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Functional Analysis of the *Erwinia herbicola tutB* Gene and Its Product

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The *tutB* gene, which lies just downstream of *tpl*, has been cloned from *Erwinia herbicola*, and its product was analyzed. Despite its high sequence similarity to tryptophan transporters, TutB was found to be a tyrosine-specific transporter. Tryptophan acted as a competitive inhibitor of tyrosine transport. Unlike the tryptophanase operon, the *tpl* and *tutB* genes do not constitute an operon.

Tyrosine phenol-lyase (Tpl) is a tyrosine-inducible enzyme distributed in some enteric bacteria (8). Tpl catalyzes the α,β-elimination of L-tyrosine to produce pyruvate, ammonia, and phenol (16), thereby allowing bacteria to utilize L-tyrosine as carbon and nitrogen sources. One of the most notable features of Tpl is its ability to synthesize 3,4-dihydroxyphenylalanine (L-DOPA) from pyruvate, ammonia, and catechol through the reversal of α,β-elimination (15). L-DOPA is used in the treatment of Parkinson's disease, which afflicts 1 out of every 1,700 individuals.

During the course of study of *tpl*, an open reading frame, designated as *tutB*, was found just downstream of the *tpl* gene (9). It was predicted to encode a protein of 416 amino acids with many hydrophobic regions. Since the genetic organization of the *tpl* and *tutB* loci (Fig. 1A) is quite similar to that of the tryptophanase operon (*tna* consisting of *tnaA* [tryptophan indole-lyase] and *tnaB* [tryptophan-specific permease]) of *Escherichia coli* (7), and also since the physiological role of Tpl as to tyrosine is considered to be equivalent to that of TnaA as to tryptophan, the *tutB* gene was assumed to constitute an operon with *tpl* and to encode a tyrosine-specific transporter.

In this study, we cloned the *tutB* gene from *Erwinia herbicola* and determined the properties of its product by using *Escherichia coli* cells. In addition, to elucidate the role of the Tpl-TutB system in the tyrosine degradation pathway, *lac* fusions were constructed and their expression was monitored. The bacterial strains used in this study were derivatives of *E. coli* K-12 or *E. herbicola* AJ2985. The strains and plasmids are listed in Table 1 with their characteristics. Standard recombinant DNA procedures were used (23), and the results of in vitro manipulations such as PCR and mutagenesis were confirmed by DNA sequencing (24).

Cloning and sequencing of *tutB* of *E. herbicola*. An open reading frame situated 155 nucleotides downstream of the coding region of *tpl* has already been found and designated *tutB* although its function has not been elucidated. A plasmid, pEBT, obtained in our previous study (31) contains the *tpl* gene and a C-terminally truncated *tutB* gene. The intact *tutB* gene was cloned from an *E. herbicola* genomic library constructed as described previously (13), and the resulting plasmid was named pTK928. The 0.6-kb *Mfe*I-*Hpa*I fragment excised from pTK928 and the 3.3-kb *Sma*I-*Mfe*I fragment cut off from pEBT were annealed at their *Mfe*I-protruding ends, and the resulting 3.9-kb fragment containing the entire *tpl* and *tutB* genes in their native configurations was cloned into the *Sma*I site of pUC19 to generate pTK1016. The structure of the *tpl-tutB* gene is schematically shown in Fig. 1A. The DNA sequence of *tutB* was determined by the method described by Sanger et al. (24).

Computer analysis of the deduced amino acid sequence suggested that the product is a membrane protein. A homology search using FASTA (21) revealed that the TutB protein belongs to a family of aromatic amino acid transporters with 11 membrane-spanning segments (25, 26). A detailed discussion as to the similarity among them is presented later.

Transport studies. In a preliminary experiment, we observed that the introduction of pACYC177 carrying the *tutB* gene into an *E. coli* strain with the Φ(*tpl'-'lac*) gene (14) enhanced the tyrosine-induction ratio of *tpl* by twofold, but neither the phenylalanine- nor the tryptophan-mediated induction ratio was elevated. Since the expression of *tpl* is positively regulated by the TyrR protein (22) and its coeffectors (three aromatic amino acids), even though the magnitude of activation is quite different among the three ligands (13, 28), the above observation implied that the *tutB* gene might encode a tyrosine transporter. In order to characterize the *tutB* gene product, an *E. coli* strain deficient in the uptake of all aromatic amino acids (5) was constructed (MG1655 Δ*aroP mtr24* Δ*pheP* Δ*tna* Δ*tyrP*) and used for transport assays. AroP is a general aromatic transporter with high affinity for the three aromatic amino acids (22), whereas Mtr and TyrP are high-affinity and tryptophan- and tyrosine-specific transporters, respectively (10, 33). The *tnaB* gene, which constitutes the *tna* operon with *tnaA*, encodes a low-affinity, tryptophan-specific transporter (7). The PheP protein, which was initially described as a phenylalanine-specific transporter, has been recently shown to cause the accumulation of tyrosine in cells, although K_m for tyrosine was more than 10-fold higher than that for phenylalanine (5). The *aroP*, *pheP*, *tna*, and *tyrP* genes were disrupted by the method described by Datsenko and Wanner (6). The primers used (aroP-3, aroP-4, pheP-1, pheP-2, tna-1, tna-2, tyrP-3, and

