*

pSH768	*tpl*$^{+}_{E.herbicola}$ Amp^{r} ColE1*ori*	Laboratory stock (Suzuki *et al.* 1993)
pTK304	pTZ19R derivative; 2.2 kb *Sma*I-*Pst*I fragment of pSH768 was inserted into pTZ19R.	This chapter
pTK306	pTK304 derivative; *Bam*HI site (Primer No. 187) was introduced by *in vitro* mutagenesis, which enable to fuse with the *'lac* gene of pRS552 in frame.	This chapter
pTZ19R	*lacZα*$^{+}$ Amp^{r} f1*ori* ColE1*ori*	Pharmacia Biotech

[a] NIG: National Institute of Genetics, Genetic Stock Research Center, Mishima 411-0801, Japan.

[b] CGSC: *E. coli* Genetic Stock Center.

cotransducible Tn*10* (Tet^{r}) using BD18 (Table 1) (Benner *et al.* 1985) as the donor, and then carbon source requirement was checked. Tetracycline sensitve mutant of TK314 was selected by the method of Bochner *et al.* (Bochner *et al.* 1980).

Oligonucleotide-directed mutagenesis. *In vitro* mutagenesis with a synthetic oligonucleotide was performed by the method of Kunkel *et al.* (Kunkel *et al.* 1987) using pTZ19R (Pharmacia Biotech) derivative containing the *tpl* gene from pSH768 (Suzuki *et al.* 1993) as a template (pTK304). The oligonucleotides used for mutageneses were synthesized at Biologica, Co., Japan. Nos. 182, 188, and 191 oligonucleotides were designed to introduce *Eco*RI sites and used to construct the *tpl'-'lac* fusions carrying a series of deletions in the upstream region. No. 187 was designed

to introduce a *Bam*HI site and used to fuse *tpl'* with *'lac* in frame on pRS552 (Simons *et al.* 1987). The entire fragments used for later manipulations were sequenced, by the method of Sanger *et al.* (Sanger *et al.* 1977), to ensure that no base change other than those planned had occurred.

No. 182 (for TK314), 5'-AGCGG CGAATTCTAATGACGTG-3'.

No. 188 (for TK334), 5'-TTAAAATCCGAATTCTTCTTCAATGTA-3'.

No. 191 (for TK348), 5'-AGCGTAACGGAATTCGGTCATAC-3'.

No. 187 (to fuse *tpl'* with *'lac* in frame), 5'-TCGGCAGGATCCTTCATGTT TA-3'.

Construction of the *tpl'-'lac* translational fusion and its transfer to the *E. coli* chromosome. All the *tpl'-'lac* translational fusions carrying various upstream extents were derivatives of pRS552 (Simons *et al.* 1987) and were created as follows. An *Eco*RI recognition site was introduced into the upstream region of *tpl* and a *Bam*HI site was introduced into the N-terminal region of *tpl*, respectively, by *in vitro* mutageneses as described above. Then the DNA fragments generated by digestion with these endonucleases were inserted into pRS552, which was predigested similarly. These translational fusions on plasmids were transferred to the *E. coli* chromosome (Table 2-1 and Fig. 2-2) as single-copy ones by the method of Elliott (Elliott 1992) using TE2680 as a host.

β-Galactosidase assay. Cultures were grown at 37 ℃ to the late-

exponential phase in the M-63 minimal medium as described above. β-Galactosidase activity was assayed according to the method of Miller (Miller 1992). Assays were performed in duplicate on two separate cultures, and the values showed less than 10 % error.

RESULTS AND DISCUSSION

Effect of the upstream region of *tpl* on the expression of *tpl'-'lac* fusion in *E. coli*. As mentioned in the previous chapter, three possible TyrR binding sites (the consensus sequence is TGTAAAN6TTTAC A, referred to as TyrR box) (Pittard and Davidson 1991) were present in the upstream region of *tpl*. Box 1 was centered at -313.5 b, Box 2 at -200.5 b, and Box 3 at -85.5 b with respect to the transcription start site (Fig. 2-1). To demonstrate that such a far upstream region can influence the expression of its structural gene, the *tpl'-'lac* fusions carrying a series of deletions in the upstream region were constructed and transferred to *E. coli* chromosome as single-copy fusions as described in Materials and Methods. As shown in Fig. 2-2, TK337, TK314, TK334, and TK348 possessed -628 ~, -350 ~, -309 ~, and -145 ~ upstream elements from the transcription start site, respectively. β-Galactosidase assays were performed as to these strains grown in the M63-minimal medium containing glycerol or glucose as a carbon source and in the presence or absence of tyrosine as an inducer. The

results shown in Table 2-2 made it obvious that the expressions of fusions carrying more than -350 b elements of *tpl* were, similarly as those of *tpl* in *E. herbicola*, induced by tyrosine (compare Gly with Gly+Tyr of TK337 and

