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A bacterial basic region leucine zipper histidine kinase regulating toluene degradation

(two-component regulatory system/signal transduction/aromatic hydrocarbons/*Pseudomonas*)

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ABSTRACT **The two-component signal transduction pathways in bacteria use a histidine–aspartate phosphorelay circuit to mediate cellular changes in response to environmental stimuli. Here we describe a novel two-component *todST* system, which activates expression of the toluene degradation (*tod*) pathway in *Pseudomonas putida* F1. The *todS* gene is predicted to encode a sensory hybrid kinase with two unique properties—a basic region leucine zipper dimerization motif at the N terminus and a duplicated histidine kinase motif. Evidence from a synthetic peptide model suggests that TodS binds as a dimer to a pseudopalindromic sequence (5′-TGACTCA), which resembles the recognition sequence of the eukaryotic transcription factors Fos and Jun. These results provide additional evidence that bacteria and eukaryotes share common regulatory motifs. The *todT* gene product, a response regulator, was overproduced as a fusion protein in *Escherichia coli*, and the purified protein was found to bind specifically to a 6-bp palindromic DNA structure in the *tod* control region. The phosphorylated form of TodT appears to be the activator of *tod* structural genes. This is the first report of a two-component system that regulates aromatic metabolism in bacteria.**

The *to*luene *d*egradation (*tod*) pathway in *Pseudomonas putida* F1 (*Pp*F1) is a paradigm of bacterial metabolism of aromatic hydrocarbons, which include the intractable polychlorinated biphenyls and some polynuclear aromatics, whereby ring fission is initiated through the formation of a "dihydrodiol" intermediate (1). The *tod* pathway is distinct from four other aerobic toluene-degrading routes of which regulation of the respective pathway falls into one of three existing families of aromatic catabolic regulators: the LysR transcriptional regulators, the σ^{54}-dependent NtrC transcriptional activators, and the AraC/XylS activators (2). Although much is known about the biochemical steps and enzymology of the *tod* system there has been a paucity of information on its regulation (1, 3).

The two-component signal transduction systems are the major routes bacteria use to detect environmental signals that mediate changes in cellular behavior or biological processes (4, 5). These systems consist typically of two proteins—a sensory histidine kinase and a response regulator. In the general model, signal perception by the sensor, either membrane-bound or cytoplasmic, promotes autophosphorylation of a conserved histidine residue usually situated at the C terminus of the protein. The phosphate is then transferred from histidine to a conserved aspartate residue, usually found at the N terminus of the cognate response regulator. The phosphorylated response regulator subsequently mediates control at the transcriptional level by binding to a target DNA site(s). To date, several subtypes of the sensory component exist; a common variant are those known as "hybrid kinases," which have both a histidine-kinase domain and a response-regulator domain in the same protein (for review see ref. 6). In addition, many of the hybrid kinases have a separate (extrinsic) response regulator. An example is the ArcAB system of *Escherichia coli*, which responds to osmolarity and redox changes. The kinase (ArcB) autophosphorylates at both the conserved histidine in the kinase domain and aspartate in the intrinsic response regulator domain. Phosphorylation of the extrinsic response regulator (ArcA) occurs from the conserved histidine and is dependent on phosphorylation of the ArcB intrinsic-response domain (7).

In this study we examined the *Pp*F1 chromosomal region downstream of *todH,* the last gene of the *tod* operon (Fig. 1). Two regulatory genes, designated *todS* and *todT,* were cloned and their functional properties examined. These genes comprise a two-component system that provides a new and interesting insight into the regulation and activation of bacterial aromatic degradative pathways and an unique example of a basic region leucine zipper (bZIP) histidine kinase.

MATERIALS AND METHODS

Localization and Inactivation of *todST* Genes. Plasmid pDTG552 (12) was used as a probe to obtain three subsequent overlapping clones, pPF1 (2.7-kb *Pst*I fragment), pPF10 (2.4-kb *Eco*RI–*Xba*I), and pPF14 (3.1-kb *Sal*I–*Hin*dIII fragment), which encompass the *todS* and *todT* genes (Fig. 1). The vectors for the three plasmids are pK194 (13), pUC18, and pUC13 (14), respectively. Strain *Pp*F1(*todS*::Km^R) was constructed by first inserting a 2.2-kb *Sal*I–*Xho*I fragment containing the kanamycin resistance (Km^R) gene from Tn*5* (15) into the *Sal*I site of pPF1. An ≈0.3-kb *Sal*I–*Sma*I DNA fragment containing the origin of transfer (*oriT*) of plasmid RK2 was inserted into the *Bam*HI site of pPF1, which was then introduced into *Pp*F1 by conjugation (9). Of 50 transconjugants analyzed, about one-half were the result of double crossovers. The double crossovers were detected by loss of the plasmid-encoded Ap^R and this result was subsequently supported by Southern hybridization (not shown). For inactivation of *todT*, a Km^R cassette from pUC4K (Pharmacia) was inserted into the unique *Eco*RV site in *todT* gene in pPF14. The DNA was then introduced into *Pp*F1 by conjugation. Transconjugants were designated *Pp*F1(*todT*::Km^R). A subclone of *todT* as a 0.66-kb PCR fragment inserted at the *Hin*cII site in pUC8 was named pUCtodT.

