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Cloning and Expression of the *s*-Triazine Hydrolase Gene (*trzA*) from *Rhodococcus corallinus* and Development of *Rhodococcus* Recombinant Strains Capable of Dealkylating and Dechlorinating the Herbicide Atrazine†

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We used degenerate oligodeoxyribonucleotides derived from the N-terminal sequence of the *s*-triazine hydrolase from *Rhodococcus corallinus* NRRL B-15444R in an amplification reaction to isolate a DNA segment containing a 57-bp fragment from the *trzA* gene. By using the nucleotide sequence of this fragment, a nondegenerate oligodeoxyribonucleotide was synthesized and used to screen a genomic library of *R. corallinus* DNA for fragments containing *trzA*. A 5.3-kb *Pst*I fragment containing *trzA* was cloned, and the nucleotide sequence of a 2,450-bp region containing *trzA* was determined. No *trzA* expression was detected in *Escherichia coli* or several other gram-negative bacteria. The *trzA* gene was subcloned into a *Rhodococcus-E. coli* shuttle vector, pBS305, and transformed into several *Rhodococcus* strains. Expression of *trzA* was demonstrated in all *Rhodococcus* transformants. *Rhodococcus* sp. strain TE1, which possesses the catabolic gene (*atrA*) for the N-dealkylation of the herbicides atrazine and simazine, was able to dechlorinate the dealkylated metabolites of atrazine and simazine when carrying the *trzA* gene on a plasmid. A plasmid carrying both *atrA* and *trzA* was constructed and transformed into three *atrA*- and *trzA*-deficient *Rhodococcus* strains. Both genes were expressed in the transformants. The *s*-triazine hydrolase activity of the recombinant strains carrying the *trzA* plasmid were compared with that of the *R. corallinus* strain from which it was derived.

The *s*-triazine herbicides have been used in a variety of weed control programs with major crops. Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) is one of the most heavily used herbicides in North America (21). The detection of atrazine in groundwater and surface water has prompted some environmental concerns (1, 14). There has been considerable interest in finding microbial activities that might be used to degrade atrazine and other *s*-triazine compounds in herbicide wastes and contaminated soils (6, 7, 12, 16, 24). Several microorganisms that can metabolize atrazine have been isolated during the last few years (2, 3, 17, 18, 25, 33). We recently reported that *Rhodococcus* sp. strain TE1 can degrade atrazine efficiently to produce the dealkylated metabolites deisopropylatrazine (2-chloro-4-ethylamino-6-amino-*s*-triazine [CEAT]) and deethylatrazine (2-chloro-4-amino-6-isopropylamino-*s*-triazine [CIAT]) and that this catabolic function was associated with an indigenous 77-kb plasmid in this bacterium (4, 28). Two recently reported *Pseudomonas* isolates (18, 33) degrade atrazine efficiently but by different pathways.

Cook and Hutter (5, 8) isolated and characterized an isolate of *Rhodococcus corallinus* that dechlorinates and deaminates the dealkylated *s*-triazine compounds CEAT and CIAT but not atrazine. The enzyme, named *s*-triazine hydrolase and encoded by the *trzA* gene, has been purified (23). The combination of the catabolic activities of both *Rhodococcus* sp. strain TE1 and *R. corallinus* would result in dealkylation and dechlorination of atrazine.

We have been involved in developing genetic methods to manipulate bacterial activities for bioremediation of atrazine- and other pesticide-contaminated wastes and spills. We constructed a new *Escherichia coli-Rhodococcus* shuttle vector, pBS305, which was successfully used in an electroporation-mediated transformation system in a number of *Rhodococcus* strains (27). We used this vector to clone the *atrA* gene encoding the enzyme for atrazine N-dealkylation from *Rhodococcus* sp. strain TE1 and reported its expression in several *Rhodococcus* strains. However, we were unable to introduce the plasmid carrying *atrA* into the *R. corallinus* strain to obtain a *Rhodococcus* recombinant capable of N-dealkylating and dechlorinating atrazine (26).

In this study, we report on the cloning of the dechlorinating (hydrolytic) activities of *R. corallinus* toward CEAT and CIAT (encoded by the *trzA* gene) and combine them with the atrazine-dealkylating capabilities of *Rhodococcus* sp. strain TE1 (or the cloned *atrA* gene), which produces the substrates (CEAT and CIAT) required by the hydrolase. The *trzA* gene was expressed in several *Rhodococcus* strains when subcloned into pBS305. A plasmid carrying both *atrA* and *trzA* was constructed and transformed into three *atrA*- and *trzA*-deficient *Rhodococcus* strains, and both genes were expressed in the recombinant cells.