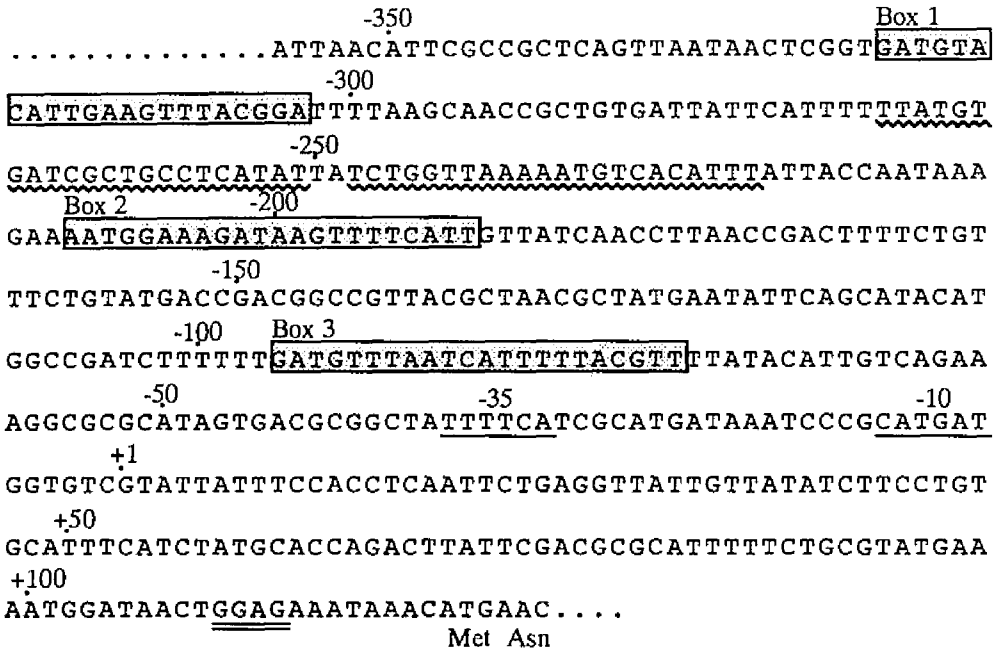

Fig. 2-1. Nucleotide sequence of the upstream region of *E. herbicola tpl* (Suzuki *et al.* 1993), which is indispensable for the intact expression of its structural gene (see Results and Discussion). Three possible TyrR binding sites (the consensus sequence is TGTAAA N_6TTTACA) (Pittard and Davidson 1991) are enclosed with stippled boxes. Two possible CRP binding sites (the consensus sequence is AAATGTGATCT/AGATCTCATTT) (Berg and von Hippel 1988; Botsford and Harman 1992) are underlined with wavy lines. The presumed -35 and -10 promoter regions (underlined), the SD sequence (double-underlined), and the initial methioninyl and second asparaginyl codons are indicated. When fused with the *'lac* operon, the upper region from the second asparaginyl codon was followed in frame by N-terminal deleted β-galactosidase, in which case a *Bam*HI recognition site was introduced by *in vitro* mutagenesis to facilitate the connection with pRS552 (Simons *et al.* 1987). The bases with dots above are positioned with respect to the transcription start site (+1) (Suzuki *et al.* 1995).

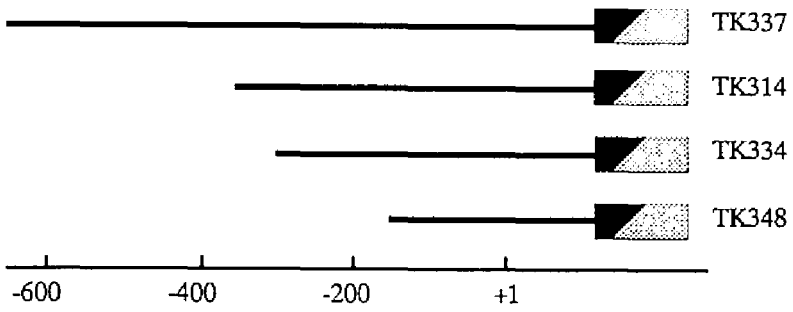

Fig. 2-2. Schematic representation of a series of *tpl'-'lac* fusions carrying various upstream lengths of *tpl*. The thin lines represent the non-coding regions and correspond to their lengths. The thick lines represent the coding regions, in which the N-terminal portion of TPL is shown in black and the fused 'Lac is shaded. The names of the strains carrying the corresponding fusions are shown on the right side. The bottom line indicates the approximate positions from the transcription start site (+1) at 200 b intervals.

Table 2-2. Effect of the upstream length on the expression of *tpl'-'lac* fusion in *E. coli*

Strain	Upstream length (bp)[a]	β-Galactosidase activities (Miller U.) of strain grown in[b]					
		Gly	Glc	Gly+Tyr	(I[c])	Glc+Tyr	(I[d])
TK337	-630 ~	41.3	33.1	1620	(39)	230	(6.9)
TK314	-350 ~	70.1	53.0	2270	(32)	352	(6.6)
TK334	-300 ~	50.0	41.5	103	(2.1)	63.9	(1.5)
TK348	-150 ~	66.8	58.9	63.5	(1.0)	56.1	(1.0)

a Approximate length of the upstream region from the transcription start point of *tpl*.

b Minimal medium containing; Gly, 0.2 % glycerol as a carbon source; Glc, 0.2 % glucose as a carbon source; Tyr, 2 mM tyrosine as an inducer of *tpl*.

c The ratio of tyrosine-induction in the presence of glycerol as a carbon source.

d The ratio of tyrosine-induction in the presence of glucose as a carbon source.

TK314 in Table 2-2) and were subject to catabolite repression (compare Gly+Tyr with Glc+Tyr of TK337 and TK314 in Table 2-2). However, TK334 and TK348, the fusions of which possessed shorter elements than that of TK314, did not show sufficient activities and, almost completely, lost their induction abilities in the presence of tyrosine (Gly and Gly+Tyr in Table 2-2). It is noteworthy that there was only a little difference between the fusions of TK314 and TK334 as to length, but there was a striking difference between them whether or not they possessed a possible TyrR box (Box 1 in Fig. 2-1). It was easily speculated that the TyrR protein bound to Box 1 might play an important role in the expression of *tpl*. Thus, we examined the mode of expression of the *tpl'-'lac* fusion in *tyrR* mutant, and the results were described in the next paragraph.

Expression of the *tpl'-'lac* fusion in *E. coli tyrR* and/or *crp* mutants. As described in the previous chapter, cAMP-dependent catabolite repression was observed in the regulation of *tpl*. Moreover, in the upstream region of *tpl*, two possible CRP-binding sites (the consensus sequence is AAATGTGATCT/AGATCACATTT) (Berg and von Hippel 1988; Botsford and Harman 1992) were present between Box 1 and Box 2 (Fig. 2-1) even though they showed good agreements only in half (left or right)-arms.

The above findings strongly suggested that both the TyrR and CRP proteins were involved in the *tpl* transcriptional regulation in *E. coli*. Thus,

the author attempted to express the *tpl'-'lac* fusion in *E. coli tyrR* and/or *crp* mutants. A tetracycline-sensitive mutant of TK314, called TK319, which was selected on a Bochner plate (Bochner *et al.* 1980), was generally transduced with P1 (JP2144; *tyrR366*) (Camakaris and Pittard 1973) lysate and/or P1 (BD18; Δ*(crp)96 zhd-732*::Tn*10*(Tet^r)) (Benner *et al.* 1985) lysate, which gave TK339 (*crp*), TK357 (*tyrR*), and TK358 (*tyrR crp*), respectively, and then, β-galactosidase assays as to these strains were performed similarly.

As for TK314 (*tyrR*$^+$ *crp*$^+$), the ratio of tyrosine-induction was 32 when glycerol was used as a carbon source. In the case of glucose as a carbon source, the induction ratio was 6.6 (Table 2-3). The low induction ratio and low expression values in the presence of glucose as a carbon source indicated that catabolite repression actually occurred in this strain. And the catabolite repression was slightly (10 %) relieved when cAMP was added to the medium (data not shown). However, in the *tyrR* mutant (TK357), whichever type of carbon source was used, the induction by tyrosine was abolished. Moreover, the β-galactosidase values were almost identical for the two carbon sources (Table 2-3). These results indicated that neither tyrosine-induction nor catabolite repression occurred in the *tyrR*$^-$ strain, that is, the *tyrR* mutation abolished the glucose effect on the regulation of *tpl*. The loss of induction ability was recovered by donating wild-type *E. coli tyrR* (pMU400) (Cornish *et al.* 1982) (data not shown). The TyrR protein and its ligand tyrosine

Table 2-3. The expression of *tpl'-'lac* fusion in *E. coli tyrR* and/or *crp* mutants

Strain	Relevant genotype[a]	β-Galactosidase activities (Miller U.) of strain grown in[b]			
		Gly	Glc	Gly+Tyr (I[c])	Glc+Tyr (I[d])
TK314		70.1	53.0	2270 (32)	352 (6.6)
TK357	*tyrR366*	51.6	43.5	55.7 (1.1)	46.0 (1.1)
TK339	Δ(*crp*)*96*	NA[e]	57.5	NA	210 (3.7)
TK358	*tyrR366* Δ(*crp*)*96*	NA	41.2	NA	43.1 (1.0)

a Relevant genotype conferred on TK319 by P1 transduction.

b Minimal medium containing; Gly, 0.2 % glycerol as a carbon source; Glc, 0.2 % glucose as a carbon source; Tyr, 2 mM tyrosine as an inducer of *tpl*.

c The ratio of tyrosine-induction in the presence of glycerol as a carbon source.

d The ratio of tyrosine-induction in the presence of glucose as a carbon source.

e Not applicable since the *crp* mutant shows a strong preference for carbon sources.