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Abbreviations: bZIP, basic region leucine zipper; Km^r, kanamycin resistance; GST, glutathione *S*-transferase; ORF, open reading frame.
Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. U72354).

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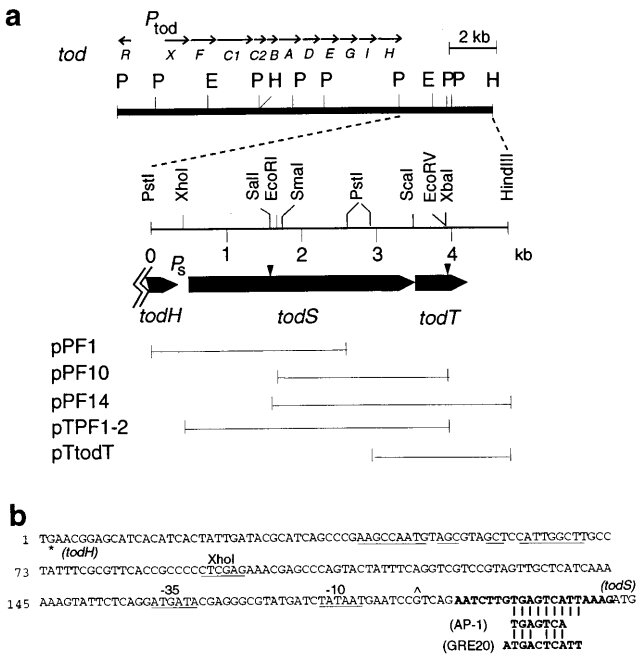

FIG. 1. (*a*) Localization of *todS* and *todT* genes in *Pp*F1 chromosome. The *todXFC1C2BADEGIH* operon encodes a membrane protein (TodX) and seven enzymes (TodABC1C2, TodD–TodI) for the total conversion of toluene to pyruvate and acetyl-CoA (8, 9). *todR* is a truncated LysR-type protein and has no apparent regulatory role in *tod* expression (9). E, *Eco*RI; P, *Pst*I; H, *Hin*dIII. P_{tod} and P_s are promoter sites. Sites of kanamycin (Km) resistance gene disruptions are indicated (arrowheads). Plasmid derivatives in the *todST* region are described in the text. (*b*) Characteristics of the *todHS* noncoding region. *, stop codon of *todH.* An inverted repeat sequence is underlined. Promoter elements resembling the *E. coli* −10 (TATAAT) and −35 (TTGACA) sequences, and *Pseudomonas rpoD*-like consensus sequences are underlined. A transcriptional start site (^) was determined in *E. coli* by primer extension analysis (data not shown). The 20-mer sequence (in boldface type) in its double-stranded form was used in the gel retardation assays as described in Fig. 5. The AP-1-binding sequence of Jun/Fos (10) and yeast GCN4 transcription factors (GRE20; ref. 11) are provided for comparison.

Overexpression of TodT and DNA Binding. TodT was overproduced as a fusion protein using a commercial glutathione *S*-transferase (GST) system. A 630-bp *Nde*I–*Hin*dIII fragment containing *todT* in pUCtodT was gel purified and inserted into pGEX-4T-3 (Pharmacia) at the *Bam*HI site by blunt-end ligation. The recombinant plasmid, which produced an in-frame TodT fusion to GST was designated pGSTtodT. A 49-kDa fusion protein was produced in *E. coli* XL-1 Blue cells induced with 0.3 mM isopropyl-β-D-thiogalactopyranoside for 3 h. The fusion protein was purified according to the manufacturer's instructions.

DNA binding was carried out as follows: primers merRX (5′-GAGCACGCACAGTATTAC) and merRP (5′-CATCGACATCAGGTTATC) were used to amplify the intergenic region of *todRX* with pRX2 (9) as the template. The gel-purified 253-bp PCR fragment was mixed in a 5:1 ratio with a 196-bp PCR DNA containing the *todHS* intergenic region (Fig. 1). Each tube containing 1 μg of DNA and varying amounts of protein (0, 1, or 3 μg) in 15 μl of binding buffer (20 mM Tris·HCl, pH 7.4/2 mM $MgCl_2$/2 mM EDTA/10 mM KCl/0.1% Nonidet P-40) was incubated on ice for 20 min. Three microliters of gel-loading buffer was added and the products separated on a 4.5% polyacrylamide gel in TAE buffer (40 mM Tris·acetate/1 mM EDTA, pH 8.0) at 12°C (see Fig. 4*a*).

DNase I Footprinting. Footprinting of TodT to the *tod* promoter was carried out by the DNase I protection assay using the 253-bp PCR template as described, except that the primer merRX was labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase. The probe was precipitated with 20 μg of sonicated herring sperm DNA as a carrier, and dissolved in 70 μl of TE buffer (10 mM Tris·HCl, pH 8.0/1 mM EDTA). Two micrograms of the labeled DNA was incubated with varying amounts of the GST–TodT protein for 20 min on ice in 40 μl of binding buffer. DNase I (0.1 unit) and $CaCl_2$ (2.5 mM) were added to the mixture and incubation was carried out on ice for 100 s. The reaction was terminated by adding 25 μl of stop solution containing 1% SDS, 125 mM NaCl, 25 mM EDTA, and 25 μg/ml tRNA. The mixture was extracted with phenol chloroform, followed by precipitation with ethanol. The pellet was dissolved in 14 μl of loading dye solution (80% formamide/1 mM EDTA/0.1% bromophenol blue/0.1% xylene cyanol). After heating at 90°C for 3 min, the samples were separated by electrophoresis on a 7% denaturing polyacrylamide gel containing 8 M urea along with a DNA sequence ladder generated with the labeled merRX as primer and plasmid pRX2 as template.