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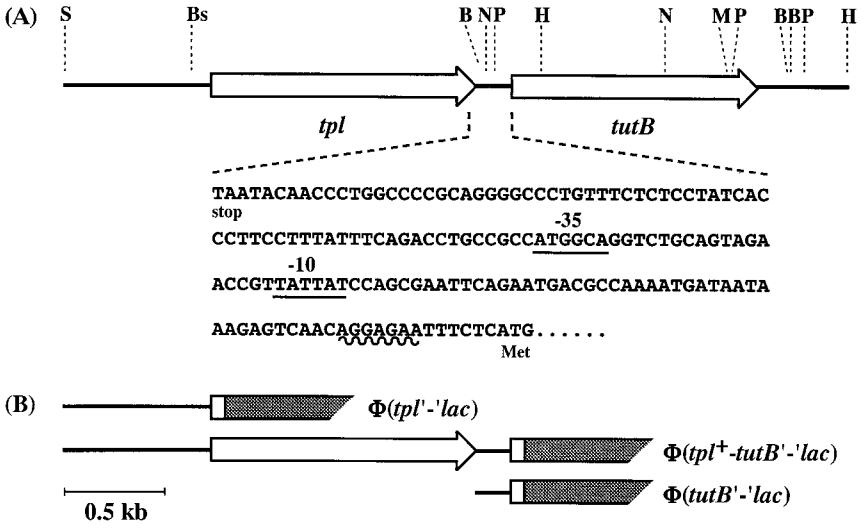

FIG. 1. Genetic organization of the *tpl-tutB* locus in *E. herbicola* (A) and structures of the *lac* fusions used in this study (B). The open reading frames corresponding to Tpl and TutB are depicted as arrows, and the restriction enzyme cleavage sites for *Ban*II (B), *Bss*HII (Bs), *Hpa*I (H), *Mfe*I (M), *Nco*I (N), *Pst*I (P), and *Sma*I (S) are also shown. The DNA sequence between the two open reading frames is shown (see text). Bar, 0.5 kb.

tyrP-4) are shown in Table 1. The *mtr24* transductant was selected based on resistance to 5-methyltryptophan (10, 11).

The transport assays were performed by the method described by Wookey et al. (33) with L-(U-^{14}C)-tyrosine (456 mCi/mmol, 50 μCi/ml) purchased from Amersham. The rate of nonspecific diffusion of tyrosine was determined using energy-starved cells that had been prepared by incubating cells in the presence of 50 μM carbonylcyanide-*m*-chlorophenylhydrazone (CCCP) for 30 min prior to starting the assays. The uptake of tyrosine was expressed as picomoles per milligram of dry cells as a function of time.

The *E. coli* Δ*aroP mtr24* Δ*pheP* Δ*tna* Δ*tyrP* strain, TK1135, was incapable of accumulating tyrosine (Fig. 2A). Strain TK1135 was transformed with either a low-copy-number (pTK974) or medium-copy-number (pTK1022) plasmid carrying the *tutB* gene and then examined for the ability to accumulate tyrosine. As shown in Fig. 2A, with an increasing copy number of *tutB* in the cells, the initial velocity of tyrosine uptake as well as the steady-state level of tyrosine in the cells increased. Under the conditions of 1 to 120 μM tyrosine in the assay mixture, K_m for tyrosine in the TutB system was determined to be 38 μM, which is about 10- and 100-fold higher than the K_ms for tyrosine in the TyrP and AroP systems (22, 33), respectively, but comparable to K_m for tryptophan in the TnaB system (70 μM) (1) and K_m for tyrosine in the PheP system (30 μM) (5).

The addition of 1 mM sodium ions did not enhance the uptake of tyrosine, and a 30-s preincubation of the cells in the presence of 50 μM CCCP completely abolished the ability to accumulate tyrosine (data not shown). Figure 2B shows the rapid expulsion of accumulated tyrosine from the cells upon the addition of CCCP. These results indicated that the active transport of tyrosine by the TutB system depends on the proton-motive force.