(Argaet *et al.* 1994; Pittard and Davidson 1991) should act as an activator of *tpl* in *E. coli*.

As for the *crp* mutant (TK339), the induction by tyrosine certainly took place at the ratio of 3.7, although this ratio was lower than that of TK314 (6.6) (compare Glc with Glc+Tyr in Table 2-3). The *crp* mutation reduced the ratio of tyrosine-induction, but could not overcome the TyrR-mediated activation of *tpl*. These results suggested that TyrR might play a direct role in the transcriptional activation of *tpl*, such as an interaction with RNA polymerase, while CRP might act indirectly as a second factor. The details were discussed in the next chapter.

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SUMMARY

The expression of *tpl'-'lac* fusion was regulated through both of the TyrR protein and cAMP receptor protein (CRP) in *Escherichia coli*. The *tyrR* mutation abolished the effect of CRP (catabolite repression) on the *tpl* regulation, while the *crp* mutation could not overcome the TyrR-mediated activation of *tpl*. These results suggested that, in the transcriptional regulation of *tpl*, TyrR-tyrosine complex might play a direct role such as an interaction with RNA polymerase, while CRP might act indirectly as a second factor.

The upstream deletion analysis of *tpl'-'lac* fusion indicated that Box 1, which located more than 300 b upstream from the transcription start site, was indispensable for the tyrosine-mediated activation of *tpl*. TyrR bound to Box 1 might play an important role in the activation of *tpl* transcription.

Chapter 3.

Erwinia herbicola TyrR as a Positive Regulator of *tpl* and Its Action on the *tpl* Regulatory Region

The expression of tyrosine phenol-lyase (TPL) is induced in the presence of tyrosine and is subject to catabolite repression (Enei *et al.* 1973), and that was transcriptionally regulated as described in Chapter 1. To elucidate the regulation mechanism of *tpl*, the *lac* reporter system was employed in *Escherichia coli*. By assessing the β-galactosidase activities and evaluating the mode of expression of the *tpl'-'lac* fusion in *E. coli*, it was suggested that the expression of *tpl* was regulated through both of TyrR and cAMP receptor protein (CRP), which was described in Chapter 2.

The primary purpose of this study was to find a tyrosine-responsive regulator of *tpl* in *Erwinia herbicola*. Even though the results obtained so far suggested that the object was TyrR, the author attempted to directly clone a tyrosine-responsive regulator of *tpl* from *E. herbicola* genomic library.

In this chapter, the author cloned a gene for tyrosine-responsive regulator of *tpl*, by using *lac* reporter system in *E. coli*. In addition, the author proposed a hypothetical model for the transcriptional activation of *tpl*, by estimating the β-galactosidase activities of the *tpl'-'lac* fusions possessing a variety of mutations in the upstream region.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used in this chapter were *E. herbicola* AJ2985 and derivatives of *E. coli* K-12. The relevant characteristics and sources of the strains and plasmids used are listed in Table 3-1.

Media and chemicals. *E. coli* strains were routinely grown at 37 ℃ in LB-medium (Sambrook *et al.* 1989). Bacto MacConkey agar base was purchased from Difco Laboratories (Detroit, USA) and used as recommended. D-Lactose was added at a final concentration of 1 % as a fermentable carbon source and L-tyrosine was added at a final concentration of 5 mM as an inducer of *tpl*. To study the regulation of *tpl'-'lac* fusions in *E. coli*, M63-glycerol and M63-glucose (Miller 1992) were used as the minimal media. L-Tyrosine was added as an inducer at a final concentration of 2 mM. L-Tryptophan, L-proline, and thiamine-HCl were added as growth requirements, if needed, at final concentrations of 20 μg/ml, 30 μg/ml, and 1 μg/ml, respectively. Ampicillin, kanamycin, chloramphenicol, and tetracycline were used at final concentrations of 100 μg/ml, 30 μg/ml, 30 μg/ml, and 15 μg/ml, respectively. The chemicals were all obtained commercially and not purified further.

Genetic techniques. Standard recombinant DNA procedures were performed essentially as described by Sambrook *et al.* (Sambrook *et al.* 1989)

unless otherwise stated. The method of generalized transduction using P1*vir* phage was that described by Miller (Miller 1992). The *tyrR*⁻ transductant was selected as to its resistance to 0.2 mM L-3-fluorotyrosine as described by Camakaris and Pittard (Camakaris and Pittard 1973).

Table 3-1. Bacterial strains and plasmids used in this work

Strain or plasmid	Relevant characteristic	Source or reference
Strains		
Erwinia herbicola AJ2985		Ajinomoto, Co., Inc.
Escherichia coli K-12		
CJ236	pCJ105 [F' Cam^r] / *dut1 ung1 thi-1 relA1*	Laboratory stock (Joyce and Grindley 1984)
CSH26	F⁻ *ara* Δ(*lac-pro*) *thi*	NIG[a] (Miller 1992)
JM107	F'[*traD36 proA*⁺*B*⁺ *lacI*^{q} Δ(*lacZ*)*M15*] / *endA1 gyrA96 thi hsdR17 supE44 relA1* Δ(*lac-proAB*)	NIG (Yanisch-Perron *et al.*1985)
JP2144	λ⁻ *tsx-84 trpA9605 tyrR366 his-85 ilv-632*	CGSC[b] (Camakaris and Pittard 1973)
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*(Tet^r)	Takara Shuzo, Co.,Ltd. (Vieira and Messing 1987)
TE2680	F⁻ λ⁻ IN(*rrnD-rrnE*)*1* Δ(*lac*)*X74 rpsL galK2 recD1903*::Tn*10d*-Tet^r *trpDC700*::*putPA1303*::[Kan^r-Cam^r-*lac*⁺]	Elliott T(Elliott 1992)
TK314	TE2680 but *trpDC700*::*putPA1303*::[Kan^r *tpl'-lac*⁺(carrying -350 element)]	Chapter 2
TK418	TK314 but *trpDC700*::*putPA1303*::[Kan^r *tpl'-'lac*⁺ (Box 1 mutation)]	This chapter

