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Characterization of the Expression of the *thcB* Gene, Coding for a Pesticide-Degrading Cytochrome P-450 in *Rhodococcus* Strains†

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A cytochrome P-450 system in *Rhodococcus* strains, encoded by *thcB*, *thcC*, and *thcD*, participates in the degradation of thiocarbamates and several other pesticides. The regulation of the system was investigated by fusing a truncated *lacZ* in frame to *thcB*, the structural gene for the cytochrome P-450 monooxygenase. Analysis of the *thcB-lacZ* fusion showed that the expression of *thcB* was 10-fold higher in the presence of the herbicide EPTC (*s*-ethyl dipropylthiocarbamate). Similar enhancement of the *thcB-lacZ* expression was found with other thiocarbamate pesticides. Atrazine, simazine, or carbofuran, although metabolized by the system, had no effect on the *thcB-lacZ* expression. The presence of glucose slightly increased the expression of *thcB-lacZ*, indicating no catabolic repression of the *thcB-lacZ* expression. The expression of *thcB-lacZ* was decreased more than twofold in Luria-Bertani medium. This was due in part to cysteine, which repressed *thcB-lacZ* expression. It was confirmed that the *thcR* gene, which is transcribed divergently from *thcB*, codes for a positive regulatory protein which is essential for the *thcB-lacZ* expression. Studies of the *thcR-lacZ* protein fusion showed that the *thcR* gene is expressed constitutively.

The *thcB* gene, which codes for a novel type of cytochrome P-450, has been cloned from *Rhodococcus* sp. strain NI86/21 and shown to be responsible for the degradation of thiocarbamate herbicides. Immediately downstream of *thcB* are *thcC* and *thcD*, which code for a 2Fe-2S ferredoxin and a ferredoxin reductase. All three gene products are required for the herbicide degradation phenotype. The *thcR* gene, which likely codes for a regulatory protein for the *thcB* gene, is transcribed divergently from *thcB* (19). The same gene cluster was also found on a 6-kb *Kpn*I DNA fragment cloned from an indigenous plasmid in *Rhodococcus* sp. strain TE1 (26, 27). By use of an *Escherichia coli-Rhodococcus* shuttle vector, *thcB* was introduced into and expressed in several *Rhodococcus* strains (26). In addition to the degradation of thiocarbamate herbicides, the *thcB* cytochrome P-450 is also involved in metabolizing the carbamate insecticide carbofuran and *s*-triazine herbicides like atrazine and simazine (18, 26, 27). We have also reported on combining the atrazine dealkylation activity of the cytochrome P-450 system with the dechlorinating activity of an *s*-triazine hydrolase from a *Rhodococcus corallinus* strain to create recombinant *Rhodococcus* strains which are capable of dealkylating and dechlorinating atrazine and simazine (29).

The expression of the *thcB* gene in *Rhodococcus* sp. strain NI86/21 is inducible by thiocarbamate herbicides (19) and to a smaller extent by atrazine (18). In *Rhodococcus* sp. strain TE1, the degradation of *s*-ethyl dipropylthiocarbamate (EPTC) is much faster than the degradation of atrazine (4, 26, 27) and the addition of EPTC to the incubation medium increases the rate of atrazine degradation (2), suggesting that the level of expression of the cytochrome P-450 system in strain TE1 is increased by EPTC.

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In spite of the diverse metabolic capabilities of rhodococci (1, 4, 5, 15, 19, 29, 33) and their potential for exploitation in industry and in bioremediation (1, 8, 33, 35), genetic studies of the genus have lagged; only a few genes have been cloned, and information on the regulation of *Rhodococcus* gene expression is extremely limited (8). Recent advances in the molecular biology of *Rhodococcus* species have greatly simplified the procedures for cloning genes from these bacteria, obtaining transformants, and studying their expression in the same or different *Rhodococcus* species (6, 12, 28), thereby making it possible to investigate and manipulate gene expression and the metabolic activities of the gene products.

In this study, we report on the construction of a *thcB-lacZ* fusion and its expression in the presence of EPTC and other pesticides which are metabolized by the cytochrome P-450 system. We also confirm that the *thcR* gene, which is constitutively expressed, codes for a *trans*-acting positive regulatory protein for *thcB*.