The rate of tyrosine uptake at the saturating concentration (100 μM) was not affected by the addition of a 20-fold molar excess of L-phenylalanine (data not shown), but it decreased in the presence of L-tryptophan in a concentration-dependent manner (Fig. 2D). We examined the ability of TutB-expressing TK1135 cells to accumulate labeled tryptophan (58.1 mCi/mmol, 0.02 mCi/ml; NEN Life Science Products, Inc.); however, no accumulation was observed within the tested range (10 μM to 1 mM). A double-reciprocal plot of the values in Fig. 2D was found to well-fit the case of competitive inhibition, in which the *K[infi]i* value for tryptophan was determined to be 300 μM. As an alternative approach for examining whether the TutB protein transports tryptophan or not, we used generalized transduction involving a P1 phage lysate prepared on an *E. coli trp* mutant. If the TutB protein transports tryptophan, the presence of a plasmid carrying *tutB* in aromatic transport-negative strain TK1135 should allow us to obtain *trp* transductants of the cells on the minimal medium supplemented with tryptophan. However, this approach did not work well because such transductants were easily obtained when strain TK1135 itself was infected with a P1 phage prepared on a *trp* mutant. Since the amount of labeled tryptophan that accumulated in the aromatic amino acid transport-negative cells (TK1135) was exactly the same as that accumulated in energy-starved cells (data not shown), the above finding implies that the nonspecific diffusion of tryptophan can support the growth of a tryptophan-auxotroph strain. In the minimal medium supplemented with tryptophan, no difference in the growth rate was observed between TK1135 with the *trp* mutation and the strain carrying the *tutB* gene on a plasmid (data not shown). On the basis of these results, we concluded that the TutB protein is a tyrosine-specific transporter and that its activity is inhibited by a competitive inhibitor, tryptophan.

Determination of translation start codon of *tutB*. Before constructing the Φ(*tutB'-'lac*) gene, the translation start site was determined. Multiple alignment of the deduced amino acid sequences of TutB and other transporters in the same family (Fig. 3) suggested that the N-terminal amino acid of