Site-Specific Mutagenesis of *todT*. pGSTtodT(D56N) was generated by mutagenesis of the Asp-56 codon of *todT* in pGSTtodT. Two complementary mutagenic primers (A1, 5′-TACGGACATTCAAAATTAGAC and A2, 5′-GTCTAATTTTGAATGTCCGTA) and two flanking primers (B, 5′-GGCTCCGGCATATGAGTGATCGG and C, 5′-GCCATGAAGAGCTCCGACTATTCCAGG) were synthesized. The underlined base is the mutation designed to give N56 (AAT) instead of D56 (GAT) in the *todT* sequence. Two PCR reactions using pGSTtodT as template were carried out separately with primers A1 and B and A2 and C by conventional methods using *Pfu* DNA polymerase. The two PCR products were purified from an agarose gel. The products were mixed and subjected to PCR for 2 cycles followed by 35 cycles after addition of the 2 flanking primers B and C. The amplified DNA was digested with *Nhe*I and *Afl*II and cloned into *Nhe*I–*Afl*II-digested pGSTtodT. The sequence of the cloned fragment and site of mutation was confirmed by DNA sequencing.

Synthesis of TodSbs Peptide. A 45-residue peptide, TodSbs, (MSSLDRKKPQNRSKNNYYNISLKEKGSEE-LTSEEHARIIFDGGGC)NH_2, was synthesized on an Applied Biosystems model 430A synthesizer and was cleaved from the resin by hydrofluoric acid using conventional Boc-chemistry strategy. An acetamidomethyl group was used to protect the cysteine side chain during synthesis and the hydrofluoric acid cleavage. The sequence is amino acid 1–42 of TodS plus a C-terminal linker Gly-Gly-Cys, which is designed to replace functionally the leucine zipper (11). The two cysteines at positions 21 and 32 were replaced by Ser (underlined), since in several bZIP sequences these amino acids were found to be interchangeable (16).

Peptide Dimerization. The peptide (25 mg) was dissolved in 10 ml of 80% acetic acid. An iodine solution (20 mg in 2 ml of 80% acetic acid) was added dropwise to deprotect the acetamidomethyl group and oxidize the cysteine residues for dimerization. The reaction solution was incubated for 2 h at room temperature and then cooled to 0°C. Unreacted iodine was neutralized by adding 0.1 N $Na_2S_2O_3$ until the iodine color disappeared. The solvent was evaporated under vacuum. The peptide was dissolved in 5% acetonitrile and 25% acetic acid, and purified by preparative HPLC (Vydac C4, 4.6 × 25 cm) using a linear gradient of 14–54% acetonitrile in 0.1% TFA (0.33% per min gradient, 33 ml/min flow rate). The purified product was lyophilized with >95% purity based on a profile of an analytical HPLC (Vydac C18, 0.46 × 25 cm column, 10–50% acetonitrile in 0.1% TFA, 1.0% per min gradient, 1.0 ml/min flow rate) at 215 nm. The peptide composition was confirmed with a Beckman model 6300 amino acid analyzer and a Sciex (Thornhill, ON, Canada) API III mass spectrometer. The peptide showed correct amino acid composition and molecular mass (M_r, 10,259.3 Da observed vs. 10,259.5 Da calculated).

RESULTS AND DISCUSSION

TodS and TodT Proteins Belong to the Family of Two-Component Regulatory Systems. Two open reading frames (ORFs), transcribed in the same direction as the core *tod* structural operon, were identified downstream of *todH*, the last gene of the *tod* operon, which encodes a 4-hydroxy-2-oxovalerate that carries out the penultimate step of toluene degradation to pyruvate (Fig. 1). The 2934-bp ORF, designated *todS*, encodes a 978-residue protein with a M_r of 108,225. Translational coupling of a second ORF of 206 residues (M_r, 23,030), designated *todT*, is suggested by an overlap of its potential start codon with the *todS* stop in the configuration ATGA. The predicted full-length sizes of TodS and TodT were verified by expression of subclones pTPF1–2 and pTtodT, respectively (Fig. 1) in the T7 polymerase/promoter system and labeling with [^{35}S]methionine (not shown).

Analysis of the predicted amino acid sequence of TodS indicates that it is a new member of the hybrid class of histidine kinases (Fig. 2). More interestingly, the N terminus of TodS contains a motif characteristic of bZIP, which is common among transcriptional factors in organisms as diverse as fungi, plants, and mammals, but rarely described in prokaryotes (17, 22). The bZIP motif typically consists of a region with several basic residues, which probably contacts DNA directly and an adjacent region containing a heptad repeat of leucine (the leucine zipper) that mediates dimerization. The hypothesis that TodS dimerizes through its bZIP motif and binds to a specific sequence was tested and this is described later.