to be continued

Table 3-1. *continued*

TK419	TK314 but *trpDC700*::*putPA1303*::[Kan^r *tpl'-'lac*$^+$ (Box 2 mutation)]	This chapter
TK420	TK314 but *trpDC700*::*putPA1303*::[Kan^r *tpl'-'lac*$^+$ (Box 3 mutation)]	This chapter
TK453	JM107 but Δ(*srl-recA*)*306*::Tn*10*(Tet^r) *tyrR366* *trpDC700*::*putPA1303*::[Kan^r *tpl'-'lac*$^+$(including the entire regulatory region)]	This chapter
TK472	TK314 but *trpDC700*::*putPA1303*::[Kan^r *tpl'-'lac*$^+$ (Box 1, 2, and 3 mutations)]	This chapter
TK481	JM107 but Δ(*srl-recA*)*306*::Tn*10*(Tet^r) *tyrR366* *trpDC700*::*putPA1303*::[Kan^r *tpl'-'lac*$^+$(including promoter but no regulatory region)]; 180 b *Bss*HII-*Bam*HI fragment from pTK368 was ligated with pRS552 .	This chapter
TK665	CSH26 but *tyrR366* Δ(*srl-recA*)*306*::Tn*10*(Tet^r) *trpDC700*::*putPA1303*::[Kan^r *tpl'-'lac*$^+$(including the entire regulatory region)]	This chapter
TK666	TK665 but *trpDC700*::*putPA1303*::[Kan^r *tpl'-'lac*$^+$ (Box 1 mutation)]	This chapter
TK667	TK665 but *trpDC700*::*putPA1303*::[Kan^r *tpl'-'lac*$^+$ (Box 2 mutation)]	This chapter
TK668	TK665 but *trpDC700*::*putPA1303*::[Kan^r *tpl'-'lac*$^+$ (Box 3 mutation)]	This chapter
TK669	TK665 but *trpDC700*::*putPA1303*::[Kan^r *tpl'-'lac*$^+$ (Box 1, 2, and 3 mutations)]	This chapter
TK680	pTK672 / TK665	This chapter
TK681	pTK672 / TK666	This chapter
TK682	pTK672 / TK667	This chapter
TK683	pTK672 / TK668	This chapter
TK684	pTK672 / TK669	This chapter
Plasmids		
pMU400	$tyrR^+_{E.coli}$ Amp^r ColE1*ori*	Pittard J (Cornish *et al.* 1982)
pMW118	*lacZα*$^+$ Amp^r pSC101*ori*	Nippon Gene, Co.,Ltd.
pRS552	Amp^r Kan^r *'lac*$^+$ ColE1*ori*	Simons RW (Simons *et al.*1987)

to be continued

Table 3-1. *continued*

pSH768	*tpl*$^{+}_{E.herbicola}$ Amp^{r} ColE1*ori*	Laboratory stock (Suzuki *et al.* 1993)
pTK-#20	*tyrR*$^{+}_{E.herbicola}$ Amp^{r} ColE1*ori*	This chapter
pTK304	pTZ19R derivative; 2.2 kb *Sma*I-*Pst*I fragment of pSH768 was inserted into pTZ19R.	Chapter 2
pTK368	pTZ19R derivative; *Eco*RI site (Primer No. 182) and *Bam*HI site (Primer No. 187) were introduced into pTK304 by *in vitro* mutageneses and then *Eco*RI-*Bam*HI 0.5 kb fragment was inserted into pTZ19R.	This Chapter
pTK672	pMW118 derivative (*tyrR*$^{+}_{E.herbicola}$); 2.4 kb *Sal*I (blunted)-*Ssp*I fragment from pTK-#20 was ligated with 3.9 kb *Pvu*II fragment of pMW118.	This chapter
pTK673	pMW118 derivative (*tyrR*$^{+}_{E.coli}$); 2.6 kb *Hin*dIII (blunted)-*Nde*I(blunted) fragment from pMU400 was ligated with 3.9 kb *Pvu*II fragment of pMW118.	This chapter
pTZ19R	*lacZα*$^{+}$ Amp^{r} f1*ori* ColE1*ori*	Pharmacia Biotech

[a] NIG: National Institute of Genetics, Genetic Stock Research Center, Mishima 411-0801, Japan.

[b] CGSC: *E. coli* Genetic Stock Center.

Determination of DNA sequences. DNA sequences were determined by the method of Sanger *et al.* (Sanger *et al.* 1977), using a Thermo sequenase fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham International plc, England), and DSQ-1000L sequencer (Shimadzu Co., Ltd., Japan).

Oligonucleotide-directed mutagenesis. *In vitro* mutagenesis with a synthetic oligonucleotide was performed by the method of Kunkel *et al.*

(Kunkel *et al.* 1987) using pTZ19R (Pharmacia Biotech) derivative containing the gene for *tpl* upstream region as a template (pTK368). The oligonucleotides used for mutageneses were synthesized at Biologica, Co., Japan. Nos. 42, 37, and 38 oligonucleotides were used as mutagenic primers which introduced mutations at three possible TyrR boxes (Boxes 1, 2 and 3, respectively) (Fig. 2-1 and Fig. 3-2) in the upstream region of *tpl*. The entire fragments used for later manipulations were sequenced to ensure that no base change other than those planned had occurred.

No. 42 (for Box 1 mutation), 5'-TTGCTTAAAATCCGTCGACTTCAATG CTGATCACCGAGTTATTA-3'.

NO. 37 (for Box 2 mutation), 5'-GTTGATAACAATGTCGACTTATCTGG CCATTTTCTTTAT-3'.

No.38 (for Box 3 mutation), 5'-ATGTATAAAACGTCGAAATGATTACGC ATCAAAAA AGAT-3'.

Construction of the *tpl'-'lac* translational fusion and its transfer to the *E. coli* chromosome. Constructions of the *tpl'-'lac* fusions and those transfers to the *E. coli* chromosome were performed similarly as described in Chapter 2. In brief, the upstream fragments of *tpl* carrying mutations at possible TyrR binding sites were fused with *'lac* on pRS552 (Simons *et al.* 1987) in frame and then transferred to the chromosome of TE2680 (Elliott 1992). The resultant strains, however, have

the *recD* mutation so that the plasmid replication may be abberant in these strains (Biek and Cohen 1986). This phenomenon might cause some defects in later experiments. Therefore, the fusions were transferred to other *E. coli* K-12 strains by P1 transductions.

Construction of an *E. herbicola* genomic library. Genomic DNA was extracted from *E. herbicola* AJ2985 according to the method of Wilson (Wilson 1998), 1.45 mg of DNA being obtained from 50 ml of culture. The 43.5 μg of DNA was partially digested with *Sau*3AI and approximately 4-8 kb fragments were recovered from low-melting agarose, SeaPlaque GTG (FMC Bio Products, USA). Then 1.7 μg of the partially digested fragments was ligated with 1.4 μg of pBR322, which had been digested with *Bam*HI and dephosphorylated with alkaline phosphatase (Toyobo, Co., Ltd., Japan) in advance. A ligation mixture was used to transform TK453 (Table 3-1 and see Results), about 20,000 transformants being obtained.