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

Strain, plasmid, or oligonucleotide	Characteristic(s)[a] or sequence	Source or reference
Strains		
E. herbicola		
AJ2985	Wild type	Laboratory stock
TK1047	Δ(*tpl-tutB*)::*kan*$^+$	This study
E. coli K-12		
CJ236	pCJ105 [F′ *cat*$^+$], *dut-1 ung-1 thi-1 relA1*	12
CSH26	F$^-$ *ara* Δ(*lac-pro*) *thi*	19
KY4124	HfrH *mtr24 met82 relA1 rpsL140*	11
MG1655	λ$^-$ *rph-1*	Laboratory stock
TK596	CSH26 Δ*tyrR*::*kan*$^+$ Δ(*srl-recA*)*306*::Tn*10*	13
TK1135	MG1655 Δ*aroP mtr24* Δ*pheP* Δ*tna* Δ*tyrP*	This study
Plasmids		
pACYC177	p15A replicon *bla*$^+$ *kan*$^+$	2
pCP20	pSC101 replicon (Ts) *bla*$^+$ *cat*$^+$ Flp(λR*p*) *c*I857	4
pEBT	ColE1 replicon *bla*$^+$ Δ*lacZ*α::*tpl*$^+$-*tutB*′	31
pKD46	*oriR101 repA101*(Ts) *bla*$^+$ *araC*$^+$ *gam*$^+$-*bet*$^+$-*exo*$^+$ (*araBp*)	6
pMBO131	Mini-F replicon *cat*$^+$	20
pRS552	ColE1 replicon *bla*$^+$ *kan*$^+$ *rrnBT1 'lac*	27
pTK#-20	ColE1 replicon *bla*$^+$ *tyrR*$_{E.\ herbicola}$$^+$	13
pTK631	pSC101 replicon *tet*$^+$	13
pTK913	Mini-F replicon *cat*$^+$ *tpl*$^+$ *tyrR*$_{E.\ herbicola}$$^+$; 2.2-kb *Sma*I-*Pst*I (blunt-ended) fragment of pEBT and 2.1-kb *Aat*II (blunt-ended)-*Ssp*I fragment of pTK#-20 were inserted into *Sal*I (end-filled) site and *Xho*I (end-filled) site of pMBO131, respectively	This study
pTK928	ColE1 replicon *rop*$^+$ *bla*$^+$ *tutB*$^+$	This study
pTK932	Mini-F replicon *cat*$^+$ *tpl*$^+$-*tutB*$^+$; 4.0-kb *Sma*I-*Sph*I (blunt-ended) fragment of pTK1016 was inserted into *Sal*I (end-filled) site of pMBO131	This study
pTK935	Mini-F replicon *cat*$^+$ *tpl*$^+$-*tutB*$^+$ *tyrR*$_{E.\ herbicola}$$^+$; 4.0-kb *Sma*I-*Sph*I (blunt-ended) fragment of pTK1016 and 2.1-kb *Aat*II (blunt-ended)-*Ssp*I fragment of pTK#-20 were inserted into *Sal*I (end-filled) site and *Xho*I (end-filled) site of pMBO131, respectively	This study
pTK967	ColE1 replicon *bla*$^+$ *lacZ*α::Δ(*tpl-tutB*)::*kan*$^+$; 3.9-kb *Bss*HII (blunt-ended)-*Mfe*I (blunt-ended) fragment of pTK1016 was ligated with 1.3-kb *Eco*RI (end-filled) fragment of pUC4K	This study
pTK974	pSC101 replicon *tet*$^+$ *tutB*$^+$; 1.6-kb *Ban*II (blunt-ended) fragment of pTK1016 was inserted into *Pvu*II site of pTK631	This study
pTK1005	pSC101 replicon *bla*$^+$ *rrnBT1* Φ(*tpl*′-′*lac*)	14
pTK1016	ColE1 replicon *bla*$^+$ *lacZ*α::*tpl*$^+$-*tutB*$^+$	This study
pTK1022	p15A replicon *kan*$^+$ *tutB*$^+$; 1.6-kb *Ban*II (blunt-ended) fragment of pTK1016 was inserted into *Sca*I site of pACYA177	This study
pTK1031	pSC101 replicon *bla*$^+$ *rrnBT1* Φ(*tpl*$^+$-*tutB*′-′*lac*)	This study
pTK1032	pSC101 replicon *bla*$^+$ *rrnBT1* Φ(*tutB*′-′*lac*)	This study
pTK1061	p15A replicon *kan*$^+$ *tutB*(ATG to CTG)	This study
pTK1143	Mini-F replicon *cat*$^+$ *tpl*$^+$-*tutB*(ATG to CTG); 0.8-kb *Nco*I fragment of pTK932 was replaced with corresponding region excised from pTK1061.	This study
pTK1144	pSC101 replicon *bla*$^+$ *rrnBT1 'lac*	This study
pTZ19R	ColE1 replicon flori *bla*$^+$ *lacZ*α$^+$	Pharmacia
pUC19	ColE1 replicon *bla*$^+$ *lacZ*α$^+$	34
pUC4K	ColE1 replicon *bla*$^+$ *lacZ*α::*kan*$^+$	Pharmacia
Oligonucleotides		
aroP-3	5′-ACGAGGTTTCATGATGGAAGGTCAACAGCACGGCGAGCAGGTGTAGGCTGGAGCTGCTTC-3′ (for disruption of the *aroP* gene; upstream end)	
aroP-4	5′-CGCCAGGCTAATCATCGCCCAGTTGATTACCAGTGCAGATATTCCGGGGATCCGTCGACC-3′ (for disruption of the *aroP* gene; downstream end)	
pheP-1	5′-CAACAAAAAAGACACACAGGGGAAAGGCGTGAAAAACGCGGTGTAGGCTGGAGCTGCTTC-3′ (for disruption of the *pheP* gene; upstream end)	
pheP-2	5′-AGATAGTTGATTAACACCACCAGCGAAGTGATCGCTCCGGATTCCGGGGATCCGTCGACC-3′ (for disruption of the *pheP* gene; downstream end)	
tna-1	5′-GTTATTCCTCAACCCTTTTTTTAAACATTAAAATTCTTACGTGTAGGCTGGAGCTGCTTC-3′ (for disruption of the *tna* operon; upstream end)	
tna-2	5′-CGGGAAGATCAGATACAACAACGCAGGTGGCAGGAAGGTTATTCCGGGGATCCGTCGACC-3′ (for disruption of the *tna* operon; downstream end)	
tyrP-3	5′-CGTCAGGACAGAAGAAAGCGTGAAAAACAGAACCCTGGGAGTGTAGGCTGGAGCTGCTTC-3-′ (for disruption of the *tyrP* gene; upstream end)	
tyrP-4	5′-CAGAGAAACACCACCACCAGCGCCGGACGACCACCTTTGAATTCCGGGGATCCGTCGACC-3′ (for disruption of the *tyrP* gene; downstream end)	
tutB-F	5′-ACGTCTCACTATTCCACGCCG-3′ [for construction of the Φ(*tutB*′-′*lac*) gene; upstream end]	
tutB-N	5′-TCTTAATAACCAGGAGAAATTCTCC-3′ (for site-directed mutagenesis of *tutB*)	
tutB-R	5′-CCGCATGCGGATCCATGAGAAATTCTCCTGTTGAC-3′ [for construction of the Φ(*tutB*′-′*lac*) gene, downstream end]	

[a] The *tyrR*$_{E.\ herbicola}$, *tpl*, and *tutB* genes originated from *E. herbicola*.