MATERIALS AND METHODS

Bacteria and growth conditions. The bacterial strains and plasmids used in this study are described in Table 1. *Rhodococcus* strains were grown in BMN (2) medium supplemented with 0.1% glycerol (BMNG) or in Luria-Bertani medium at 30°C with shaking. *E. coli* strains were grown in Luria-Bertani medium at 37°C. Luria-Bertani medium was supplemented with 1.5% (wt/vol) Bacto Agar (Difco)

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype	Origin or reference
Rhodococcus spp.		
TE1	*atrA*$^+$ *trzA*	28
TE3	*atrA* derivative of TE1	28
UP	*atrA trzA* Rif^r Km^r	4
R. erythropolis 4277	*atrA trzA*	ATCC[a]
R. corallinus NRRL B-15444R	*trzA*$^+$ *atrA*	23
E. coli DH5α	*hsdR17 recA1 thi-1 relA1*	15
Plasmids		
pUC19	*E. coli* vector, Ap^r	32
pBS305	*E. coli-Rhodococcus* shuttle vector, Ap^r Thio^r	27
pSWP1	A 5.3-kb *Pst*I fragment into pUC19	This work
pKL1	A 6.2-kb *Kpn*I fragment cloned into pBS305	26
pP18	A 5.3-kb *Pst*I fragment cloned into pBS305	This work
pP19	A 2.3-kb *Mlu*I-*Pst*I fragment cloned into pBS305	This work
pP20	A 3-kb *Pst*I-*Mlu*I fragment cloned into pBS305	This work
pKL13	A 3-kb *Pst*I-*Mlu*I fragment cloned into pKL1	This work

[a] ATCC, American Type Culture Collection, Rockville, Md.

for growth on solid medium, and BMN medium was solidified with 1% bacteriological agar (Oxoid).

Antibiotic concentrations used were as follows: ampicillin, 100 μg/ml; thiostrepton, 10 μg/ml; kanamycin, 25 μg/ml. Thiostrepton was kindly supplied by S. Lucania, Squibbs Institute of Medical Research, Princeton, N.J.

Chemicals. Atrazine, simazine (CEET), CEAT, and CIAT were purchased from ChemService, Westchester, Pa., and were at least 96% pure. Analytical standards of CEAT metabolites, *N*-isopropylammeline (OEAT), and *N*-ethylammelide (OOET) were kindly supplied by Ciba Geigy, Greensboro, N.C.

Metabolism of *s*-triazine compounds. The ability of bacteria to metabolize atrazine, CEET, CIAT, and CEAT was studied by using batch cultures in BMNG medium supplemented with 5 to 10 mg of the substrate per liter. The degradation was measured by determining the residual amount of the substrate at intervals by high-pressure liquid chromatography (HPLC) or gas chromatography (GC) as previously described (4). The *s*-triazine hydrolase activity of the cultures was assayed by measuring the disappearance of CEAT from the medium over time and correlating the decrease in the amount of CEAT with the amount of inorganic chloride released into the incubation medium. This was initially established by incubating CEAT with twice-washed and resuspended cells in 50 mM chloride-free phosphate buffer containing 5 mM magnesium sulfate. Inorganic chloride in cell-free supernatants was measured colorimetrically (30). The amount of each triazine in the sample was calculated on the basis of the peak areas obtained with authentic standard samples analyzed under the same conditions. Appropriate control incubations were simultaneously included in the assays. The nature of the metabolite(s) formed from CEAT was determined by HPLC (4), except that the mobile phase was 95% water and 5% acetonitrile at 1 ml/min with the detector set at 230 nm. The identification of CEAT metabolites was confirmed by GC-mass spectrometry (MS) analysis of the incubation extracts following methylation with diazomethane as previously described (3). The spectra were compared with those of methylated samples of authentic OEAT and OOET. The deaminase activity (deamination of melamine [2,4,6-triamino-1,3,5-triazine; AAAT] to ammeline [AAOT]) reported to be associated with *s*-triazine hydrolase was determined as described previously (23). Alternatively, the ability of the bacteria to utilize AAAT as the sole source of nitrogen for growth was used as a measure of deaminase activity.

Plasmid isolation from *Rhodococcus* strains and transformation of *Rhodococcus* strains by electroporation. A modified alkali lysis procedure was used to isolate plasmid DNA from *Rhodococcus* strains (27). All electroporations were performed with an Electro Cell Manipulator 600 electroporation system (BTX Corp., San Diego, Calif.) by following the protocol described previously (26). Cells were plated on Luria-Bertani plates containing thiostrepton.

Determination of the amino acid sequence of the first 25 residues of *s*-triazine hydrolase. An *s*-triazine hydrolase was purified from extracts of *R. corallinus* (23), and the amino acid sequence of the first 25 residues was determined from the purified protein with an Applied Biosystem model 477A peptide sequencer.