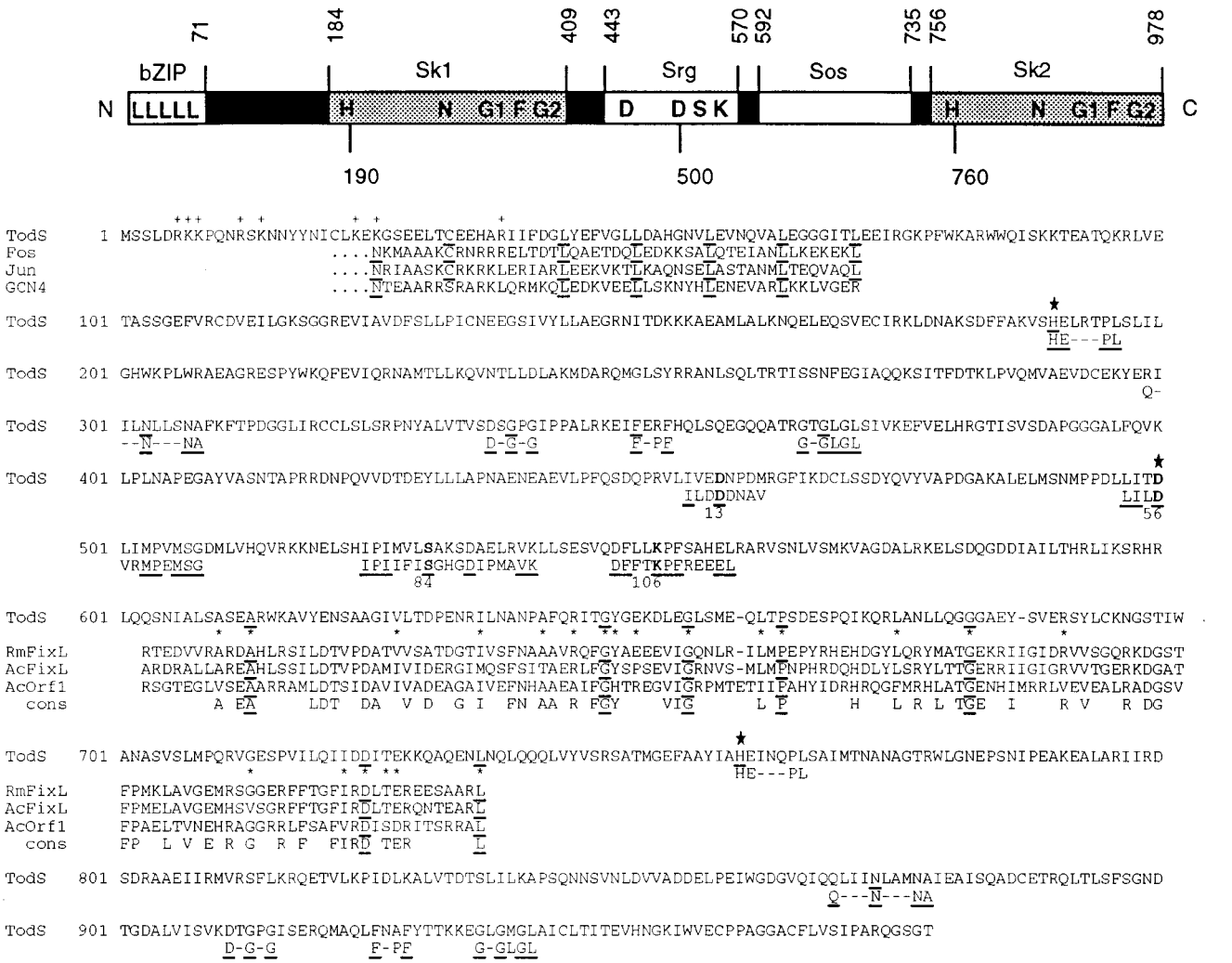

FIG. 2. (*Upper*) A schematic domain structure of TodS and its amino acid sequence characteristics. bZIP is characterized by the repeating heptad leucines (L) and the preceding charged (+) residues (17). (*Lower*) A comparison with eukaryotic Fos, Jun, and GCN4 bZIP sequences is shown; in TodS an invariant asparagine (N) found among eukaryotic bZIP proteins is replaced by a lysine (K), but a conserved cysteine (C) at position −11 from the first leucine is present. Sk1 and Sk2 are two histidine kinase domains characterized by the conserved amino acid blocks known as **H** (**H**E—PL), **N** (Q—**N**—NA), **G**1 (D-**G**-G), **F** (**F**-PF), and **G**2 (G-**GLGL**) as compiled by Parkinson and Kofoid (4). These conserved residues in TodS are underlined. Srg contains the conserved DDSK residues (in boldface type) characteristic of bacterial response regulators; the conserved Ser (S) is often replaced by a Thr (T) (18). Shown below the boldface residues, for comparison, are the corresponding regions from the TodT sequence. The large asterisks indicate potential phosphorylation sites. The Sos domain is defined by amino acid sequence similarity with the oxygen-sensing domain of *Rhizobium meliloti* FixL (19) and related proteins from *Azorhizobium caulinodans* (AcORF1) (20). Invariant residues are underlined; cons, consensus residues. The top three similarity scores as defined by the BLAST program (ref. 21; National Center for Biotechnology Information, Bethesda) for the Sk1 domain are: ORFX18 and PhoR of *Bacillus subtilis* (GenBank accession nos. L09228 and M23549, respectively); CpxA of *E. coli* [Protein Identification Resource (PIR) no. A29549]; for Sk2: NodV and NswA of *Bradyrhizobium japonicum* (GenBank accession nos. M31765 and Z22637, respectively); C4-dicarboxylate sensor kinase of *Rhodobacter capsulatus* (PIR no. S30288); for Srg: PhoB of *R. meliloti* (GenBank accession no. M96261), phosphate acceptor regulatory protein (CheY) of *P. aeruginosa* (GenBank accession no. X61231), and PhoB of *Klebsiella pneumoneae* (GenBank accession no. M31794).