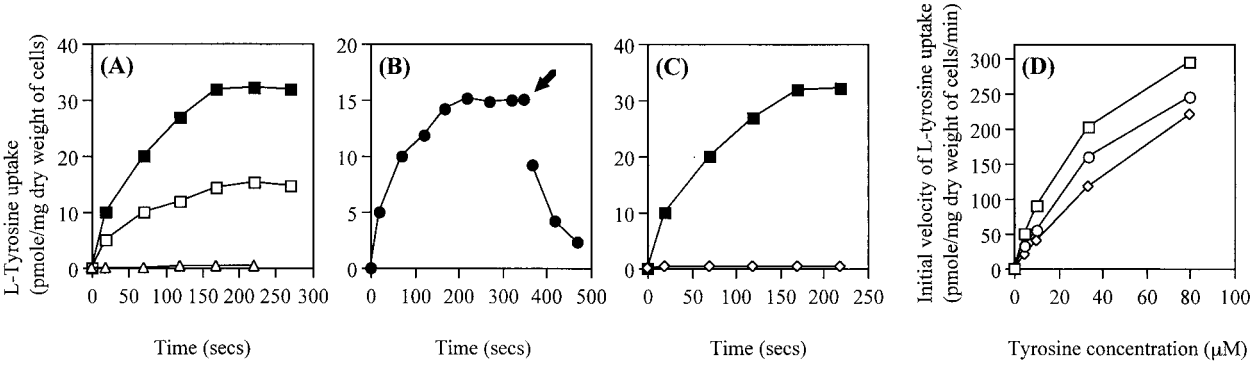

FIG. 2. Uptake studies with TutB-expressing *E. coli* cells. (A) The gene dosage effect on the accumulation of L-(U-^{14}C)-tyrosine in cells. An aromatic amino acid transport-negative strain, TK1135 (△), was transformed with either a low-copy-number plasmid (pTK974 [□]) or a medium-copy-number plasmid (pTK1022 [■]) carrying the *E. herbicola tutB* gene. Cell suspensions were incubated in the presence of 1 μM labeled tyrosine and samples were withdrawn at the indicated times. (B) Uptake and efflux of tyrosine. Strain TK1135 with pTK974 was used for assaying in the presence of 1 μM labeled tyrosine. At the time indicated by the arrow (350 s), CCCP was added to the final concentration of 50 μM. Samples were withdrawn at the indicated times (●). (C) Inability of the strain carrying the mutant *tutB* (ATG to CTG) allele to accumulate tyrosine. The assay was carried out in the presence of 1 μM labeled tyrosine and at the times indicated samples were removed. The accumulation of tyrosine in the cells carrying the mutant allele (pTK1061 [◇]) was compared to that in the cells carrying the wild-type allele (pTK1022 [■]). (D) Inhibition of tyrosine transport by tryptophan. Strain TK1135 transformed with pTK974 was used for assaying. The tyrosine concentration was varied from 5 to 80 μM in the absence (□) or presence (○) of 0.2 mM or 0.5 mM (◇) cold tryptophan. All experiments were repeated twice with essentially the same results, and data for a representative experiment being shown.

TutB is methionine followed by valine, isoleucine, and two consecutive lysine residues. To verify this assumption, site-directed mutagenesis, in which ATG for this methionine was replaced with CTG, was carried out by the method of Kunkel et al. (17). tutB-N in Table 1 was used as a mutagenic primer. The aromatic amino acid transport-negative *E. coli* strain, TK1135, transformed with pACYC177 carrying either the wild-type (pTK1022) or mutant (pTK1061) *tutB* allele, was subjected to the transport assay. As shown in Fig. 2C, the cells carrying the mutant allele were incapable of accumulating tyrosine, demonstrating that this methionine is an actual initiation codon. A possible ribosome-