Strategy for cloning the *trzA* gene from *R. corallinus* NRRL B-15444R. Two highly degenerate oligodeoxyribonucleotides, WS-3 and WS-4, were synthesized for a DNA amplification reaction which would amplify a 73-bp DNA segment encoding the N-terminal sequence of the *Rhodococcus s*-triazine hydrolase. The sequence of WS-3 corresponds to residues 1 to 6 of the N-terminal sequence of the hydrolase with an *Eco*RI linker at its 5′ end. The sequence of WS-4 corresponds to the complement of residues 13 to 19 of the N-terminal sequence of the protein with a *Pst*I linker at its 5′ end. An amplification reaction with WS-3, WS-4, and genomic DNA from *R. corallinus* yielded the predicted 73-bp DNA segment. This DNA was isolated from the gel, digested with *Pst*I and *Eco*RI, and ligated to the vector pUC19. Nucleotide sequencing of a plasmid containing this segment yielded the unambiguous sequence coding for N-terminal residues 6 to 13. The nondegenerate oligodeoxyribonucleotide WS-6 was synthesized according to this unambiguous sequence and used as a probe for *trzA* (Fig. 1). Southern blots of restriction endonuclease-digested genomic DNA from *R. corallinus* were probed with WS-6 to choose the appropriate enzyme for cloning DNA fragments containing *trzA*. Subsequently, subgenomic libraries containing *Pst*I fragments from *R. corallinus* were screened by colony hybridization to WS-6. A 5.3-kb *Pst*I fragment containing *trzA* was cloned into *E. coli* by using pUC19 to yield pWSP1. A restriction map of pWSP1 is shown in Fig. 2A.

Detailed procedures used for DNA amplification reactions, DNA fragment isolation, subcloning, DNA sequencing, and colony hybridization have been described previously (22).

Subcloning of the *trzA* gene into *E. coli-Rhodococcus* shuttle vector pBS305. Plasmid pSWP1 was digested with *Pst*I, and the 5.3-kb fragment containing the *trzA* gene was isolated from an agarose gel by the Geneclean protocol (Bio101, La Jolla, Calif.) (29). This fragment was ligated into the *Pst*I site of pBS305, resulting in pP18. To further subclone the *trzA* gene, a 2.2-kb *Hin*dIII-*Mlu*I fragment from pSWP1 was blunt ended with Klenow enzyme and then cloned into pBS305 that had been digested with *Hin*dIII and blunt ended with Klenow enzyme, to give pP19. Also, a 3-kb *Xba*I-*Mlu*I fragment from pSWP1 was isolated, treated with Klenow enzyme, and ligated to pBS305 which had been digested with *Hin*dIII and blunt ended with Klenow, to form pP20 (Fig. 2B).

Construction of a plasmid carrying both *atrA* and *trzA*. The *atrA* gene has been shown to be located on a 6.2-kb *Kpn*I fragment cloned on pBS305 (pKL1) (26). The results of subcloning of *trzA* demonstrated that this gene resides on the 3-kb *Pst*I-*Mlu*I fragment cloned in pBS305 (pP20). To construct a plasmid carrying both *atrA* and *trzA*, a 3-kb *Xba*I-*Mlu*I fragment was isolated from pSWP1, treated with Klenow enzyme, and ligated to pKL1 digested with *Hin*dIII and blunt-ended with Klenow, forming pKL13 (Fig. 2B).

