Cloning and Characterization of *tfdS*, the Repressor-Activator Gene of *tfdB*, from the 2,4-Dichlorophenoxyacetic Acid Catabolic Plasmid pJP4

BRYAN KAPHAMMER AND RONALD H. OLSEN*

Department of Microbiology and Immunology, University of Michigan
Medical School, Ann Arbor, Michigan 48109

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Plasmid pRO101, a derivative of plasmid pJP4 which contains Tn1721 inserted into a nonessential region, is inducible for 2,4-dichlorophenol hydroxylase (DCPH) encoded by *tfdB*. Plasmid pRO103, which has a deletion in the *Bam*HI-F-*Bam*HI-E region of plasmid pRO101, has elevated basal levels of DCPH but is uninducible. The regulatory gene for *tfdB*, designated *tfdS*, was cloned as an 8.3-kilobase-pair *Eco*RI-E fragment. When the cloned *tfdS* gene was in *trans* with plasmid pRO103, the baseline DCPH levels were repressed to normal uninduced levels and were fully induced when this strain was grown in the presence of 2,4-dichlorophenoxyacetic acid, 2,4-dichlorophenol, or 4-chlorocatechol. However, when *tfdS* was in *trans* with *tfdB* in the absence of *tfdCDEF*, *tfdB* was repressed but could not be induced. When *tfdS* and *tfdC1*, which encodes chlorocatechol 1,2-dioxygenase, are in *trans* with *tfdB*, *tfdB* remained uninduced, indicating that a downstream metabolite of chloro-*cis,cis*-muconate, either 2-*cis*-chlorodiene lactone or chloromaleylacetic acid, is the effector. Collectively, these data demonstrate that the gene product of *tfdS* acts as a repressor of *tfdB* in the absence of an effector and as an activator of *tfdB* when an effector is present.

The physiology, biochemistry, and genetics of 2,4-dichlorophenoxyacetic acid (TFD) degradation have been studied by many laboratories. Initial studies have focused on the delineation of the pathway for TFD degradation (8, 9, 25, 28). These studies have shown that TFD is first converted to 2,4-dichlorophenol (DCP) and subsequently hydroxylated to chlorocatechol. Chlorocatechol is then metabolized through a modified *ortho*-cleavage pathway, resulting in the production of chloromaleylacetic acid (Fig. 1) (9).

Plasmid pJP4 from *Alcaligenes eutrophus* JMP134 encodes genes for TFD degradation (5, 6, 11). Studies of the genetic organization of plasmid pJP4 showed that there were at least three operons (7, 10, 12, 15, 30). The first operon consists of *tfdA*, encoding 2,4-dichlorophenoxyacetic acid monooxygenase (12, 30). Genes of the modified *ortho*-fission pathway (*tfdCDEF*) make up the second operon (7, 10, 15), and *tfdB*, which encodes 2,4-dichlorophenol hydroxylase (DCPH), constitutes the third operon.

The regulation of *tfdA* and *tfdCDEF* was studied in the heterogenetic background of *Pseudomonas aeruginosa* PAO1c by using glucose or Casamino Acids as a carbon source for growth. The regulatory gene *tfdR* was shown to encode a negative regulatory element for *tfdA* (12) and the *tfdCDEF* operon (15). However, *tfdR* did not regulate the expression of *tfdB* (15).

This work involves the identification of the regulatory gene for *tfdB*, designated *tfdS*. Data presented here suggest that the gene product of *tfdS* acts as both a positive and a negative regulator of *tfdB*. Its location corresponds to the open reading frame transcribed divergently from the *tfdA* promoter, which has been described in the literature as *tfdO* (13, 26). The effectors which presumably interact with *tfdS* are discussed, and a regulatory model for *tfdB* is presented.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were routinely maintained on tryptone-yeast extract-glucose medium (TNA) described elsewhere (22). The growth temperature for all experiments was 37°C. Strains carrying plasmids which conferred trimethoprim resistance were routinely cultured on Vogel-Bonner basal salts medium (31) with 0.3% Casamino Acids (Difco Laboratories). Cultures for 2,4-dichlorophenol hydroxylase (DCPH) and chlorocatechol 1,2-dioxygenase assays were grown by inoculating 25 ml of basal salts medium (MMO) (29) containing 0.3% Casamino Acids with the appropriate *P. aeruginosa* strain. Antibiotics carbenicillin (500 µg/ml), trimethoprim (600 µg/ml), tetracycline (50 µg/ml), and gentamicin (5 µg/ml) were added when appropriate to maintain selection for the various plasmids. Where indicated, TFD, DCP, and 4-chlorocatechol (4CC) were added to a final concentration (wt/vol) of 0.025, 0.007, and 0.008%, respectively. After 14 h of growth, 25 ml of culture was inoculated into 100 ml of identical media and allowed to grow to mid-log phase.