Another novel feature of TodS is that it contains a duplicated histidine kinase domain, each of which is characterized by the five short sequence blocks, known as **H**, **N**, **G1**, **F**, and **G2**, that are highly conserved. Recently, the well-characterized ArcB and BarA sensory kinases regulating osmolarity in *E. coli* were reported to possess a second phosphorylatable histidine but lack the remaining conserved amino acid blocks (**N**, **G1**, **G2**, and **F**) in this second domain (23). The duplicated kinase regions (designated Sk1 and Sk2, spanning ≈aa 184–409 and ≈aa 756–978, respectively), have an overall 25% sequence similarity, which is a normal value when the sequences of different histidine kinase domains are compared. By analogy with known systems, His-190 and His-760 are the predicted autophosphorylated sites in TodS. Asp-458, Asp-500, Ser-530, and Lys-552 define the conserved residues in the "intrinsic" response regulator domain (designated Srg; spanning aa ≈443–570) of TodS. This ≈128-residue domain is reminiscent of CheY, which is a prototype response regulator of the bacterial chemotaxis pathway and of which the first three-dimensional structure of a response regulator has been determined (for review see ref. 18).

In between Srg and Sk2, spanning aa 592–735, is a putative oxygen-sensing region designated Sos. This assignment is based on its sequence similarity to a known oxygen-sensing/heme-binding domain of the *R. meliloti* FixL protein (19, 20). The low sequence homology was not unexpected, since heme-binding regions are known to be poorly conserved (19). The seven invariant amino acids as shown, in fact, exceed those of a previous alignment of sequences from two oxygenases, cytochrome P450 IID1 and isopenicillin synthase, which have similarities with the FixL heme-binding sequence (19).

Many histidine kinases contain a variable number of transmembrane domains along the polypeptide chains. TodS does not contain a region of sufficient hydrophobicity to suggest that it is a transmembrane protein (RAOARGOS and HELIXMEM analysis, PC/Gene, IntelliGenetics).

Analysis of the amino acid sequence of TodT reveals that it is most homologous (46% identity; 65% overall similarity) to the NodW response regulator of *B. japonicum* (24). Asp-13, Asp-56, Ser-84, and Lys-106 are the conserved amino acids typically found in response regulators (18). The C terminus of TodT contains a sequence (between aa 144 and 186 inclusive) that has 17 of 19 consensus residues of the DNA-binding domains of class 3 response regulators (25). These consensus residues are LSxREx$_2$VLx$_5$Gx$_2$NKxIAx$_2$Lx2Sx$_2$TVx$_2$Hx$_2$-Nx$_3$KL, where the underlined sequences are conserved in TodT and x specifies other amino acids.

***todST* Inactivation and Positive Regulation by *todT*.** Several lines of evidence indicate that the *todST* gene products positively regulate expression of the *tod* structural genes. Both *todS* and *todT* genes were selectively interrupted at their chromosomal loci by insertion of a Km^r gene from Tn5 or pUC4K into the unique *Sal*I and *Eco*RV sites, respectively, thus interrupting the TodSk1 domain and the putative DNA-binding region of TodT (Fig. 1). Mutants *Pp*F1(*todS*::Km^R) and *Pp*F1(*todT*::Km^R) resulting from double cross-over recombination were unable to use toluene as the sole carbon source. These strains also were incapable of converting indole to indigo, a reaction that is carried out by the toluene dioxygenase (TodABC1C2) complex (26). There also was no detectable catechol dioxygenase activity, indicating that the *todE* gene in these mutants was not expressed. To ascertain that the insertion of Km^R in *todS* did not have a polar effect on the expression of *todT*, mutant *Pp*F1(*todS*::Km^R) was complemented with pAP13, a *todS*-containing plasmid in the broad-host range pVLT33 vector (27). The *todS* gene was carried on a 3.2-kb *Sca*I–*Sca*I fragment and cloned in the blunt-ended *Eco*RI site of pVLT33. As a result, *Pp*F1(*todS*::Km^R)[pAP13] was able to use toluene as a carbon source.

Further evidence for the regulatory role of *todS* and *todT* came from the absence of a toluene-inducible β-galactosidase (*lacZ*) activity when a reporter plasmid, pMR149, was mobilized into either mutant strain (Table 1). The pMR149 plasmid contains a toluene-inducible promoter element in front of the *todX* gene, which was transcriptionally fused to *lacZ* in the correct orientation to drive *lacZ* transcription (9). In toluene-grown *Pp*F1 cells, pMR149 increased the *lacZ* activity nearly 5-fold, compared with no induction of the promoterless control plasmid pHRP311 (Table 1).