Other genetic methods. Plasmid isolation from *E. coli*, DNA manipulations,

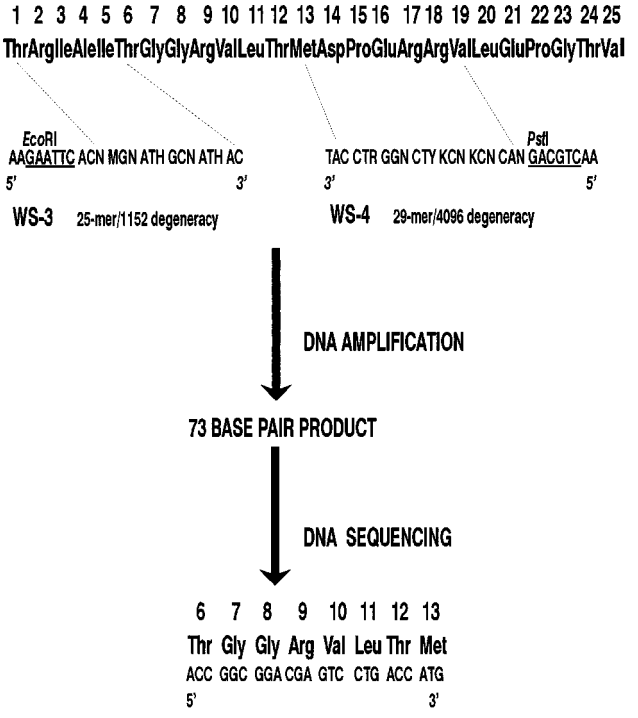
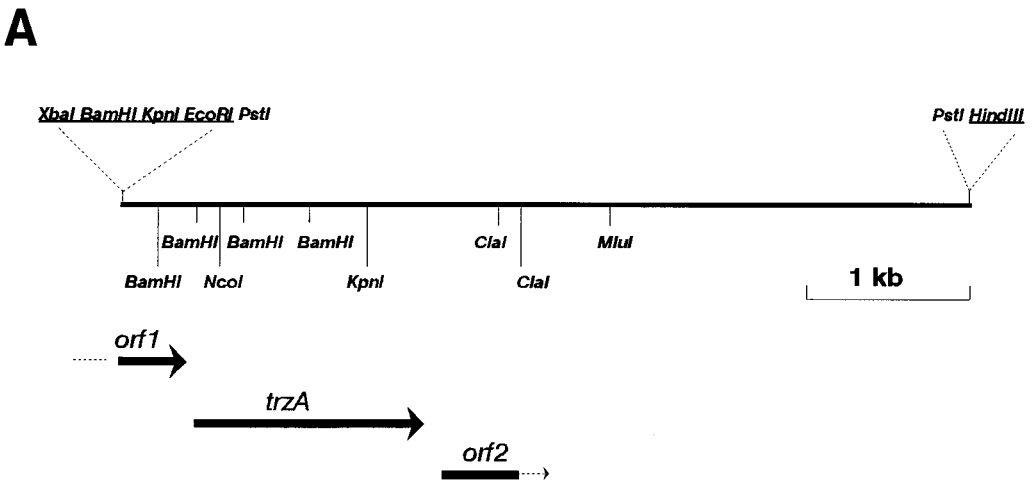


FIG. 1. Strategy for cloning the *trzA* gene from *R. corallinus* NRRL B-15444R.



B

Host strains	Plasmid	Insert	*trzA* phenotype	*atrA* phenotype
E.coli DH 5 α	pSWP1	PstI MluI PstI	−	−
Rhodococcus sp. TE3	pP18	PstI MluI PstI	+	−
Rhodococcus sp. TE1	pP18	PstI MluI PstI	+	+
Rhodococcus sp. TE3	pP19	MluI PstI	−	−
Rhodococcus sp. TE3	pP20	PstI MluI	+	−
Rhodococcus sp. TE3	pKL13	PstI MluI	+	+

FIG. 2. (A) Partial restriction map of the 5.3-kb *Pst*I fragment cloned on pSWP1. Restriction sites from the polylinker site of pUC19 are underlined. (B) Subcloning of the *trzA* gene and its expression.

and plasmid transformation into *E. coli* were performed as in the cloning manual (19).

Nucleotide sequence accession number. The nucleotide sequence of the *trzA* gene has been submitted to GenBank as no. L16534.

RESULTS

Cloning of the *trzA* gene from *R. corallinus*. Previous purification of the *s*-triazine hydrolase from *R. corallinus* demonstrated that the enzyme is a tetramer composed of four identical subunits of approximately 54 kDa (23). With 36 μg of purified, desalted *s*-triazine hydrolase, N-terminal analysis yielded the sequence of the first 25 amino acids (Fig. 1). With degenerate oligonucleotides derived from the N-terminal sequence, an amplification reaction was used to isolate a DNA segment containing a 57-bp fragment from the *trzA* gene. By using the sequence of this fragment, a nondegenerate oligonucleotide (WS-6) was synthesized and used to screen a subgenomic library of *R. corallinus* DNA for fragments containing *trzA*. A 5.3-kb *Pst*I fragment containing *trzA* was cloned into *E. coli* by using pUC19 to yield pWSP1. The general cloning strategy used for isolating the *Rhodococcus trzA* gene is shown in Fig. 1, and a restriction map of the 5.3-kb *Pst*I fragment on pWSP1 is shown in Fig. 2A.

Expression of the cloned *trzA* gene in gram-negative bacteria. The *E. coli* host strain DH5α containing pWSP1 showed no detectable *s*-triazine hydrolase activity. Reversal of the orientation of the insert (so that *trzA* is in the same orientation as the *lac* promoter in pUC19) had no effect. Attempts to express *trzA* by placing it under control of other *E. coli* promoters were unsuccessful (data not shown). The broad-host-range plasmid pMMB277 (20) was used to transfer the DNA fragment containing the *trzA* gene into other gram-negative bacterial hosts, *Klebsiella pneumoniae* and *K. planticola*, and into the *s*-triazine-degrading *Pseudomonas* strain NRRL B-12227 (9, 10); however, no *trzA* hydrolase activity was detected in these recombinant strains (data not shown).