Extract preparation and enzyme assays. Cell extracts for DCPH assays were prepared by harvesting mid-log-phase cultures by centrifugation at 8,000 × *g* for 10 min at room temperature. The cell pellets were washed once with 10 ml of 100 mM potassium phosphate, pH 7.6, containing 1 mM 2-mercaptoethanol, 0.1 mM EDTA, and 2 µM flavin adenine dinucleotide. DCPH activity was measured by following the disappearance of the DCPH cosubstrate, NADPH, by a modification of the method of Liu and Chapman (18). The 1-ml assay cuvette contained 960 µl of buffer, 10 µl of 10 mM NADPH, and 20 µl of cell extract. Endogenous oxidation of NADPH was monitored by following the decrease at A₃₄₀ for 5 min, followed by the addition of 10 µl of 10 mM DCP.

* Corresponding author.

FIG. 1. Pathway of TFD degradation to chloromaleylacetic acid. The gene designation is shown in parentheses for each enzyme.

DCPH activity was calculated by using the absorbance coefficient of Liu and Chapman (18). Protein concentrations were determined by the method of Bradford (1) by using bovine serum albumin as the standard.

Chlorocatechol 1,2-dioxygenase assays were performed as described elsewhere (8, 15).

Genetic techniques. Plasmids pRO1727 (3), pRO1769 (4), and pRO2321 (33) were used as cloning vectors. TFD pathway genes were cloned from plasmid pRO101 (12) or pJP4 (6). Techniques for DNA isolation and purification (21), restriction endonuclease cleavage, and ligation of purified DNA (4) and for bacterial transformation (19) have been described previously.

Chemicals and reagents. 4CC was purchased from Helix Biotech Ltd., Vancouver, Canada. TFD and DCP were purchased from Sigma Chemical Co., St. Louis, Mo. Restriction endonucleases and other enzymes involved with DNA manipulations were purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind. or Bethesda Research Laboratories, Inc., Gaithersburg, Md.

RESULTS

Expression and regulation of *tfdB*. *P. aeruginosa* PAO1c containing plasmid pRO101 (Fig. 2) shows regulated expression of *tfdB* (Table 2). However, *P. aeruginosa* containing plasmid pRO103, a deletant of pRO101 (Fig. 2), has elevated basal levels of DCPH independent of the presence of inducing compounds TFD, DCP, or 4CC (Table 2). This pattern of inducer-independent, elevated basal levels of DCPH was also observed (Table 2) in *P. aeruginosa* PAO1c containing the cloned *tfdB* gene on plasmid pRO2338 (Fig. 2). Uninducible *tfdB* activity in strains containing either pRO103 or pRO2338 indicates that these plasmids lack a regulatory gene for *tfdB*. Furthermore, lack of this regulatory gene results in elevated basal levels of DCPH independent of an inducing compound. Also, these observations suggest that the regulatory locus for *tfdB* is located in the deleted region of plasmid pRO103 (Fig. 2).