MATERIALS AND METHODS

Bacteria, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Rhodococcus* strains were grown in BMN medium (3) supplemented with 0.1% glycerol (BMNG medium) or in Luria-Bertani (LB) medium at 30°C with shaking. *E. coli* strains were grown in BMNG medium with required supplements or in LB medium at 37°C with shaking. BMN medium was solidified with 1% (wt/vol) bacteriological agar (Oxoid) and supplemented with 60 to 75 μg of filter-sterilized EPTC per ml to assess the ability of bacterial strains to grow on EPTC as a sole source of carbon. LB medium was supplemented with 1.5% Bacto Agar (Difco) for growth on solid medium. The antibiotics used and their concentrations were as follows: ampicillin, 100 μg/ml; thiostrepton, 10 μg/ml. Thiostrepton was kindly supplied by S. Lucania of Squibbs-Bristol Meyers, Princeton, N.J.

Determination of EPTC and atrazine metabolism. The ability of the cells to metabolize EPTC and atrazine was tested in batch cultures in BMNG medium supplemented with 60 μg of EPTC per ml or 10 μg of atrazine per ml. The degradation of EPTC was determined by assay of the residual amount of EPTC in the medium over time by gas chromatography as described previously (32). Atrazine metabolism was determined by high-pressure liquid chromatography (4). The herbicides were quantified by comparing the peak areas on the chromatograms with those of authentic standards (>97% pure) run under identical conditions.

β-Galactosidase assay. For the β-galactosidase assay, *E. coli* cultures were grown for 4 h after subculture from overnight cultures, and *Rhodococcus* sub-

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant phenotype	Origin or reference
Rhodococcus sp.		
TE1	Isolated from EPTC-treated soil, *lacZ*	32
TE3	thcR thcB thcC thcD derivative of TE1	32
Escherichia coli		
XL-1	*recA lac hsdR* (F′ *lacI*^{q} *lacZ*ΔM15 Tn*10*)	Stratagene Co.
MC4100	*araD139* Δ(*argF-lac*) U169 *rpsL150 relA1 deoC1 ptsF25 rbsR*	31
Plasmids		
pBlueScript (KS$^+$)	Cloning vector in *E. coli*; Ap^r	Stratagene Co.
pMC1871	pBR322 carrying a truncated *lacZ*	30
pBS305	*E. coli-Rhodococcus* shuttle vector; Ap^r Thio^r	26
pKL1	6-kb *Kpn*I fragment cloned into pBS305	26
pKLB1	6-kb *Kpn*I fragment cloned into pBlueScript	26
pKLB2	*Bgl*II fragment deleted from pKLB1	26
pKLB4	3.5-kb *Mlu*I fragment cloned into pBlueScript	26
pEPT6	3.9-kb *Eco*RV-*Kpn*I fragment cloned into pBS305	26
pKLZ10	pKLB1 with *lacZ* fused to *thcB*	This study
pKLZ11	8.3-kb *Kpn*I fragment from pKLZ10 subcloned into pBS305	This study
pKLZ12	1.5-kb *Sph*I fragment removed from pKLZ11	This study
pKLZ13	1.4-kb *Bgl*II-*Bam*HI fragment from pKLB4 inserted into pKLZ12	This study
pKMZ10	pKLB4 with *lacZ* fused to *thcR*	This study
pKMZ11	5-kb *Xba*I fragment from pBS305 inserted into pKMZ10	This study
pKLP2	1.5-kb *Sph*I fragment removed from pKL1	This study

cultures were grown for 12 h. β-Galactosidase assays were performed in duplicate as described by Miller (17), and enzyme activity was expressed in Miller units.

Plasmid isolation from *Rhodococcus* strains and transformation by electroporation. A modified alkaline lysis procedure was used to isolate plasmid DNA from *Rhodococcus* strains (26). Electroporations were performed by the protocol described previously (28).