Table 1. β-Galactosidase activities expressed from *lacZ* promoter fusion plasmids in P_pF1 wild-type and mutant strains

Strain	Plasmid	β-Galactosidase activities		
		Uninduced	Induced	Fold induction
P_pF1 (*todS*::Km^R)	pMR149	240	300	1.0
P_pF1 (*todT*::Km^R)	pMR149	370	470	1.2
P_pF1	pMR149	350	1660	4.7
P_pF1	pHRP311	250	260	1.0

Plasmid pHRP311 contains a promoterless *lacZ* gene (28) and was used as a negative control. Plasmid pMR149 has been described (9). Cultures were grown to midexponential phase in minimal salts medium with 20 mM pyruvate (uninduced) or 20 mM pyruvate plus toluene supplied in the vapor phase (induced). β-Galactosidase activity was assayed using chloroform and SDS to permeabilize cells and expressed in Miller units (9).

TodT is capable of activating the otherwise dormant *tod* operon in a heterologous system. *E. coli* HB101 cells containing plasmid pDTG301 were used as host. pDTG301 contains an intact *todXFC1C2BADEGIH* operon but lacks *todT* and the 3′ end of *todS* in the broad-host-range mobilizable pLARF1 vector (12). Despite the presence of the toluene dioxygenase-encoding genes (*todC1C2BA*) necessary for the indole–indigo conversion, cells of *E. coli* HB101(pDTG301) on Luria–Bertani plates were unable to carry out this reaction. When recombinant *todT* was provided in *trans*, indigo-forming colonies were obtained (Fig. 3). TodT (*todT*^{DN}), in which Asp-56 has been changed to Asn-56 by site-specific mutagenesis, was unable to impart indigo production under the same assay conditions (Fig. 3*b*). The TodT^{DN} variant, however, was still capable of specific DNA binding (not shown). Loss of function as a consequence of amino acid substitution at the conserved Asp (e.g., Asp-57 in CheY) of response regulators has been well documented (18). Consistent with the majority of response regulators (18), it appears that the phosphorylated form of TodT is the activator of the *tod* structural genes.

The *trans*-acting effect of TodT as a positive regulator of the *tod* operon in *E. coli*, in the absence of an intact *todS*, implies activation by metabolic cross-talk carried out either by a noncognate sensor or a chemical phosphorylating agent such as acetyl phosphate (29, 30). Alternatively, the truncated TodS (aa 1–375) encoded in pDTG301 is active in phosphorylation even though

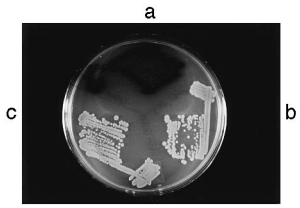

FIG. 3. Positive transactivation action of TodT by indole assay and essentiality of Asp-56. The plasmids pGSTtodT (*a*), pGSTtodT(D56N) (*b*), and their parental vector pGEX-4T-3 (*c*) were transformed individually into *E. coli* HB101(pDTG301). A transformant from each was streaked on a Luria–Bertani plate containing 1 mM indole and the appropriate antibiotics. The plate was incubated overnight at 37°C, followed by an overnight incubation at room temperature. The strain containing wild-type TodT but not the mutant TodT in pGSTtodT(D56N) produced indigo colonies.

the predicted Sk1 domain, which extends to amino acid position 409 (Fig. 2) is incomplete.

Specific Binding of TodT to *todX* Promoter Region. To study the DNA binding of TodT to the promoter region of *todX*, TodT was overexpressed as a GST fusion protein in *E. coli*, and purified using the glutathione-affinity chromatography (data not shown). Electrophoretic mobility-shift experiments were carried out using the purified protein and two PCR-generated DNA fragments; a 253-bp fragment upstream of the *todX* gene and a 196-bp fragment upstream of the *todS* gene as an internal control (Fig. 4*a*). A mixture of the two DNA fragments was used in the binding studies by incubating them with the purified TodT. As shown, TodT specifically shifted the 253-bp fragment and not the 190-bp fragment. In a separate experiment, specific DNA binding was also obtained with a 622-bp *Pst*I–*Nco*I fragment containing the *todRX* intergenic region (data not shown). These results indicate that the TodT protein specifically binds to the *todX* promoter region.

To further localize the TodT-binding site, DNase I footprinting assays were performed using the 253-bp fragment labeled with ^{32}P at one end (Fig. 4*b*). The studies showed that the TodT protected a small region encompassing a 6-bp inverted repeat (*tod* box) centered at −105 to −106 bp from the *todX* transcriptional start site (Fig. 4*c*). Two hypersensitive sites (marked ΔA and ΔC) are located at the base of the inverted repeat. These findings are consistent with the TodT being a positive regulator of the *tod* operon. One may also infer from the existence of the dyad symmetry that TodT binds to the protected region as a dimer.