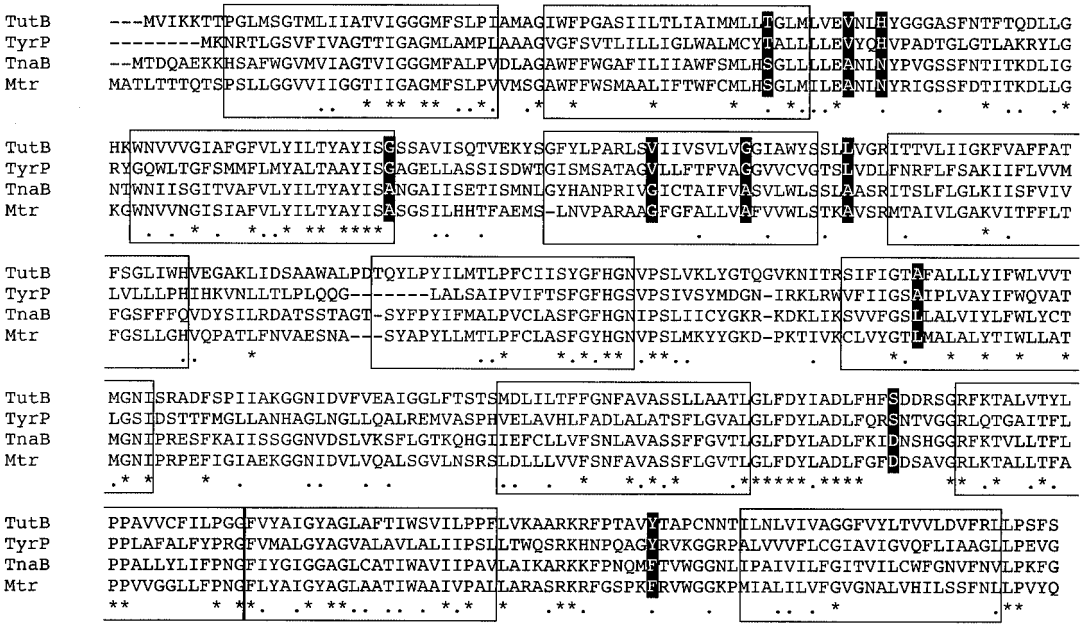

FIG. 3. Comparison of amino acid sequences of TutB and *E. coli* aromatic amino acid transporters in the same family. TyrP, a high-affinity, tyrosine-specific transporter (33); TnaB, a low-affinity, tryptophan-specific transporter of the *tna* operon (26); and Mtr, a high-affinity, tryptophan-specific transporter (10). Alignment was constructed with the use of the ClustalW 1.6 program (32), and identical residues (∗) and conservative amino acid changes (•) in the four sequences are shown. The amino acid residues distinctively conserved in the tyrosine or tryptophan transporters at the same positions on alignment are highlighted. Eleven membrane-spanning regions were deduced according to the model proposed for Mtr by Sarsero and Pittard (25), and are enclosed by boxes.

binding site was found 6 nucleotides upstream, which is indicated by a wavy line in Fig. 1A.

Structural comparison of TutB with other aromatic amino acid transporters. One unexpected finding was that, when the primary structure of TutB was compared to those of other transporters in the same family, the TutB protein showed higher similarity to tryptophan transporters (Mtr and TnaB) (7, 10) than a tyrosine transporter (TyrP) (33). The TutB protein exhibited 48 and 46% identity with Mtr and TnaB in amino acid sequence, respectively; however, it showed only 31% identity with TyrP. The fact that the TutB protein is capable of recognizing both tryptophan and tyrosine but is active only in tyrosine transport might indicate that this protein should be a powerful tool for investigating the molecular mechanism underlying the active transport of aromatic amino acids.

The membrane topology of the TutB protein was deduced from the model proposed for the Mtr protein (25), the 11 membrane-spanning segments enclosed by boxes in Fig. 3. Scrutiny of the four sequences revealed residues distinctively conserved in the tyrosine- or tryptophan-specific transporters at the same positions on alignment, which are highlighted in Fig. 3. Some of these residues were predicted to exist in loop regions, but this is not so surprising because, as has been reported previously, in the case of AroP-PheP chimeras, replacement of a cytoplasmic loop significantly influenced their transport activity (5).

Expression of the Φ(*tpl′-′lac*), Φ(*tpl*$^+$*-tutB′-′lac*), and Φ(*tutB′-′lac*) genes in *E. coli*. For a better understanding of the role of the *tpl-tutB* gene in the tyrosine degradation pathway, we constructed the Φ(*tpl′-′lac*), Φ(*tpl*$^+$*-tutB′-′lac*), and Φ(*tutB′-′lac*) translation fusions (Fig. 1B) and monitored their expression in *E. coli* cells. Construction of the Φ(*tpl′-′lac*) gene was described elsewhere (14), and the Φ(*tutB′-′lac*) gene was created by connecting the initiation codon of the *tutB* gene with the seventh codon of the *′lac* gene on pRS552 (27). The primers used (tutB-F and tutB-R) are shown in Table 1. A plasmid, pTK1144, carrying the truncated *′lac* gene was also constructed and used as a control for β-galactosidase assaying. An *E. coli* strain, TK596, was transformed with two compatible plasmids; one was a low-copy-number plasmid containing one of the *lac* reporter genes (pTK1005, pTK1031, pTK1032, or pTK1144) and the other was a single-copy plasmid carrying either the *tpl* or *tpl-tutB* gene (pTK913 or pTK935). Cells were grown in M63-glucose minimal medium (19) in the absence and presence of 1 mM L-tyrosine, and then subjected to the β-galactosidase assay (19). The results are presented in Table 2.