Construction of *thcB-lacZ* fusion. Plasmid pMC1871 was digested with *Bam*HI, and a 3-kb fragment was isolated from agarose gel with GeneClean (Bio 101, Inc., La Jolla, Calif.) (34). The purified DNA fragment was treated with Klenow enzyme to make blunt ends before ligation with pKLB1 (26) which had been digested with *Bgl*II and then treated with Klenow enzyme. The ligation mixture was transformed into MC4100 and plated on LB ampicillin plates with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Plasmid pKLZ10 was isolated from a transformant which showed a light-blue colony on an X-Gal plate, and restriction digestion verified that the 0.7-kb *Bgl*II fragment in pKLB1 had been replaced by a 3-kb DNA fragment in pKLZ10 (Fig. 1). This resulted in an in-frame fusion of *lacZ* at codon 67 of *thcB*. The junction of *thcB* and *lacZ* was further confirmed by sequencing with a synthesized oligonucleotide as a primer.

To test the expression of the *thcB-lacZ* fusion in *Rhodococcus* strains, we subcloned a 8.3-kb *Kpn*I fragment from pKLZ10 into the *Kpn*I site of the *E. coli-Rhodococcus* shuttle vector pBS305 (28), forming pKLZ11.

Construction of the *thcR-lacZ* fusion. The 3.5-kb *Mlu*I fragment which carries the *thcR* gene has been cloned in pBluescript (26). This plasmid, pKLB4, was digested with *Eco*RV and ligated with the Klenow-enzyme-treated 3-kb *Bam*HI fragment carrying truncated *lacZ*. The ligation mixture was transformed into MC4100. Plasmid pKMZ10 was isolated from a transformant which formed a light-blue colony on an X-Gal plate, and restriction digestion verified that the 3-kb fragment had been inserted into the *Eco*RV site of pKLB4. This resulted in an in-frame fusion of *lacZ* at codon 19 of *thcR* (Fig. 1). The junction of *thcR* and *lacZ* was confirmed by sequencing with a synthesized oligonucleotide as a primer.

A 5-kb *Xba*I fragment from pBS305, which carries the *Rhodococcus* origin of replication and the *tsr* (thiostrepton resistance) gene, was isolated from an agarose gel and inserted into pKMZ10 which had been digested with *Xba*I, producing pKMZ11.

Other plasmid constructions. A 1.5-kb *Sph*I DNA fragment was removed from pKL1 by digesting the plasmid with *Sph*I and religating the 12.4-kb fragment isolated from an agarose gel with GeneClean. This results in a plasmid, pKLP2, which carries only part of *thcR*, its last 160-bp DNA deleted from the coding region. The same *Sph*I DNA fragment was removed from plasmid pKLZ11, and the resulting plasmid, pKLZ12, was used to study the expression of *thcB-lacZ* in the absence of the products of *thcR*. A 1.4-kb *Bgl*II-*Bam*HI fragment, which contains *thcR*, was isolated from pKLB4 and treated with Klenow enzyme. This fragment was then inserted into pKLZ12 digested with *Hin*dIII and treated with Klenow enzyme, forming pKLZ13.

DNA sequencing and other genetic methods. DNA sequences were determined by the dideoxy chain termination methods of Sanger et al. (23). Plasmid isolation from *E. coli*, restriction digestion, ligation, and plasmid transformation into *E. coli* cells were performed as described by Maniatis et al. (16).

RESULTS AND DISCUSSION

Effect of EPTC and other thiocarbamate pesticides on *thcB-lacZ* expression. The effect of thiocarbamate herbicides on *thcB-lacZ* expression was studied with bacterial cells carrying plasmids with a *thcB-lacZ* fusion growing in BMNG medium with or without the addition of thiocarbamate herbicides.