AP-1 Binding. The bZIP motif in transcriptional factors, such as the oncogene products Fos and Jun, and yeast GCN4 is known to mediate protein dimerization and bind DNA in a

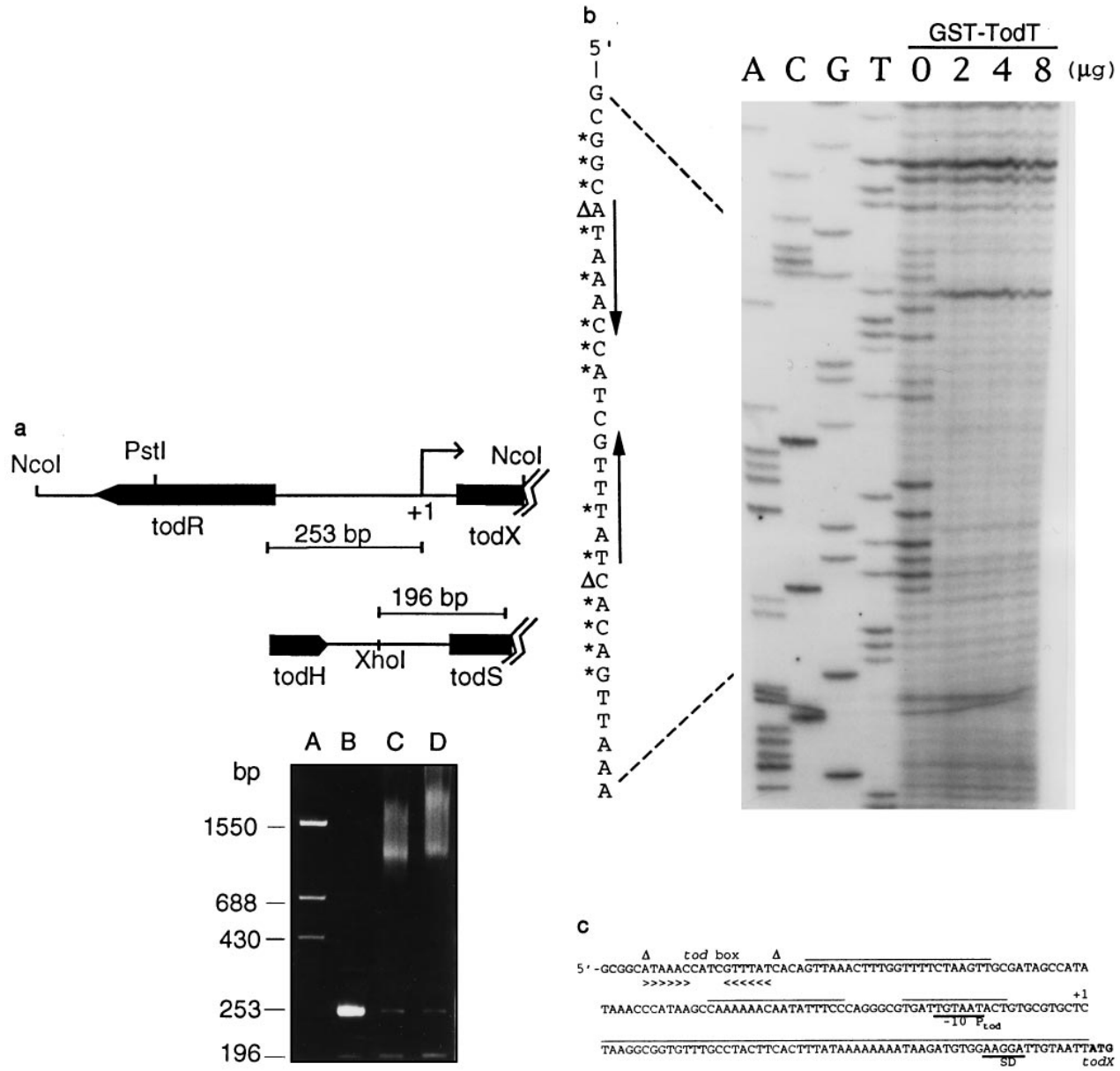

FIG. 4. (*a*) Specific binding of TodT to *todRX* intergenic region. DNA fragments (253 bp and 196 bp) used in gel retardation assays (lanes B–D) are as marked. Lanes: A, DNA standard; B, no protein; C, 1 μg protein; D, 3 μg protein (see *Materials and Methods*). (*b*) DNase I footprint of TodT binding at the *tod* promoter. The complement of the sequence surrounding the protected region is written alongside; * and Δ indicate DNase I protected and hypersensitive sites, respectively; converging arrows indicate a 6-bp inverted repeat labeled as *tod* box in *c*, together with other sequence characteristics of the *tod* regulatory region. +1, transcription start site and −10 promoter element (P_{tod}) were as determined previously (4). SD, Shine–Dalgarno sequence; A+T rich sequence is overlined.

sequence-specific manner (10, 31). We asked whether this is the case for TodS, especially when the "pseudopalindromic" sequence, 5′-TGACTCA, identical to the recognition sequence of Fos and Jun (commonly known as the AP1-binding site; ref. 10), was uniquely found upstream of the *todS* start codon (Fig. 1). DNA-binding assays incorporated a short dimerized peptide provided by a Gly-Gly-Cys linker designed to mimic the full-length protein (11). Mobility-shift and competition assays indicate specific DNA-binding activity of the TodSbs dimerized peptide to two DNA substrates, each containing the AP-1 target sequence (Fig. 5). Binding to the 196-bp restriction fragment (Fig. 5*b*) was more pronounced than to the double-stranded 20-mer (Fig. 5*a*). In the latter case, although the binding appears weak (estimated to be 30%), molar excess of nonspecific poly(dI-dC) did not compete with the target DNAs and bovine serum albumin did not bind to the 20-mer. Reduction of the disulfide bond in TodSbs, by addition of 10 mM DTT, substantially decreased the amount of mobility-retarded DNA. This latter result lends support to the importance of dimerization for DNA binding, and provides the basis for further detailed investigations using purified TodS protein. TodS may be unique among known histidine kinases in incorporating a bZIP motif to effect protein dimerization for its function. Mutations that disrupt the bZIP motif in the full-length protein would test this hypothesis.