As for the Φ(*tpl′-′lac*) gene, its expression was increased by 7-fold upon the addition of tyrosine in the absence of the *tutB* gene (460 to 3,300) and by 10-fold in the presence of the *tutB* gene (440 to 4,400). Under tyrosine-induced conditions, the β-galactosidase activity of the cells carrying *tutB* was 1.3-fold as high as that of the cells not carrying *tutB* (4,400 against 3,300). The mechanism underlying TyrR-mediated tyrosine induction of *tpl* has been well established (14, 28). These results indicated that introduction of the TutB system actually increased the intracellular level of tyrosine. It is known that the expression of two major tyrosine transporters, AroP and TyrP, is severely repressed by tyrosine (22); however, both systems played significant roles in the accumulation of tyrosine in the cells grown in the medium supplemented with tyrosine, which resulted in a sevenfold induction of *tpl* in the absence of *tutB*. In our assay system, AroP-expressing *E. coli* cells (MG1655 *mtr24* Δ*pheP* Δ*tna* Δ*tyrP*) grown in the presence of 1 mM tyrosine accumulated as much tyrosine as was observed in TutB-expressing cells (pMBO131 carrying *tutB*/TK1135) grown under the same conditions (data not shown), which may account for the relatively low effect of *tutB* on the tyrosine-induction of *tpl*.

TABLE 2. Specific β-galactosidase activity expressed from the Φ(*tpl′-′lac*), Φ(*tpl*$^+$*-tutB′-′lac*), and Φ(*tutB′-′lac*) genes in *E. coli* cells grown under tyrosine-induced or noninduced conditions

Fusion[a]	Relevant genotype[b]	Sp act (Miller units) of β-galactosidase of various *lac* fusions in *E. coli* cells grown in[c]:	
		MM	MM + tyrosine
-′lac	*tpl*$^+$*-tutB*$^+$	<0.1	<0.1
Φ(*tpl′-′lac*)	*tpl*$^+$	460	3,300
	tpl$^+$*-tutB*$^+$	440	4,400
Φ(*tpl*$^+$*-tutB′-′lac*)	*tpl*$^+$	1.1	1.0
	tpl$^+$*-tutB*$^+$	1.1	1.1
Φ(*tutB′-′lac*)	*tpl*$^+$	1.2	1.2
	tpl$^+$*-tutB*$^+$	1.3	1.2

[a] The structures of the fusions present on low-copy-number plasmids are shown in Fig. 1B. The *-′lac* gene was used as a control.

[b] An *E. coli* strain (TK596) carrying one of the fusions was transformed with either pTK913 (mini-F replicon *cat*$^+$ *tpl*$^+$ *tyrR*$_{E.\ herbicola}$$^+$) or pTK935 (mini-F replicon *cat*$^+$ *tpl*$^+$*-tutB*$^+$ *tyrR*$_{E.\ herbicola}$$^+$).

[c] *E. coli* cells were grown in M63-glucose minimal medium (MM) or MM supplemented with 1 mM tyrosine and then subjected to the β-galactosidase assay (19). Assays were performed in duplicate for three separate cultures, and the values obtained showed an error of less than 5%.

The β-galactosidase activity of the strain carrying the Φ(*tpl*$^+$*-tutB′-′lac*) gene was extremely low but significant compared to that of the strain carrying the *′lac* gene (control), indicating that the *tutB* gene is actually expressed in *E. coli* cells. The expression of *tutB* was unaffected by the addition of tyrosine to the medium. Changing the carbon source from glucose to glycerol had no effect on expression (data not shown). Introduction of the *tutB* gene did not have any effect on the expression of the fusion either. The low-level expression of *tutB* was not due to the lowered concentration of tyrosine in the cells on the introduction of extra copies of the *tpl* (tyrosine-degrading enzyme) gene because introduction of pUC19 carrying *tpl* into the cells had only a partial effect on the tyrosine induction of the Φ(*tpl′-′lac*) gene (data not shown). β-Galactosidase activity as well as the mode of expression of the Φ(*tutB′-′lac*) gene were essentially the same as those in the case of the Φ(*tpl*$^+$*-tutB′-′lac*) gene. These results demonstrate that the expression of *tutB* is very low and constant, and unlike the *tna* operon of *E. coli* (7, 29), the *tpl* and *tutB* genes are transcribed separately. This notion was supported by the results of Northern hybridization analysis (23), with total RNA extracted from *E. herbicola* cells grown under tyrosine-induced conditions, where an intense signal corresponding to 1.6 kb in length was observed when the *tpl*-specific probe was used (30), but no distinct band was observed when the *tutB*-specific probe was used (data not shown). The DNA sequence between the stop codon of *tpl* and the start codon of *tutB* is shown in Fig.