E. coli MC4100 cells carrying pKLZ10 or pKLZ11 showed slightly blue colonies on X-Gal plates, suggesting some expression of the *thcB-lacZ* fusion. The β-galactosidase assay showed that the basal level of *thcB-lacZ* expression was very low (22 U with plasmid pKLZ11) (Table 2). The presence of EPTC had no effect on *thcB-lacZ* expression. This was not unexpected since it has been shown previously that *E. coli* cells carrying a plasmid containing a 6-kb *Kpn*I fragment encoding EPTC and atrazine-degrading genes had no detectable herbicide degradation capability (26). Besides *thcB*, other attempts to express *Rhodococcus* genes in *E. coli*, in most cases, have also failed (5, 11, 14, 29). However, it was not clear whether the proteins were not made or whether they were inactive in *E. coli* cells. Our results reported here clearly suggest that very little, if any, P-450 is produced in *E. coli* cells carrying the cloned *thcB* gene, since extremely low amounts of β-galactosidase were produced from the *thcB-lacZ* fusion. This may be caused by the absence of the *trans*-acting factor between EPTC and the *thcB* promoter or the poor functioning of the *thcB* promoter in *E. coli* cells. This appears to be the main reason why the pesticide degradation system is not expressed in *E. coli*. It does not, however, exclude the possibility that ThcB proteins produced in *E. coli* are not functional, since β-galactosidase activity was present in cells carrying the *thcB-lacZ* fusion. Only a low level of activity and light blue colonies were seen.

Rhodococcus sp. strain TE3, a derivative of TE1 which lacks the cytochrome P-450 system, showed no β-galactosidase activity (less than 2 Miller units). When the strain was used as a recipient strain for plasmid pKLZ11, *thcB-lacZ* expression was 20-fold higher than that in *E. coli* MC4100 (Table 2) and reached the highest level at the early log phase. The addition of EPTC (50 μg/ml) in the medium increased *thcB-lacZ* expression by more than 10-fold compared with the level without