Concluding Remarks. We have described TodS, a novel histidine kinase that regulates toluene degradation in *Pp*F1. The unusual features of TodS portray an ever-increasing diversity of the two-component systems in prokaryotes as well as those recently described homologues in plants and fungi (4–6). Indeed, much is to be learned about the prokaryotic devices for propagating dual, if not multiple, signals. The presence of a duplicated histidine kinase motif in TodS implies that it is likely a dual sensor responding to either toluene as a primary environmental signal or sensing oxidative stress in the cell milieu. Other possibilities exist. Because the *tod* pathway is oxygen-dependent, the presence of a putative oxygen-sensing domain in TodS may not be a fortuitous event. Because TodS is a cytoplasmic protein, an interaction(s) with a separate transmembrane (receptor) protein or another periplasmic protein(s) may be necessary. Preliminary evidence shows that TodX, an outer-membrane protein encoded by *todX*, the first gene of the *tod* operon, is somehow linked to the signal transduction process, which results in specific response of the *tod* promoter to toluene (Y.W. and P.C.K.L., unpublished work). We believe that greater specificity or fine tuning is bestowed upon catabolic promoter by the interplay of signal–transduction components in which transmission of the input and output signals have to be precise. This is in sharp contrast to promoters of other pseudomonad catabolic pathways in which a remarkable level of nonspecificity (leakiness) has been noted for the "single" transcriptional activators that control them. For example, the upper pathway promoter of the toluene/xylene (TOL) system in *P. putida* mt-2 can be efficiently cross-regulated by DmpR, the regulator of a different (phenol) catabolic pathway. Also, the toluene-responsive regulator of the TOL pathway (XylR), can recognize as effectors a whole variety of aromatic structures that are quite different from those substrates of the pathway (32).

It is evident that the basic principles of signal transduction involving protein phosphorylation in response to aromatic hydrocarbons are remarkably similar between toluene action in *Pp*F1 and induction of cytochrome P450 A1 enzyme by dioxin (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) in eukaryotes (for review see ref. 33). The existence of common regulatory features among bacteria and eukaryotes indicates that prokaryotes offer a rich ground for exploring and understanding key modes of cellular regulation, such as protein phosphorylation.

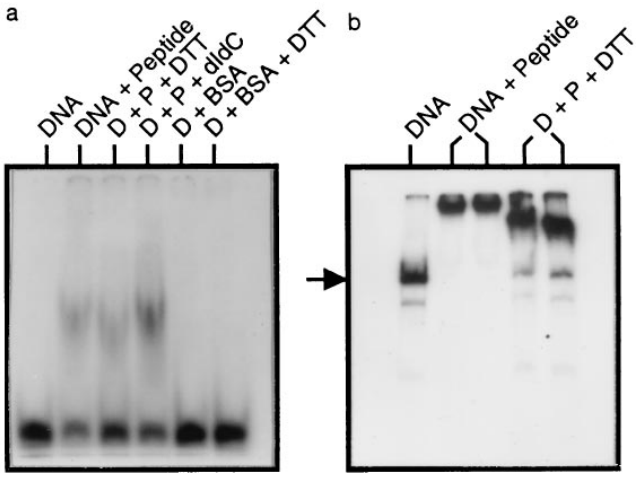

FIG. 5. Gel retardation assays showing DNA binding to a dimerized synthetic peptide. (*a*) The DNA substrate was a ^{32}P-labeled double-stranded 20-mer, 5′-AATCTTGTGAGTCATTAAAG; the underlined portion is the eukaryotic AP-1 recognition sequence (see Fig. 1). P, peptide TodSbs; D, DNA samples; DTT, 10 mM dithiothreitol; BSA, bovine serum albumin; dIdC, poly(dI-dC) (Pharmacia). (*b*) The DNA substrate was a ^{32}P-labeled 196-bp restriction fragment prepared by PCR amplification of the DNA region from an *Xho*I site in the *todHS* intergenic region to the nucleotides corresponding to the first leucine of the bZIP sequence. The arrow indicates the position of the 196-bp amplified DNA fragment. The minor faster migrating bands are unpurified PCR products. DNA binding was performed as described (6): 320 nM peptide/3 fmol of 20-mer/≈25 ng of PCR DNA (0.3 fmol). Binding was performed on ice for 30 min in buffer containing 20 mM Tris (pH 7.4), 2 mM $MgCl_2$, 2 mM EDTA, 5 mM KCl, 0.1% Nonidet P-40. Mixtures were separated in 8% polyacrylamide gels in 0.5× Tris-borate EDTA buffer at 12°C.

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