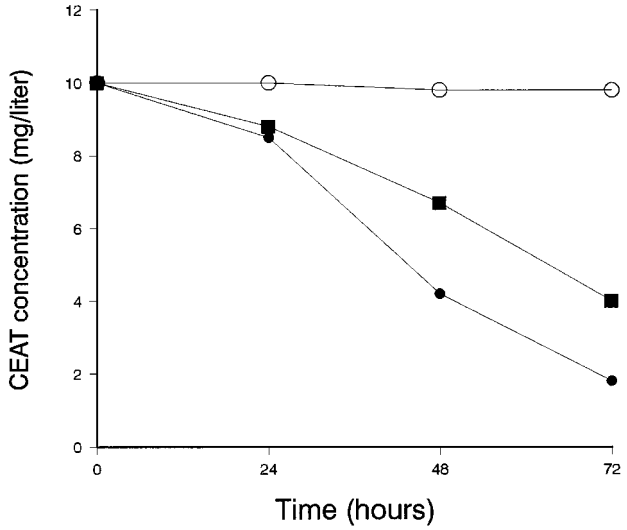


FIG. 3. CEAT degradation by *R. corallinus* and *Rhodococcus* sp. strain TE3 carrying the cloned *trzA* gene. All the cultures were adjusted to the same optical density (0.16 at 600 nm) at time zero. Symbols: ○, with *Rhodococcus* sp. strain TE3; ■, with *R. corallinus* NRRL B-15444R; ●, with strain TE3 carrying the cloned *trzA* gene (pP18).

Expression of the cloned *trzA* gene in *Rhodococcus* sp. strain TE3. In subsequent experiments, we subcloned the 5.3-kb *Pst*I fragment on pSWP1 into an *E. coli-Rhodococcus* shuttle vector pBS305 (27) and transformed the recombinant plasmid, pP18, into *Rhodococcus* sp. strain TE3 by electroporation. This strain, a derivative of *Rhodococcus* sp. strain TE1, lacks the capability to metabolize atrazine and other *s*-triazines because of the absence of the 77-kb plasmid that is present in *Rhodococcus* sp. strain TE1 and that was shown previously to be associated with the catabolic functions (26). The results of the degradation of CEAT by *Rhodococcus* sp. strain TE3 carrying pP18 and the *R. corallinus* strain, both adjusted to the same cell density (optical density at 600 nm) in BMNG medium, are shown in Fig. 3. The recombinant strain degraded CEAT at a higher rate than did *R. corallinus* from which the *trzA* gene was cloned.

The *s*-triazine hydrolase activity in *R. corallinus* is inducible, with optimal activity present in cells grown with 2-chloro-4,6-diamino-1,2,3-triazine (CAAT), AAAT, or 1,3,5-triazine-2,4,6-triol (OOOT) as the nitrogen source. Addition of these compounds to media in which ammonia was used as the nitrogen source also induced triazine hydrolase activity but at lower levels (23). Unlike *R. corallinus*, *Rhodococcus* sp. strain TE3 cannot use OOOT or AAAT as the nitrogen source for growth. We found that the degradation of CEAT by *R. corallinus* was greatly induced by the presence of OOOT in the incubation medium while the efficiency of CEAT degradation by *Rhodococcus* sp. strain TE3 carrying pP18 remained the same (data not shown). That the decrease in CEAT concentration actually represented *s*-triazine hydrolase activity was confirmed by stoichiometric release of inorganic chloride during incubation of the cells in chloride-free phosphate buffer-magnesium sulfate medium. Colorometric measurement showed that 5.7 μmol of Cl was released when 6.0 μmol of CEAT was degraded. The hydrolase activity does not require any addition of cofactors (23). The results clearly show that the chloride moiety of CEAT is removed by *Rhodococcus* sp. strain TE3 cells carrying pP18 and that the gene cloned on pP18 codes for *s*-triazine hydrolase.

Plasmid pP18 was also introduced into *Rhodococcus* sp. strain UP and *R. erythropolis* 4277 by electroporation. All of the recombinant cells showed the capability to dechlorinate CEAT (Table 2). The amount of CEAT dechlorinated by the recombinants, similar to the results in Fig. 3, was greater than the amount dechlorinated by *R. corallinus.*

TABLE 2. Expression of *atrA* and *trzA* in *Rhodococcus* strains

Strain	Plasmid	% of wild-type AtrA activity[a]	% of wild-type TrzA activity[b]
Rhodococcus sp. strain TE1	None	100	0
R. corallinus NRRL B-15444R	None	0	100
Rhodococcus sp. strain TE1	pP18	108	165
	pKL13	NT[c]	NT
Rhodococcus sp. strain TE3	pP18	0	160
	pKL13	106	158
Rhodococcus sp. strain UP	pP18	0	160
	pKL13	102	160
R. erythropolis	pP18	0	145
ATCC 4277	pKL13	97	152

[a] The cultures were adjusted to the same cell density (0.15 at 600 nm) at zero time. 100% of wild-type AtrA activity represents the amount of atrazine dealkylated after 48 h of incubation with *Rhodococcus* strain TE1.
[b] 100% wild-type TrzA activity represents the amount of CEAT dechlorinated after 48 h of incubation with *R. corallinus* NRRL B-15444R.
[c] NT, not tested.