The region corresponding to the deletion in plasmid pRO103 was cloned as an 8.3-kilobase-pair *EcoRI*-E fragment from pRO101 into cloning vector pRO1769 and desig-

TABLE 1. Bacterial strains and plasmids, characteristics, and sources

Strain/plasmid	Relevant markers ^a	Reference
<i>P. aeruginosa</i> PAO1c	Prototroph	14
Plasmids		
pRO1727	Cb ^r Tc ^r	3
pRO1769	Sm ^r Gm ^r	4
pRO2321	Tp ^r Tc ^r	33
pJP4	Hg ^r <i>tfdA</i> ⁺ <i>tfdB</i> ⁺ <i>tfdCDEF</i> ⁺ <i>tfdR</i> ⁺ <i>tfdS</i> ⁺	5
pRO101 ^b	Hg ^r <i>tfdA</i> ⁺ <i>tfdB</i> ⁺ <i>tfdCDEF</i> ⁺ <i>tfdR</i> ⁺ <i>tfdS</i> ⁺	12
pRO103 ^c	Hg ^r <i>tfdA</i> ⁺ <i>tfdB</i> ⁺ <i>tfdCDEF</i> ⁺ <i>tfdR</i> <i>tfdS</i>	12
pRO1956	Cb ^r <i>tfdS</i> ⁺	This study
pRO1968	Cb ^r	This study
pRO1969	Cb ^r	This study
pRO1970	Gm ^r <i>tfdS</i> ⁺	This study
pRO1971	Cb ^r <i>tfdCDEF</i> ⁺ <i>tfdB</i> ⁺	This study
pRO2338	Tp ^r <i>tfdB</i> ⁺	This study
pRO2344	Tp ^r <i>tfdS</i> ⁺	This study

^a Abbreviations: Cb, carbenicillin; Tc, tetracycline; Tp, trimethoprim; Gm, gentamicin; Sm, streptomycin.

^b Plasmid pRO101 is plasmid pJP4::Tn1721.

^c Plasmid pRO103 is a deletion mutant of plasmid pRO101.

nated plasmid pRO1956 (Fig. 2). *P. aeruginosa* PAO1c containing plasmids pRO103 and pRO1956, when grown in the absence of inducer, showed basal DCPH levels 40% lower than uninduced *P. aeruginosa* PAO1c(pRO101) (Table 2). This may occur because plasmid pRO1956, which contains the regulatory gene, is in higher copy number (R. H. Olsen, unpublished data) than plasmid pRO103. However, presence of TFD, DCP, or 4CC in the growth medium of *P. aeruginosa* PAO1c(pRO103, pRO1956) allowed full induction of *tfdB* to levels comparable to those of induced *P. aeruginosa* PAO1c(pRO101) (Table 2). These data demonstrate that plasmid pRO1956 contains the regulatory gene for *tfdB* and in the presence of this regulatory gene product, *tfdB* is repressed. Moreover, when this regulatory gene product and its effector are present, *tfdB* is fully induced to levels corresponding to those of induced *P. aeruginosa*(pRO101) (Table 2). These observations suggest three levels of *tfdB* expression: a repressed level in the presence of the regulatory protein but not its effector; a derepressed level in the absence of the regulatory gene, resulting in elevated baseline levels of DCPH; and a fully induced level of DCPH when both the regulatory gene product and its effector are present in the cell.

We have designated the locus on plasmid pRO1956 which regulates *tfdB* expression as *tfdS*. It was further localized by subcloning to create the plasmids shown in Fig. 3. Neither plasmid pRO1968 nor plasmid pRO1969 regulated the expression of *tfdB* when in *trans* with plasmid pRO103 in *P. aeruginosa* PAO1c (Fig. 3 and Table 3). However, plasmid pRO1970, which contained a *Bgl*II deletion in plasmid pRO1956, when maintained in *trans* with plasmid pRO103 in *P. aeruginosa* PAO1c, resulted in the regulated expression of *tfdB* (Table 3).

Previously we showed that *tfdR* encoded on plasmids pRO1949 and pRO1956 negatively regulates the expression of *tfdA* (12) and *tfdCDEF* (15). However, the *tfdR* gene product did not regulate the expression of *tfdB* (15), and therefore the portion of the *Bam*HI-E fragment on plasmids pRO1956 and pRO1970 does not contain *tfdS* (Fig. 3). These

trans complementation tests localize *tfdS* to the 1.3-kilobase-pair region shown in Fig. 3 and indicate that *tfdS* maps to the open reading frame adjacent to *tfdA* (30), previously designated by others as *tfdO* (13).