β-Galactosidase assay. Cultures were grown at 37 ℃ to the late-exponential phase in the M-63 minimal medium as described above. β-Galactosidase activity was assayed according to the method of Miller (Miller 1992). Assays were performed in duplicate on two separate cultures, and the values showed less than 10 % error.

RESULTS

Cloning of a gene for tyrosine-responsive positive regulator of *tpl* from an *E. herbicola* genomic library. For constructing a useful host to clone the gene(s) for regulator(s) of *tpl*, JM107 was transduced with P1 (TK314; *trpDC700*::*putPA1303*::[Kan^r *tpl'-'lac*$^+$]), the fusion of which was considered to possess an enough upstream region for the expression of *tpl*. The resultant strain was also transduced with P1 (JP2144; *tyrR366*) (Camakaris and Pittard 1973), which would hold the expression of the fusion at a basal level, and P1 (MV1184; Δ(*srl-recA*)*306*::Tn*10*(Tet^r)) (Vieira and Messing 1987), which would prevent the gene conversion. Finally, TK453 (Table 3-1) was obtained as a cloning host and made it competent.

TK453 transformed with an *E. herbicola* genomic library (see Materials and Methods) was spread on a MacConkey-lactose plate containing 5 mM tyrosine as an inducer. Then the colonies colored red on the indicator plate were selected. At this point, however, at least three possible reasons should be considered for these phenotype changes, (i) the gene(s) for positive regulator(s) of *tpl* was(were) cloned, the product(s) of which activated the expression of the *tpl'-'lac* fusion, (ii) the gene for β-galactosidase itself was cloned, the activity of which was expressed, and (iii) unknown factors such as one causing a pH decrease. To exclude the latter two possibilities,

plasmids extracted from red color-forming colonies obtained in the first screening were introduced into another strain, *i.e.* TK481. As shown in Table 3-1, the *tpl'-'lac* fusion in TK481 contained only a *tpl* promoter but no regulatory region. Therefore, even if the factor responsible for the *tpl* regulatory region was donated to TK481, the factor no longer had the element on which it act so that the expression of *tpl'-'lac* fusion remained basal (forming a white colony). On the contrary, in the latter two cases described above, TK481 transformed with plasmids showed the red color again.

In this way, 20 positive clones were obtained, those were thought to encode the tyrosine-responsive positive regulator(s) of *tpl* on plasmids.

***E. herbicola* TyrR as a positive regulator of *tpl*.** Genetic analyses revealed that all the plasmids extracted from the 20 clones gave the same length (1.6 kb) DNA fragments on *Eco*RI digestion. In addition, all clones complemented the TyrR$^-$ phenotype (Camakaris and Pittard 1973) of the host strain (TK453). These results suggested that one species of gene was cloned as a tyrosine-responsive positive regulator of *tpl*, and that it was *tyrR* of *E. herbicola*. One of these plasmids, named pTK-#20, which had the shortest (but 6 kb) insert, was chosen for later genetic operations. In enzymatic manipulations with pTK-#20, it was convinced that a 3.5 kb *Sal*I fragment certainly contained the gene of interest, so the DNA sequence of it was determined. The DNA sequence and the predicted amino acid sequence

revealed that it was *tyrR*, as thought (Fig. 3-1).

Multiple amino acid sequence alignment of TyrRs. The DNA sequence analysis gave two potential initiation codons seperated by 46 codons at the 5' end, and it was assumed that the second ATG was the actual initial codon for the following two reasons. One was that the deletion of the upstream region including the first potential initiation codon did not alter its activation function on the *tpl'-'lac* fusion (data not shown). The other is that the amino acid sequence of the N-terminus from the second ATG showed good agreement with other bacterial TyrRs, as shown in Fig. 3-1.

E. coli tyrR has already been sequenced by Cornish *et al.* (Cornish *et al.* 1986), and recently, Bai and Somerville cloned and sequenced the *tyrR* genes from both *Salmonella typhimurium* and *Citrobacter freundii* (Bai and Somerville 1997, Genebank accession numbers; U90140 and U90141, respectively). Comparison of their primary structures may allow to acquire more general and comprehensive knowledge of TyrRs, so that multiple amino acid sequence alignment was executed with the Clustal W 1.6 program (Thompson *et al.* 1994) (Fig. 3-1). *E. herbicola* TyrR showed relatively low (less than 72 % identity) similarity with the others, while the TyrRs of *E. coli*, *S. typhimurium*, and *C. freundii* showed high similarities (more than 90 % identity) to each other. All TyrRs had the identical helix-turn-helix motif in the C-terminal domain so they must recognize the same DNA sequence