Identification of CEAT metabolites. No accumulation of OEAT formed from CEAT was found in the incubation medium, presumably because of its deamination to OOET as shown previously with *R. corallinus* (8). We did observe the formation of OEAT from *R. corallinus* and from *Rhodococcus* sp. strain TE3 carrying pP18 when the washed suspensions of the bacterial cultures were incubated in CEAT-supplemented chloride-free phosphate buffer at low cell densities of 0.2 (at 600 nm). HPLC analysis of the supernatants showed the presence of OEAT and OOET in addition to CEAT. OEAT and OOET were eluted at 6.5 and 3.5 min, respectively (see Materials and Methods). The formation of the metabolites was further supported by cochromatography with authentic standards of OEAT and OOET. The metabolites were then extracted, methylated, and analyzed by GC-MS, and the spectra and fragmentation patterns were compared with those of the methylated authentic compounds.

Three peaks with retention times of 17.5, 11.6, and 5.3 min were seen on the gas chromatogram. The GC-MS of the peak with the retention time of 17.5 min showed a molecular ion at *m/e* 173 in addition to the chlorine isotopic peak ($M^{\cdot+}$ + 2). Furthermore, the molecular ion decomposed with loss of CH_3 and C_2H_4 to give *m/e* 158 and 145. The spectrum and further fragmentation pattern were analogous to those of the authentic deisopropylatrazine. The mass spectrum of the methylated material represented by the GC peak (R_t 11.6 min) exhibited a molecular ion at *m/e* 184. The molecular ion decomposed with loss of CH_3, C_2H_4, and C_2H_5N to give *m/e* 169, 156, and 141, respectively. The spectrum and subsequent fragmentation pattern were analogous to those of the authentic methylated OOET. The GC-MS results for the peak with the retention time of 5.3 min showed a molecular ion at *m/e* 169 which decomposed with the loss of CH_3 to give *m/e* 154 and a loss of C_2H_5N from the molecular ion with the formation of *m/e* 126. The spectrum and the fragmentation pattern were analogous to those of the authentic methylated OEAT. The mass spectra of the methylated metabolites represented by molecular ions at *m/e* 184 and 169 showed no chlorine isotopic peaks, indicating the elimination of Cl from the 2 position of CEAT.