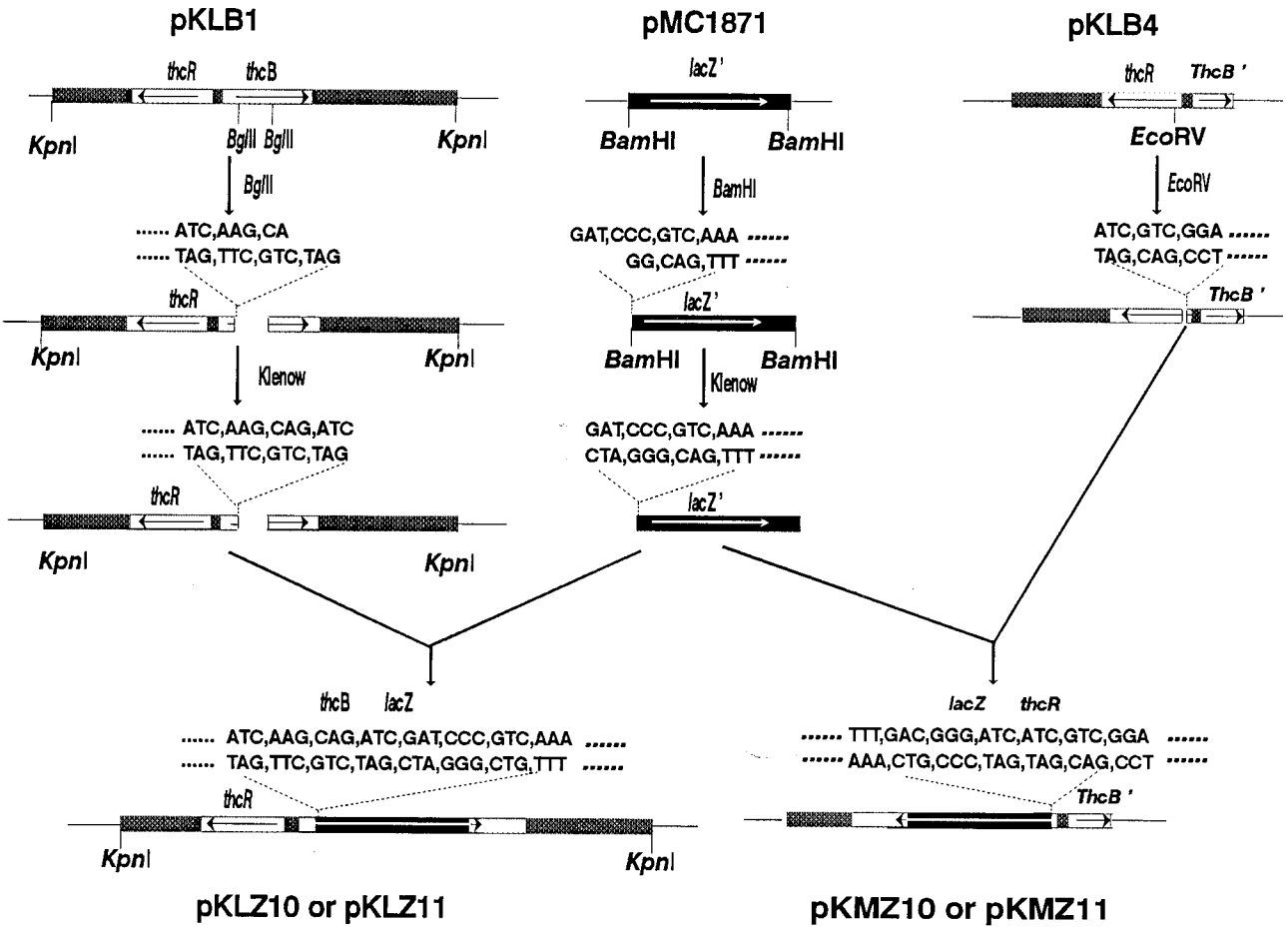

FIG. 1. Strategy for the construction of pKLZ10 or pKLZ11 and pKMZ10 or pKMZ11. Arrows indicate the direction of the transcript of each gene. The sequences of the ends of the fragments spliced to form the *thcB-lacZ* fusion are shown.

EPTC. The addition of butylate, molinate, cycloate, and pebulate showed similar effects (Table 2). In separate experiments, the optimal concentration of EPTC required (tested at 0, 0.05, 0.5, 5, 25, 50, 75, and 100 μg/ml) to obtain the maximum expression of β-galactosidase activity was determined. The effect was evident even at 0.05 μg of EPTC per ml. The activity at 5 μg of EPTC per ml was almost half of that assayed at the optimal level of 50 μg/ml. No further increase in the activity was evident when 75 or 100 μg of EPTC per ml was used.

Effects of atrazine, simazine, and carbofuran on *thcB-lacZ* expression. It was shown previously that the same cytochrome P-450 system which degrades the herbicide EPTC is involved in the dealkylation of several *s*-triazine compounds (26, 29) and also in the hydroxylation of the carbamate insecticide carbofuran. The effect of these compounds on *thcB-lacZ* expression was tested as described above with *Rhodococcus* sp. strain TE3 carrying pKLZ11 in the BMNG medium containing 15 μg of atrazine or simazine per ml or 50 μg of carbofuran per ml. The β-galactosidase assay results showed that none of the *s*-triazines or carbofuran had any effect on *thcB-lacZ* expression (Table 2).

It has been shown previously that the addition of EPTC stimulates the degradation rate of atrazine by *Rhodococcus* sp. strain TE1 carrying the cytochrome P-450 system (2). Similar activation of atrazine dealkylation was obtained with strain TE3 carrying pKL1. The results shown above are consistent with these observations and show that the cytochrome P-450 level is much higher in the presence of EPTC (Table 2). However, atrazine degradation was increased by only 2-fold in the presence of EPTC although the enzyme level was 10-fold higher as measured by the expression of the *thcB-lacZ* fusion. This may be due, in part, to the fact that EPTC, as an inducer, is also rapidly degraded by cytochrome P-450 and, as the substrate, may compete for the enzyme with atrazine.

The involvement of cytochrome P-450 enzymes in the degradation of a variety of chemicals is well established (7, 10, 15, 20–22, 24, 25). The expression of these enzymes has been found to be well regulated and, in many cases, induced by the substrates (15, 21). Early results of two-dimensional protein electrophoresis showed that the expression of *thcB* is greatly induced by EPTC (19) and to a lesser extent by atrazine (18). However, we did not find any effect of the *s*-triazine herbicides atrazine or simazine on *thcB-lacZ* expression. Our results suggest that the regulation of *thcB* expression is designed to respond to thiocarbamates, while the product of *thcB* has a wide range of substrates.