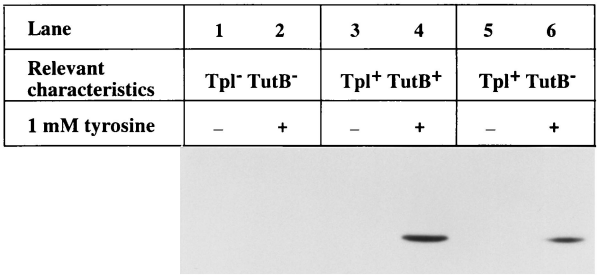

FIG. 4. The Tpl contents of various *E. herbicola* strains. *E. herbicola* Δ(*tpl-tutB*)::*kan*$^+$ cells transformed with pMBO131 (lanes 1 and 2) or pMBO131 carrying either the *tpl-tutB* gene (pTK932, lanes 3 and 4) or the *tpl-tutB*(ATG to CTG) gene (pTK1143, lanes 5 and 6) were grown in M63 minimal medium containing 0.02% yeast extract in the presence and absence of 1 mM tyrosine. When OD_{600} reached 0.5, the cells were harvested from 1 ml of culture and then suspended in 100 μl of cracking buffer (13). After boiling for 5 min, 10 μl of each whole-cell extract was applied and separated on a sodium dodecyl sulfate–12.5% polyacrylamide gel (18). Immunoblotting with anti-Tpl antibodies was performed as described previously (13). The image on X-ray film was analyzed with a Fujifilm ImageGauge program, and the densitometric values were estimated within the linear range.

1A, and a possible promoter of the *tutB* gene is indicated by underlining.

These results revealed that in contrast to the physiological equivalence of Tpl-TutB and TnaA-TnaB, both of which generate pyruvate (carbon source) and ammonia (nitrogen source) from aromatic amino acids, the mode of expression was quite different between them. At present, we do not have a clear answer as to why the expression level of *tutB* remained extremely low even in the presence of tyrosine. However, too much accumulation and degradation of tyrosine via the Tpl-TutB system may be unfavorable for cells because of the toxic effect of phenol liberated from tyrosine.

Effect of TutB on expression of Tpl in *E. herbicola*. The chromosomal *tpl-tutB* locus in *E. herbiocola* was deleted and replaced with the kanamycin resistance gene (*kan*$^+$) by means of a homologous recombination event using linearized pTK967, and the resulting Δ(*tpl-tutB*)::*kan*$^+$ strain was transformed with a mini-F-derived plasmid (pMBO131) or pMBO131 carrying either the *tpl-tutB* gene (pTK932) or the *tpl-tutB*(ATG to CTG) gene (pTK1143). It has been shown that the F plasmid is stably maintained in *Erwinia* species (3). The Tpl contents of the cells grown under tyrosine-induced and non-induced conditions were determined by immunoblotting (13), and the results are presented in Fig. 4. Apparently, the induction of Tpl by tyrosine was observed regardless of the presence or absence of the active TutB system (lanes 3, 4, 5, and 6); however, the presence of the functional *tutB* gene actually enhanced the tyrosine-induction ratio of Tpl (twofold; lane 4 versus lane 6). These results indicated that, as in the case of the *lac* reporter assay results for *E. coli* (Table 2), the TutB system is necessary for full induction of Tpl and also revealed the existence of other tyrosine transport system(s) in *E. herbicola*.

In conclusion, we cloned the *tutB* gene from *E. herbicola* and analyzed its product in *E. coli* cells. Although the amino acid sequence of TutB exhibited significant similarity to those of tryptophan transporters, it specifically transported L-tyrosine. Tryptophan acted as a competitive inhibitor of tyrosine transport. Unlike the *tna* operon, the *tpl* and *tutB* genes did not constitute an operon.

Nucleotide sequence accession number. The DNA sequence of *tutB* was deposited in GenBank under accession number AF418598.

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