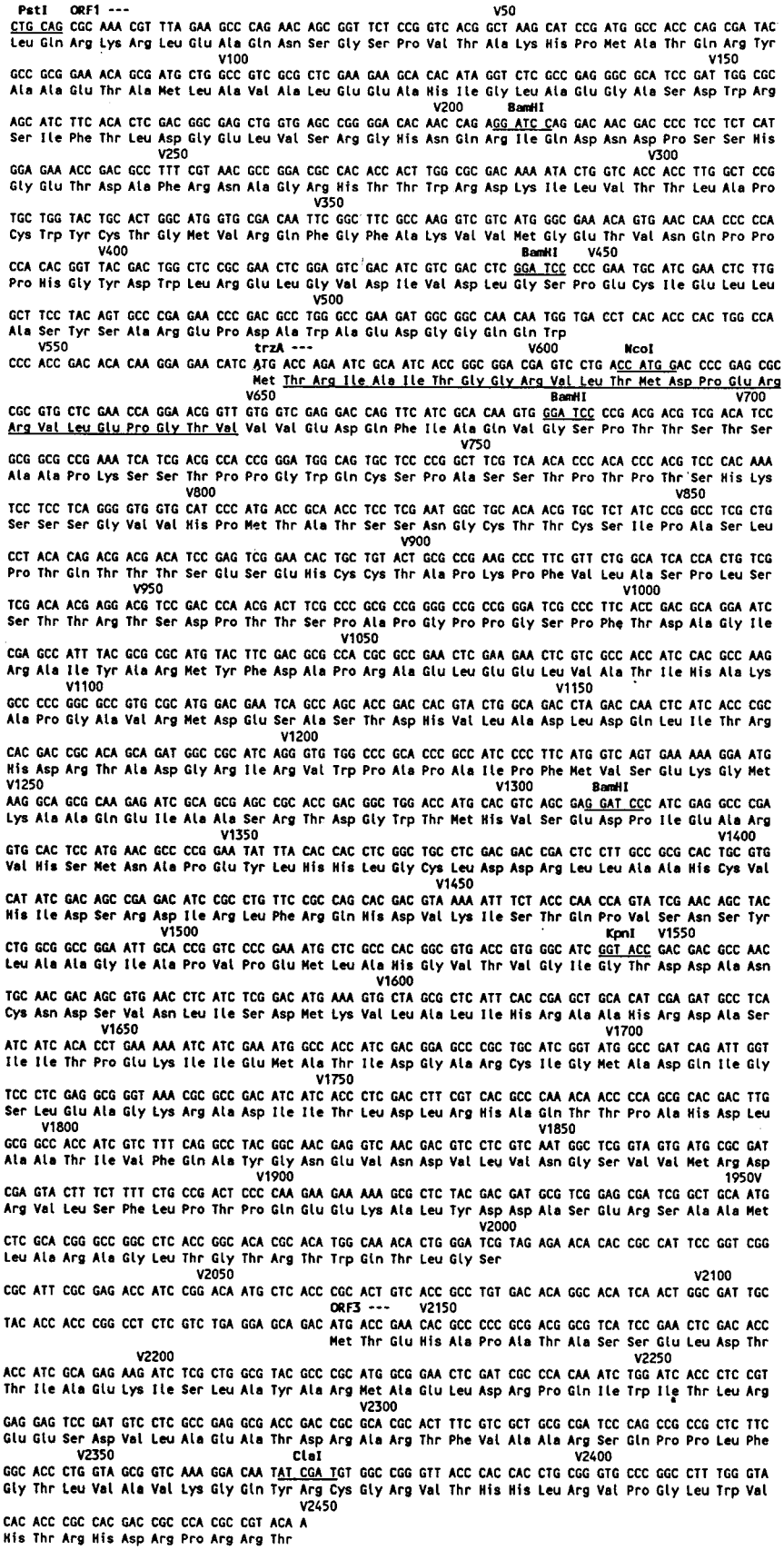


FIG. 4. Nucleotide and deduced amino acid sequences of the *trzA* gene and hypothetical polypeptides from ORF1 and ORF2. The N-terminal residues of the hydrolase protein that was purified from *R. corallinus* extracts are underlined. Restriction sites shown in Fig. 3 are underlined.

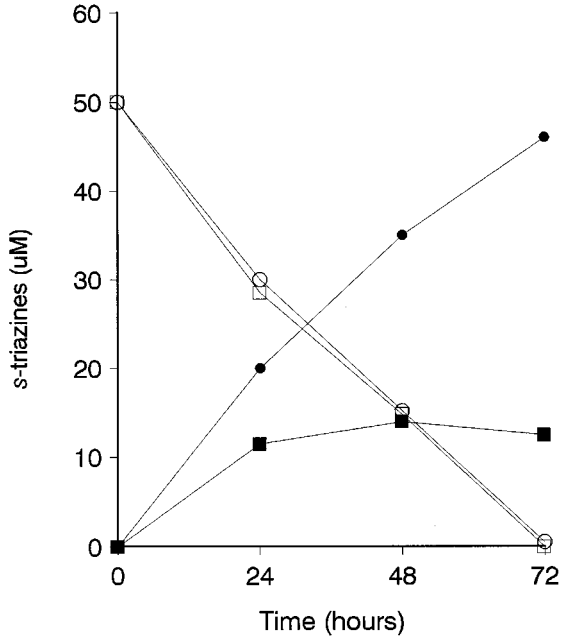


FIG. 5. CEET dealkylation by *Rhodococcus* sp. strain TE1 and dealkylation and dechlorination by strain TE1 carrying pP18. ○, CEET degradation by *Rhodococcus* sp. strain TE1; ●, CEAT production from CEET by strain TE1; □, CEET degradation by strain TE1 carrying pP18; ■, CEAT production from CEET by strain TE1 carrying pP18.