***thcB-lacZ* expression in the presence of glucose and in LB medium.** The addition of 1 mg of glucose per ml slightly increased *thcB* gene expression (Table 3), suggesting that expression of the cytochrome P-450 system is not under the control of catabolic repression. Lack of catabolic repression in the

TABLE 2. Effect of pesticides on *thcB-lacZ* expression[a]

Strain	Pesticide added[b]	β-Galactosidase activity (Miller units)
E. coli MC4100(pKLZ11)	NA[c]	22
	EPTC	23
Rhodococcus sp. strain TE3(pKLZ11)	NA	429
	EPTC	5,539
	Butylate	5,366
	Molinate	5,207
	Cycloate	5,342
	Pebulate	3,944
	Atrazine	435
	Simazine	409
	Carbofuran	431

[a] *E. coli* cells were grown at 37°C in BMNG medium containing 50 μg of ampicillin per ml, and *Rhodococcus* cells were grown at 30°C in BMNG medium containing 10 μg of thiostrepton per ml.
[b] Thiocarbamate pesticides and carbofuran were added at concentrations of 50 μg/ml. Atrazine and simazine were added at 10 μg/ml.
[c] NA, no addition.

Rhodococcus genus has been cited as an advantage for the potential of the bacteria in bioremediation strategies (8, 35).

It is interesting to notice that the expression of *thcB-lacZ* in LB medium was less than half of that seen in BMNG medium (Table 3). This indicated that the *thcB-lacZ* expression is also under negative control by one or more compounds present in LB medium. To determine which compound(s) in LB medium represses *thcB-lacZ* expression, we tested the effect of all 20 amino acids and several vitamins on *thcB* expression. Only cysteine showed a significant effect (Table 3). Cysteine also showed a similar inhibitory effect on the degradation of atrazine by strain TE3 carrying pKL1 (Fig. 2). The addition of propionaldehyde or propionic acid, which may be produced by N-dealkylation of EPTC (19) by the cytochrome P-450, also repressed *thcB-lacZ* expression by 30 to 40%.

The *thcR* gene codes for a positive regulatory protein for *thcB*. The *thcR* gene, which is divergently transcribed from *thcB*, codes for a regulatory protein of the AraC-XylS family, most members of which represent positive transcriptional regulators (9, 19). In a previous report, we have shown that the 3.9-kb *Eco*RV-*Kpn*I fragment, which contains *thcB*, *thcC*, and *thcD* but not *thcR*, does not confer the herbicide EPTC or atrazine degradation capability on *Rhodococcus* transformants (26), suggesting that the *thcR* gene product was needed for *thcB* expression. Since the *Eco*RV-*Kpn*I fragment carries only a very short upstream region of *thcB* (the *Eco*RV site is 216-bp upstream from the translational start site of *thcB*), it is possible that this fragment may not contain a complete or functional *thcB* promoter. To verify this, we constructed another plasmid, pKLP2, by removing a 1.5-kb *Sph*I fragment from pKL1. This plasmid carries *thcB*, *thcC*, and *thcD* and most of *thcR*, with only 160 bp deleted from the 3′ end of the *thcR* coding region. However, like pEPT6, this plasmid conferred no pesticide degradation activity. By using a strain carrying plasmid pKLZ12, we found that *thcB-lacZ* expression was almost completely abolished by the absence of the function product of *thcR* when cells were grown in BMNG medium. The β-galactosidase activity was only 25 Miller units, compared with 429 Miller units found in the presence of the *thcR* gene. The addition of EPTC did not increase *thcB-lacZ* expression, suggesting that the presence of the ThcR protein is essential for *thcB-lacZ* expression and the induction by thiocarbamate herbicides. We also constructed the plasmid pKLZ13 by reinserting a DNA fragment containing the *thcR* gene into pKLZ12 at a new position. Results showed that *thcB-lacZ* expression recovered to its wild-type level, indicating that *thcR* encodes a *trans*-acting positive regulatory protein for *thcB*.