The effector for *tfdB* regulation. The effector for *tfdB* regulation was studied by examining the expression of *tfdB* on plasmid pRO2338 in *trans* with plasmid pRO1956 in *P. aeruginosa* PAO1c. When this strain was grown in the absence of 4CC, elevated basal levels of DCPH were repressed (compared with *P. aeruginosa* PAO1c(pRO2338)); however, *tfdB* could not be induced by 4CC (Table 2). Repressed levels of DCPH in *P. aeruginosa* PAO1c (pRO2338, pRO1956) are low, perhaps because *tfdS* is on a high-copy-number plasmid and *tfdB* is on a low-copy-number plasmid. The lack of induction of this strain by 4CC suggests that the effector of *tfdB* regulation is a downstream metabolite of chloro-*cis,cis*-muconate. Chloro-*cis,cis*-muconate was eliminated as a possible effector of *tfdB* regulation by taking advantage of a duplication of the *tfdC* gene, designated *tfdC1*, on plasmid pRO1956. Ghosal and You (11) demonstrated that a region of the *Bam*HI-E fragment of pJP4 has homology to *tfdC*, and we demonstrated that there is an active *tfdC1* gene, but not *tfdD* or *tfdE*, on this *Bam*HI-E fragment (15). Since *P. aeruginosa* PAO1c(pRO1956) was inducible for chlorocatechol 1,2-dioxygenase (data not shown) when grown in the presence of 4CC, chloro-*cis,cis*-muconate was produced, yet no induction of *tfdB* was obtained. These data, when interpreted with the results in Table 2 showing that *tfdB* is uninducible in *P. aeruginosa* PAO1c(pRO2338, pRO1956) grown in the presence of 4CC, suggest that chloro-*cis,cis*-muconate, the product of *ortho*-cleavage of 4CC catalyzed by chlorocatechol 1,2-dioxygenase, is not the effector of *tfdB* regulation.

The downstream metabolite, which induces *tfdB*, is not produced in *P. aeruginosa* PAO1c(pRO2338, pRO1956) because the structural genes for the metabolism of 4CC to chloromaleylacetic acid, *tfdCDEF*, are not active on plasmid pRO2338 or present on pRO1956 (data not shown). Recent DNA sequence data confirm that *tfdCDEF* could not be active on plasmid pRO2338 (10). In contrast, *P. aeruginosa* PAO1c(pRO101) and *P. aeruginosa* PAO1c(pRO103, pRO1956) metabolize TFD, DCP, and 4CC to downstream metabolites of the TFD pathway, allowing the induction of *tfdB* (Table 2).

Induction of *tfdB* by a downstream metabolite of 4CC was demonstrated in *P. aeruginosa* containing plasmids pRO1971 and pRO2344. Plasmid pRO1971 contains *tfdCDEF* and *tfdB* (Fig. 2) and showed the same regulatory pattern as the other strains containing the unregulated *tfdB* gene, that is, it had elevated baseline, but not fully induced, levels of DCPH independent of the inducer 4CC (Table 4). Plasmid pRO2344 contains *tfdS* cloned as an *Eco*RI-*Bgl*II fragment into vector plasmid pRO2321 (Fig. 3). *P. aeruginosa* PAO1c containing plasmids pRO1971 and pRO2344 restored regulation of *tfdB*. Absence of the inducer resulted in repression of the high basal levels seen in strains containing only plasmid pRO1971, and presence of inducer allowed full activation of *tfdB* (Table 4). In this case, since *tfdCDEF* is active in these strains (data not shown), the effector of *tfdB* regulation can be metabolically produced from 4CC, resulting in the full activation of *tfdB*. The DCPH levels in these strains are higher than those of *P. aeruginosa* PAO1c(pRO101) and *P. aeruginosa* PAO1c (pRO103, pRO1956) due to the copy number effect of plasmids pRO1971 and pRO2344. In this case, *tfdB* is on a high-copy-number plasmid and *tfdS* is on a low-copy-number plasmid.

FIG. 2. (A) Restriction maps of pJP4, pRO101, and pRO103. Plasmids pRO2338 and pRO2344 are clones in vector plasmid pRO2321. Plasmid pRO1956 is a clone in vector plasmid pRO1769. Plasmid pRO1971 is a clone in vector plasmid pRO1727. The hatched boxes on plasmids pJP4 and pRO101 indicate the location of the structural genes. The dark box in plasmid pRO103 indicates the area of deletion. (B) Restriction maps of vectors pRO1727, pRO2321, and pRO1769.