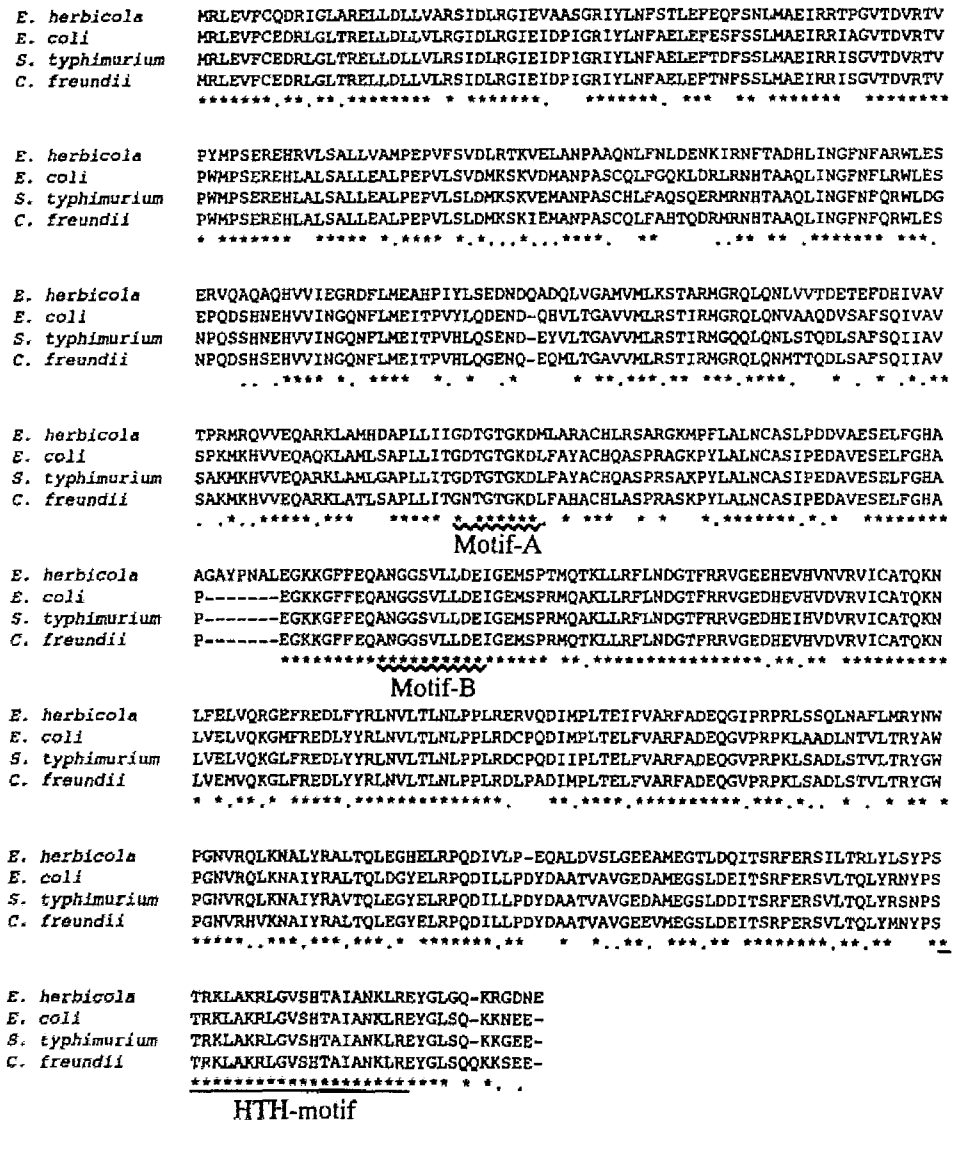

Fig. 3-1. Multiple amino acid sequence alignment of the TyrR proteins of *E. herbicola*, *E. coli* (Comish *et al.* 1986), *S. typhimurium* (Bai and Somerville 1997, Genebank accession number, U90140), and *C. freundii* (Bai and Somerville 1997, Genebank accession number, U90141) was excuted with the Clustal W 1.6 progaram (Thompson *et al.* 1994). The astrisks indicate residues conserved in all TyrR proteins, and the dots those of which only a conservative change has occurred. The amino acid residues in the helix-turn-helix (HTH-) motif in the C-terminal domain are conserved in all TyrR proteins and are underlined. The wavy underlines indicate ATP binding motifs-A and -B. Only TyrR of *E. herbicola* possessed seven extra amino acid residues between the two ATP binding motifs.

(TGTAAAN$_6$TTTACA) (Pittard and Davidson 1991). It should be mentioned that only *E. herbicola* TyrR possessed seven extra amino acid residues in the central domain (Fig. 3-1). This was disscussed later.

Significance of each TyrR box as to the expression of the *tpl'-'lac* fusion. As mentioned in the earlier chapters, there were three possible TyrR boxes in the upstream region of *tpl* (Fig. 2-1 and 3-2). And the results shown in Chapter 2 (Table 2-2) suggested that Box 1 played an important role in the expression of *tpl'-'lac* fusion. To elucidate the mechanism of TyrR-mediated activation of *tpl*, a series of mutations were introduced in three possible TyrR boxes (Fig. 3-2) in the upstream region of *tpl* and then fused with *'lac* operon similarly. By means of oligonucleotide-directed mutagenesis, as described in Materials and Methods, the nucleotide sequence of every possible TyrR box was changed not to be recognized by the TyrR protein, and then transferred to the *E. coli* chromosome as the single-copy *tpl'-'lac* fusion. The strains which had mutations in Box 1, Box 2, Box 3, and Boxes 1, 2, and 3 were named TK418, 419, 420, and 472, respectively (Table 3-1). CSH26 was transduced with the P1 (TK314, 418, 419, 420, or 472; *trpDC700*::*putPA1303*::[Kan^r *tpl'-'lac*$^+$(wild-type or Box mutations)]) lysate, P1 (JP2144; *tyrR366*) (Camakaris and Pittard 1973) lysate, and P1 (MV1184; Δ(*srl-recA*)*306*::Tn*10*(Tet^r)) (Vieira and Messing 1987) lysate, respectively. Finally, these strains were transformed with low-

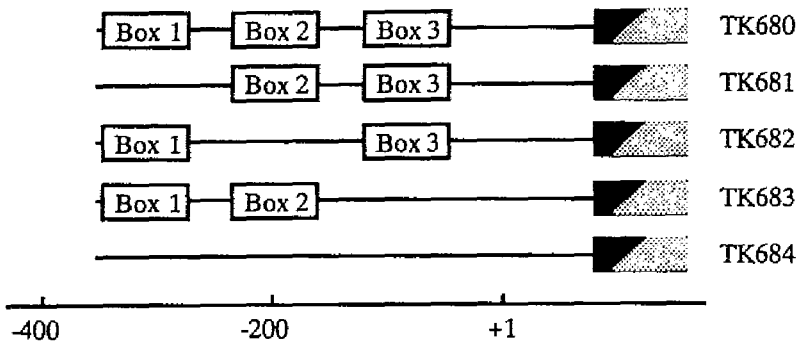

Fig. 3-2. Schematic representation of the *tpl'-'lac* fusions possessing mutations in the TyrR boxes. The thin lines represent the *tpl* upstream region. The possible TyrR boxes (Box 1, Box 2, and Box 3) are boxed, and were centered at -313.5 b, -200.5 b, and -85.5 b from the transcription start site, respectively. In the cases those boxes were destroyed by *in vitro* mutageneses not to be recognized by TyrR protein (see Results), no box are given at their corresponding regions. The names of the strains carrying the corresponding fusions are shown on the right side. The bottom line indicates the approximate positions from the transcription start point (+1) (Suzuki *et al.* 1995).

Table 3-2. Significance of each TyrR box as to activation of the expression of *tpl'-'lac* fusion

Strain	Relevant characteristic[a]	β-Galactosidase activities (Miller U.) of strain grown in[b]					
		Gly	Glc	Gly+Tyr	(I[c])	Glc+Tyr	(I[d])
TK680		98.9	68.2	1813	(18)	786	(12)
TK681	Box 1 mutation	66.0	56.7	149	(2.3)	136	(2.4)
TK682	Box 2 mutation	92.2	56.7	204	(2.2)	86.4	(1.5)
TK683	Box 3 mutation	93.6	57.5	323	(3.5)	160	(2.8)
TK684	Box 1, 2, 3 mutations	70.2	55.4	71.7	(1.0)	59.5	(1.1)

a Relevant characteristic conferred on TK680 by *in vitro* mutagenesis.

b Minimal medium containing; Gly, 0.2 % glycerol as a carbon source; Glc, 0.2 % glucose as a carbon source; Tyr, 2 mM tyrosine as an inducer of *tpl*.

c The ratio of tyrosine-induction in the presence of glycerol as a carbon source.

d The ratio of tyrosine-induction in the presence of glucose as a carbon source.

copy number pTK672 ($tyrR^{+}_{E.\ herbicola}$) (Table 3-1), and named TK680, 681, 682, 683, and 684, respectively (Table 3-1 and Fig. 3-2). β-Galactosidase assays were performed as to these strains grown in the M63-minimal medium containing glycerol or glucose as a carbon source and in the presence or absence of tyrosine as an inducer (Table 3-2).