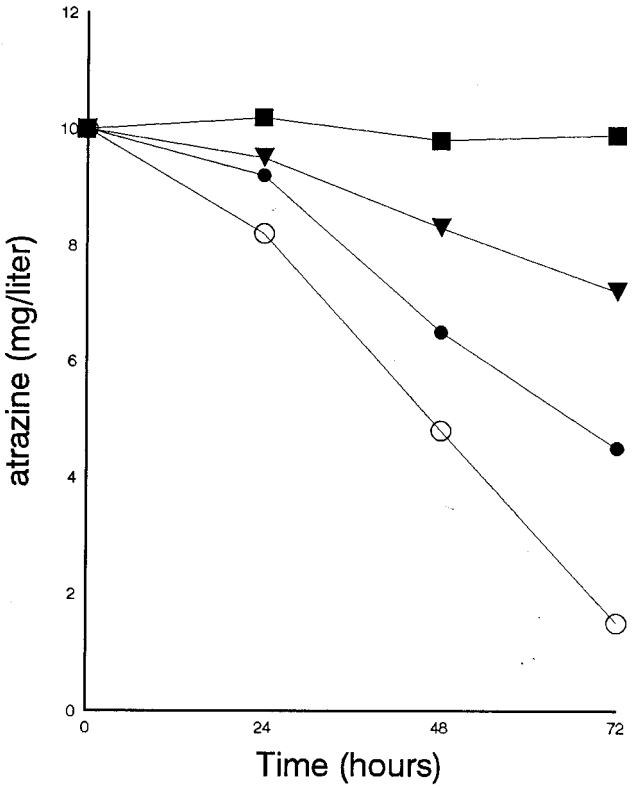

FIG. 2. Effect of growth media on atrazine degradation by *Rhodococcus* sp. strain TE3 carrying pKL1. Symbols: ■, uninoculated control; ▼, in LB medium; ●, in BMNG medium plus cysteine (100 mg/liter); ○, in BMNG medium.

TABLE 3. Effects of carbon type, rich medium, and cysteine on *thcB-lacZ* expression in *Rhodococcus* sp. strain TE3(pKLZ11)[a]

Growth medium[b]	β-Galactosidase activity (Miller units)
BMNG	429
BMNG + EPTC	5,489
BMN + glucose	605
LB	205
LB + EPTC	2,478
BMNG + cysteine	256
BMNG + cysteine + EPTC	2,660

[a] *Rhodococcus* cells (strain TE3/pKLZ11) were grown at 30°C in medium containing 10 μg of thiostrepton per ml.
[b] EPTC was added at a concentration of 50 μg/ml. Cysteine was added at a concentration of 100 μg/ml.

Characterization of the expression of *thcR*. To determine the regulation of the regulatory gene itself, we constructed a *thcR-lacZ* fusion and determined the effects of various growth conditions on *thcR-lacZ* expression. *Rhodococcus* sp. strain TE3 cells carrying pKMZ10 showed 96 Miller units of β-galactosidase activity when grown in BMNG medium, and the enzyme level was slightly lower in LB medium (Table 4). However, unlike the effect on *thcB-lacZ* expression, the addition of EPTC had no effect on *thcR-lacZ* expression (Table 4). In *E. coli* MC4100, the *thcR* promoter is not fully functional, al-

TABLE 4. Effects of growth medium on *thcR-lacZ* expression[a]

Strain	Growth medium	β-Galactosidase activity (Miller units)
E. coli MC4100(pKLZ11)	BMNG	42
	BMNG + EPTC	38
	BMNG + atrazine	39
	LB	29
	LB + EPTC	27
Rhodococcus sp. strain TE3(pKLZ11)	BMNG	96
	BMNG + EPTC	91
	BMNG + atrazine	85
	LB	75
	LB + EPTC	81

[a] Growth conditions and concentrations of additives are described in Table 2, footnotes *a* and *b*.

though the *thcR-lacZ* fusion showed higher expression than the *thcB-lacZ* fusion did (Tables 2 and 4).

Rhodococci are ubiquitous in the environment, possess a wide range of metabolic diversity, lack catabolic repression, can persist in the environment under harsh conditions, and, therefore, have been considered of great potential in bioremediation applications (1, 8, 35). Although some progress has been made in understanding the genetics of the bacteria and a few *Rhodococcus* genes have been cloned, the regulation of gene expression in the genus is at present poorly characterized. This study provides the details on the regulation of the expression of a pesticide-degrading cytochrome P-450 system. By genetically manipulating this system, we are currently attempting to obtain mutants which are as effective for atrazine degradation without EPTC addition as those with EPTC added.

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