Therefore, our observations suggest that either 2-*cis*-chlorodiene lactone or chloromaleylacetic acid is the effector of *tfdB* regulation.

DISCUSSION

A model for *tfdB* regulation is shown in Fig. 4. The *tfdB* gene product, protein S (S), in the absence of effector acts to repress the transcription of *tfdB*. S protein, in the presence of effector, forms an S protein-effector complex (SE) which activates transcription of *tfdB*. This model predicts that in the absence of *tfdS*, *tfdB* would no longer be repressed nor would it be inducible. This would result in high basal levels of DCPH independent of the presence of the effector. The

results of DCPH assays with *P. aeruginosa* PAO1c containing plasmids pRO103, pRO2338, and pRO1971, none of which contain *tfdS*, show higher basal activity, as the model predicts. When *tfdS* is added in *trans* to these strains, regulation is restored, basal levels of DCPH are lowered, and in the presence of effector, *tfdB* is fully induced.

Recently, Pieper et al. (24) discussed the regulation of the TFD pathway on the basis of growth of *A. eutrophus* JMP134 and a mutant *A. eutrophus* JMP134-1 on TFD, phenoxyacetic acid, phenol, or fructose. On the basis of DCPH activities of these two strains grown on these four compounds, they concluded that the mutant *A. eutrophus* JMP134-1 expressed *tfdB* "semi-constitutively," following

TABLE 2. Regulation of *tfdB* by the *EcoRI*-E fragment of pRO101

Strain	Inducer	DCPH activity ^a (μ mol/min/mg of protein)
PAO1c(pRO101)	— ^b	25.9
	TFD	68.4
	DCP	62.4
	4CC	70.0
PAO1c(pRO103)	—	36.6
	TFD	37.8
	DCP	31.9
	4CC	35.6
PAO1c(pRO103, pRO1949) ^c	—	41.3
	TFD	37.8
PAO1c(pRO103, pRO1956)	—	15.2
	TFD	57.2
	DCP	59.4
	4CC	86.6
PAO1c(pRO1956)	—	0.0
	TFD	0.0
	DCP	0.0
	4CC	0.0
PAO1c(pRO2338)	—	28.9
	4CC	29.2
PAO1c(pRO2338, pRO1956)	—	6.1
	4CC	4.6

^a Enzyme activities are characteristic of at least three independent experiments.

^b —, No inducer used.

^c Data taken from reference 15.

FIG. 3. Subcloning of *tfdS* from *EcoRI*-E. The location of *tfdS* is indicated by the hatched box. Plasmids pRO1968 and pRO1969 are clones in vector plasmid pRO1727. Plasmids pRO1956 and pRO1970 are clones in vector plasmid pRO1769. Plasmid pRO2344 is a clone in vector plasmid pRO2321. Plasmid pRO1949 is described elsewhere (15).

TABLE 3. Regulation of *tfdB* by subclones of pRO1956

Strain	Inducer	DCPH activity ^a (μ mol/min/mg of protein)
PAO1c(pRO103)	— ^b	36.6
PAO1c(pRO103, pRO1956)	4CC	35.6
	—	15.2
PAO1c(pRO103, pRO1968)	4CC	86.6
	—	46.0
PAO1c(pRO103, pRO1969)	4CC	41.1
	—	49.0
PAO1c(pRO103, pRO1970)	4CC	44.1
	—	12.0
PAO1c(pRO1956)	4CC	80.0
	—	0.0
PAO1c(pRO1968)	4CC	0.0
	—	0.0
PAO1c(pRO1969)	4CC	0.0
	—	0.0
PAO1c(pRO1970)	4CC	0.0
	—	0.0

^a Enzyme activities are characteristic of at least three independent experiments.

^b —, No inducer used.

the classification of Collins et al. (2). We interpret the data of Pieper et al. (24) to mean that *A. eutrophus* JMP134-1 has high basal levels of DCPH, like *P. aeruginosa* PAO1c containing plasmid pRO103. Presumably, *A. eutrophus* JMP134-1 contains a defect in *tfdS*.