The expression of *tpl'-'lac* fusion carrying the wild-type Boxes (TK680) was induced by tyrosine at the ratio of 18 in the presence of glycerol as a carbon source and was subject to catabolite repression (compare Gly+Tyr with Glc+Tyr in Table 3-2), although these ratios were lower than those observed for TK314 (Table 2-2 or 2-3 in Chapter 2). This disparity was considered to be derived from the differences in the genetic background between them, but not from the inferiority of *E. herbicola* TyrR as to activation of *tpl*, since lower ratios were also obtained when the *tyrR* gene of *E. coli* was similarly donated (pTK673) (data not shown).

Only the addition of tyrosine to the medium greatly induced the expression of *tpl'-'lac* fusion, while the addition of phenylalanine or tryptophan, which was also known to be ligand of TyrR (Pittard and Davidson 1991; Argaet *et al.* 1994), induced its expression at the ratio of 1.7 or 1.0, respectively (data not shown). Thus, L-tyrosine is the major factor in the TyrR-mediated activation of *tpl*.

Every Box mutation severely reduced the ratio of tyrosine-induction of *tpl*,

and no induction was seen when all Boxes were destroyed (Table 3-2). The results indicated that each Box actually functioned as a TyrR binding site *in vivo* and played a significant role in TyrR-mediated activation of *tpl*. It should be mentioned that, as to the expression of *tpl'-'lac* fusion in the Box 1 mutant, not only the ratio of tyrosine-induction but also the β-galactosidase values were almost equal for the two carbon sources, while those in Box 2 and 3 mutants were different and were considered to be subject to catabolite repression (compare Gly+Tyr with Glc +Tyr in Table 3-2). These results indicated that the Box 1 mutation overcame the CRP effect, which was discussed later. It should also be stressed that the β-galactosidase activity of the fusion in the Box 2 mutant grown in the presence of glucose and tyrosine was considerably lower than those of the fusions in other Box mutants (Glc+Tyr in Table 3-2). This was also discussed later.

DISCUSSION

Cloning of *E. herbicola tyrR* as a gene for tyrosine-responsive positive regulator of *tpl*. In this chapter, the author cloned a gene for tyrosine-responsive positive regulator of *tpl* from an *E. herbicola* genomic library using the *lac* reporter system. The ability of the gene product to complement the *E. coli* $TyrR^-$ phenotype and determination of its DNA sequence revealed that it was *tyrR*.

The primary structure of *E. herbicola* TyrR exhibited a few differences from those of other TyrRs. As described above, TyrR of *E. herbicola* possessed seven 'extra' amino acid residues in its central domain, compared with other bacterial TyrRs (Fig. 3-1). However, when compared with other regulators, such as NtrC (Miranda-Rios *et al.* 1987) and NifA (Buikema *et al.* 1985), known to be homologues of *E. coli* TyrR (Yang *et al.* 1993), these 'extra' amino acids were found to be 'not extra' (data not shown). The role of the central domain of *E. coli* TyrR has been the subject of study. The necessities for the monomer-monomer interaction (Cui and Somerville 1993), ATP binding (Pittard and Davidson 1991; Yang *et al.* 1993; Argaet *et al.* 1994), and the conformational change to a hexamer from a dimer (Wilson *et al.* 1994; Kwok *et al.* 1995) have been discussed. In addition, the ATPase activity of *E. coli* TyrR was reported by Cui *et al.*, although its turnover number was considerably low and its contribution remained unknown (Cui *et al.* 1993). On the other hand, it is known that NtrC possesses high ATPase activity in its phosphorylated form and that its activity are required for transcriptional activation (Popham *et al.* 1989; Wedel *et al.* 1990; Kustu *et al.* 1991; Weiss *et al.* 1991). At present, these 'extra' amino acid residues of *E. herbicola* TyrR cannot be attributed to specific features of the protein. Characterization of *E. herbicola* TyrR is in progress.

Roles of TyrR and CRP in the regulation of *tpl*. As can be seen

in Table 3-2, the mutation of each possible TyrR box severely decreased the ratio of tyrosine-induction of *tpl*, which indicates that every box acts functionally *in vivo* as a TyrR recognition site. As mentioned above, the tyrosine-mediated conformational change of the *E. coli* TyrR protein from a dimer to a hexamer was reported by Wilson *et al.* (Wilson *et al.* 1994). Indeed, the tyrosine-mediated repression of *aroLM* and *aroF-tyrA* of *E. coli* occur through this mechanism, in which the TyrR hexamer inhibits an RNA polymerase to access their promoters (Cobbett 1988; Pittard and Davidson 1991; Lawley and Pittard 1994; Kwok *et al.* 1995). From this coupled with above results, the author established a hypothetical model for the TyrR-mediated activation of *tpl*, as depicted in Fig. 3-3. The TyrR dimers bound to the three TyrR boxes upstream of *tpl* convert its configuration to a hexamer in the presence of tyrosine, and the distal Box 1-TyrR complex will come up to the downstream promoter region. And then, the Box 1-TyrR complex is involved in the interaction with RNA polymerase to trigger *tpl* transcription. Actually, the expression of *tpl'-'lac* fusion in the Box 1 mutant was the lowest (Table 3-2; except Glc+Tyr in the Box 2 mutant, discussed later), and the basal expression (Gly in Table 3-2) was almost the same as that of the fusion in the Box1, 2, and 3 mutant. The low ratio of tyrosine-induction may depend on the Box 1-free, Box 2- and Box 3-TyrR hexamer formed in the presence of tyrosine. The Box 3 mutation, which is the nearest site to the *tpl*

promoter, had the least effect on the expression of *tpl*. The role of Box 1 is to arrange the TyrR protein in the correct position where it can contact with the polymerase effectively.