Data presented here eliminate all intermediates of the TFD pathway as effectors of *tfdB* regulation, with the exception of *cis*-2-chlorodiene lactone and chloromaleylacetic acid (Fig. 1). Clearly one of these two compounds is the effector of *tfdB* regulation. Kukor et al. demonstrated that *P. aeruginosa* PAO lacks a maleylacetic acid reductase on its chromosome (17). This results in the accumulation of chloromaleylacetic acid in strains of *P. aeruginosa* PAO containing the structural genes of the TFD pathway when grown in the presence of TFD (17). We show here that *P. aeruginosa* PAO1c(pRO101), *P. aeruginosa* PAO1c(pRO103, pRO1956), and *P. aeruginosa* PAO1c(pRO1971, pRO2344) can be fully induced when 4CC is in the growth media (Tables 2, 3, and 4). However, when *tfdS* on plasmid pRO1956 is in *trans* with *tfdB* on plasmid pRO2338, which lacks *tfdCDEF*, *tfdB* is repressed and cannot be induced by 4CC in the growth media. We have demonstrated that the *Bam*HI-E fragment of plasmid pJP4 contains *tfdC1* but not *tfdD* or *tfdE* and that *tfdC1* is induced by 4CC, encoding chlorocatechol 1,2-dioxygenase (15). Plasmid pRO1956 also contains the region of *Bam*HI-E which encodes *tfdC1* (data not shown). Therefore, one of the downstream metabolites of the product of

TABLE 4. Regulation of cloned *tfdB* by *tfdS*

Strain	Inducer	DCPH activity ^a (μ mol/min/mg of protein)
PAO1c(pRO1971)	— ^b	65.7
PAO1c(pRO1971, pRO2344)	4CC	64.7
	—	43.3
PAO1c(pRO2344)	4CC	139.0
	—	0.0
PAO1c(pRO2344)	4CC	0.0
	—	0.0

^a Enzyme activities are characteristic of at least three independent experiments.

^b —, No inducer used.

FIG. 4. Model of *tfdB* regulation. The *tfdS* gene product, protein S (S), in the absence of an effector, acts to repress the transcription of *tfdB*. The S protein, in the presence of effector, forms an S protein-effector complex (SE) which activates transcription of *tfdB*. The hatched boxes indicate the locations of the structural genes of the TFD pathway.

the chlorocatechol 1,2-dioxygenase reaction must be the effector. However, the unavailability and instability of these compounds make determination of the actual effector experimentally difficult. There are examples of the end product of the pathway acting as the inducer of some of the genes of the catabolic pathway. The best characterized of these is the β -ketoadipate pathway of *Pseudomonas putida* in which β -ketoadipate acts as the inducer of *pcaB*, *pcaC*, and *pcaD* (32). Extending this to the TFD pathway, chloromaleylacetic acid may be the inducer of *tfdB*.

The regulatory gene, *tfdS*, maps to the same location as the open reading frame of unknown function *tfdO* (13). This open reading frame was of interest to other researchers because it had a high degree of sequence homology to the class of regulatory proteins of the LysR group (13, 26). This open reading frame was speculated to code for a positive regulatory element of *tfdB* regulation (23). Our data demonstrates that *tfdS* is a regulatory gene, but its gene product acts both to repress and activate *tfdB*.

An additional complexity of *tfdB* regulation is possible. Our data do not exclude the regulation of *tfdS* by *tfdR*. However, the behavior of plasmid pRO2344 clearly shows regulation of *tfdB* by *tfdS* in the absence of *tfdR*, demonstrating that *tfdR* is not essential for *tfdS* expression. Genetic regulation by a single protein acting as both a positive and negative regulatory element has been observed for the *mer* and the arabinose systems (20, 27) in *Escherichia coli*. However, regulatory mechanisms of other aromatic catabolic pathways have been shown to have either simple positive regulation (16) or simple negative regulation, as is the case for the rest of the genes in the TFD pathway (12, 15). More detailed studies of the regulatory mechanisms of other catabolic pathways are needed to determine whether the regulatory mechanism seen for *tfdB* is unique to the TFD pathway or present in other aromatic pathways.

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