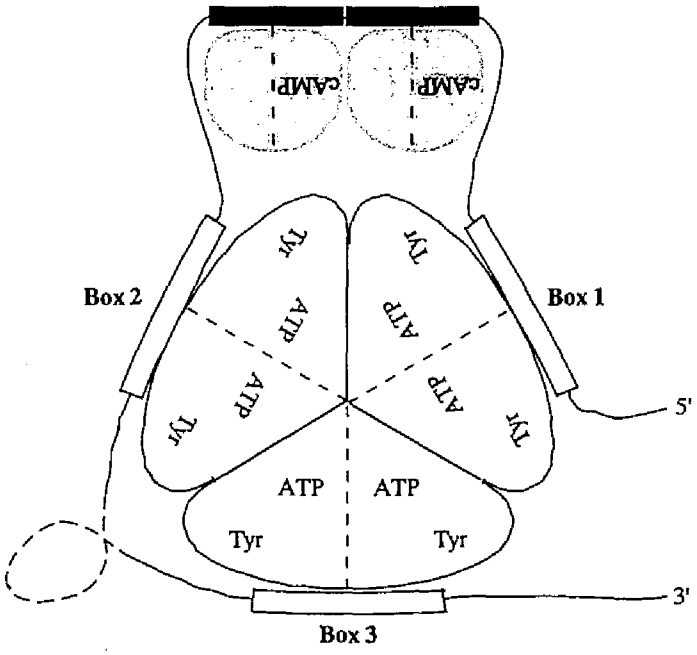

Fig. 3-3. The hypothetical model proposed for the TyrR- and CRP-mediated regulation of *E. herbicola tpl*. In the presence of L-tyrosine, the tyrosine-mediated hexamerization of TyrR (Wilson *et al.* 1994; Kwok *et al.* 1995) occurs in the upstream region of *tpl*, which may be facilitated by CRP-mediated DNA bending (Liu-Johnson *et al.* 1986; Dripps and Wartell 1987; Botsford and Harman 1992). Each TyrR dimer is shown in sector-form and is divided into subunits by a broken line. Both ATP and L-tyrosine (Tyr) bind to one mole per subunit (Argaet *et al.* 1994). Three TyrR binding sites are shown as outlined boxes denoted as Box 1, 2, and 3, respectively (see Results). CRP is shaded and is divided into subunits by a broken line. cAMP is known to bind to one mole per dimer as an active CRP(cAMP) form (Botsford and Harman 1992). The boxes in black represent two possible CRP binding sites. The DNA is shown as a thin line, but the broken line between Box 2 and Box 3 indicates that no specific structure is concerned.

Evaluating the modes of expressions of *tpl'-'lac* fusions as to catabolite repression revealed that the Box 1 mutation abolished the effect of CRP, while the Box 2 or 3 mutation did not. The catabolite repression mediated through CRP actually occurred in the wild-type strain (TK680) and even in the Box 2 or Box 3 mutant, however, when Box 1 was mutated, CRP did not give any effect on the expression of *tpl*.

Current understanding of the CRP function led the author to a possible mechanism for CRP-mediated *tpl* activation (Fig. 3-3). It is known that the binding of the CRP(cAMP) complex to its recognition site leads to DNA bending at an angle of 90-130 degrees (Liu-Johnson *et al.* 1986; Dripps and Wartell 1987; Botsford and Harman 1992). And Richet *et al.* showed, in their excellent works, that the DNA bending caused by the CRP(cAMP) complex is indispensable for MalT-mediated transpcriptional activation of the *malE-malK* promoter, in which CRP only participates to cause DNA bending, but not contact with the RNA polymerase (Richet *et al.* 1991; Richet and Sogaard-Andersen 1994; Richet 1996). In the upstream region of *tpl*, DNA bending may occur between Box 1 and Box 2 due to the binding of the CRP(cAMP) complex. And this bending will pull the TyrR-Box 1 complex to the downstream region, which will facilitate the contact among the three TyrR dimers bound to distant boxes and promote the configuration change to a hexamer. Only the model shown in Fig. 3-3 can effectively explain the

results obtained in this chapter. In the case of the Box 1 mutant, even though the CRP(cAMP) complex binds and causes the bending in that region, it has no meaning since the TyrR-Box 1 complex to be drawn to downstream does not exist. Therefore, the expression of *tpl* in the Box 1 mutant was not affected by the kind of carbon source used (Table 3-2), *i.e.* the CRP(cAMP) complex. The low ratio of tyrosine-induction depends on the Box 1-free TyrR hexamer, as mentioned above. In the Box 2 mutant, the bending caused by the CRP(cAMP) complex actually facilitates the contact of the TyrR dimers bound to Box 1 and Box 3, therefore an appropriate level of expression could be seen in the presence of glycerol as the carbon source (Table 3-2; Gly+Tyr). However, when glucose was used as a carbon source, the bending does not occur, so the most distal TyrR dimers bound to Box 1 and Box 3 can hardly gain access to each other. This is the reason for especially low expression value of the fusion in Box 2 mutant grown in the presence of glucose and tyrosine (Table 3-2; Glc+Tyr). In this chapter, the author constructed a hypothetical model for TyrR- and CRP-mediated activation of *tpl*. Although it may include many speculations, the data obtained so far assisted the hypothesis.

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SUMMARY

The gene for tyrosine-responsive positive regulator of *tpl* was cloned from *Erwinia herbicola* genomic library using *lac* reproter system in *Escherichia coli*. The DNA sequence and the predicted amino acid sequence revealed that it was *tyrR* of *E. herbicola*. The primary structure of *E. herbicola* TyrR showed relatively low similarity with the other bacterial TyrRs, while the others showed high similarities to each other.

To elucidate the mechanism of TyrR-mediated activation of *tpl*, a series of mutations were introduced in the upstream region. By examining the effects of those mutations on the expression of *tpl'-'lac* fusion in *E. coli*, the author proposed a hypothetical model for the transcriptional activation of *tpl*. It was suggested that the tyrosine-mediated configuration change of TyrR from dimer to hexamer, which might be facilitated by CRP-mediated DNA bending, was an important process for the activation of *tpl*.

Conclusions

The author studied the regulation of the expression of tyrosine phenol-lyase (TPL; EC 4.1.99.2) of *Erwinia herbicola*.

The findings in each chapter are summarized as follows.

Chapter 1.

The induction of TPL in the presence of tyrosine and the repression of it in the presence of glucose were mainly due to the transcriptional regulation of its gene. Increased amount of *tpl* mRNA in the presence of tyrosine and cAMP-dependent catabolite repression were observed. The 5'-end of *tpl* mRNA was determined using primer extension mapping and the upstream region was scrutinized. Three possible TyrR-binding sites and two possible cAMP receptor protein (CRP)-binding sites were found in the upstream region from the transcription start site.

Chapter 2.

The expression of *tpl'-'lac* fusion was regulated through both of the TyrR protein and cAMP receptor protein (CRP) in *Escherichia coli*. The *tyrR* mutation abolished the effect of CRP on the *tpl* regulation, while the *crp* mutation could not overcome the TyrR-mediated activation of *tpl*. The results

suggested that TyrR might play a direct role such as an interaction with RNA polymerase, while CRP might act indirectly as a second factor.

Upstream deletion analysis of the *tpl'-'lac* fusion indicated that the region located more than 300 b upstream from the transcription start site was indispensable for the tyrosine-mediated activation of *tpl*.

Chapter 3.

The gene for tyrosine-responsive positive regulator of *tpl* was cloned from *Erwinia herbicola* genomic library using *lac* reproter system. The DNA sequence and the predicted amino acid sequence revealed that it was *tyrR* of *E. herbicola*.

A hypothetical model for the transcriptional activation of *tpl* was proposed. It was suggested that the tyrosine-mediated configuration change of TyrR from a dimer to a hexamer, which might be facilitated by CRP-mediated DNA bending, was an important process for the transcriptional activation of *tpl*.

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