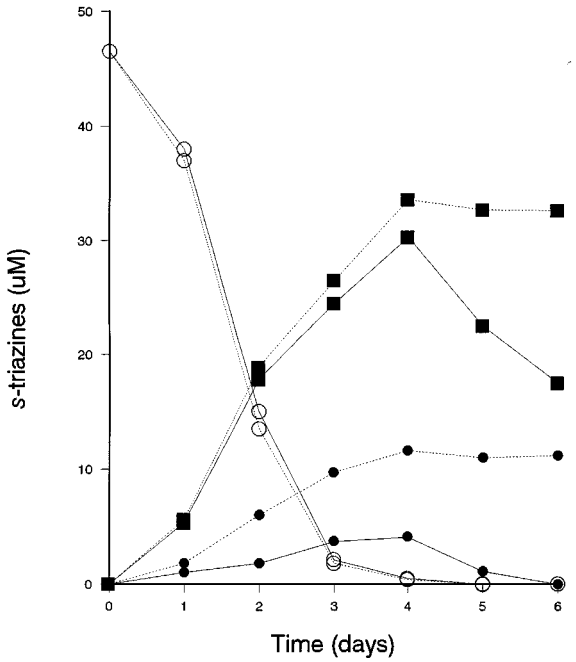


FIG. 6. Atrazine dealkylation and dechlorination by *Rhodococcus* sp. strain TE1 carrying pP18. ○, Atrazine degradation by *Rhodococcus* sp. strain TE1 carrying pP18; ■, CIAT production from atrazine by strain TE1 carrying pP18; ●, CEAT production from atrazine by strain TE1 carrying pP18. The corresponding dotted lines represent the amounts of *s*-triazines degraded or formed by strain TE1 not carrying pP18.

Subcloning of the *trzA* gene. The 2.2-kb *Hin*dIII-*Mlu*I fragment and 3-kb *Xba*I-*Mlu*I fragment from the 5.3-kb insert on pSWP1 were subcloned into pBS305, forming pP19 and pP20, respectively (Fig. 2B). Both plasmids were transformed into *Rhodococcus* sp. strain TE3 and tested for *s*-triazine hydrolase activity with CEAT as the substrate. *Rhodococcus* sp. strain TE3 carrying pP20 possessed the CEAT dechlorination activity, while the strain carrying pP19 did not, indicating that the *trzA* gene is located on the 3-kb *Xba*I-*Mlu*I fragment.

Nucleotide sequence of the *trzA* gene. Figure 4 shows the nucleotide sequence of a 2,450-bp region of DNA from the 3-kb *Xba*I-*Mlu*I fragment. Within this region, there is an open reading frame (ORF) which starts with an ATG at position 572 and ends with a TAG at 2002. A putative ribosome-binding site (Shine-Dalgarno sequence), -GGAG-, is present 10 bp upstream of the ATG codon. Analysis of this ORF revealed that immediately following the ATG start codon is the threonine codon ACC, which corresponds to the N-terminal residue of the TrzA protein as determined by protein sequencing. Therefore, it is likely that translation of the *trzA* gene is initiated at the ATG codon at position 572 and that the resulting polypeptide is subsequently processed to remove the N-terminal methionine residue. The predicted translation product of the *trzA* gene is 476 amino acids and has an isoelectric point of 5.9. The predicted polypeptide size (50,800 Da) is somewhat smaller than the 54,000 Da determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified protein (23). Analysis of codon usage within *trzA* reveals a pronounced preference for GC-rich codons as a result of the high (62%) G+C content of the gene. Incomplete ORFs were found upstream and downstream of *trzA* (Fig. 4). In addition, a 1,006-bp ORF was found on the opposite strand from *trzA* (positions 1023 to 17).

Dealkylation and dechlorination of CEET. *Rhodococcus* sp. strain TE1 dealkylates CEET to produce CEAT, which is most probably catalyzed by the same enzyme which dealkylates atrazine to form CEAT and CIAT (4). We introduced plasmid pP18 into *Rhodococcus* sp. strain TE1 to generate a recombinant strain and tested its capability for dealkylation and dechlorination of simazine. As shown in Fig. 5, CEET is degraded as efficiently by strain TE1 as by strain TE1 carrying pP18. The presence of pP18 in *Rhodococcus* sp. strain TE1, therefore, does not affect its CEET-dealkylating capability. However, the amount of CEAT formed with the recombinant strain at 48 and 72 h was only about 30% of the amount formed with strain TE1, indicating that 70% of CEAT is degraded further by the recombinant strain, presumably dechlorinated by triazine hydrolase as a result of the inclusion of pP18 in cells. It has previously been shown that CEAT is not degraded further by *Rhodococcus* sp. strain TE1 and accumulates in the incubation medium (4).

Dealkylation and dechlorination of atrazine. Dealkylation of atrazine (by the product of the *atrA* gene) in *Rhodococcus* sp. strain TE1 yields CEAT and CIAT at a ratio of approximately 1:3 (see Fig. 7, series 3). Both CEAT and CIAT have been reported to be dechlorinated by the *s*-triazine hydrolase, with the rate of CEAT dechlorination being much higher than that of CIAT (23). When atrazine was incubated with strain TE1 carrying pP18, both CEAT and CIAT were present in the medium in amounts similar to those found with strain TE1 alone until about 24 h (Fig. 6). However, the ratio of CEAT and CIAT decreased to about 1:7 after 4 days of incubation and to more than 1:10 after 5 days. There appears to be a lag between dealkylation of atrazine and dechlorination of CEAT and CIAT. This lag may partly be explained by the presence of atrazine in the incubation medium. The competitive inhibition of CEAT dechlorination by atrazine has been reported (23). The results also show that CEAT was degraded faster than

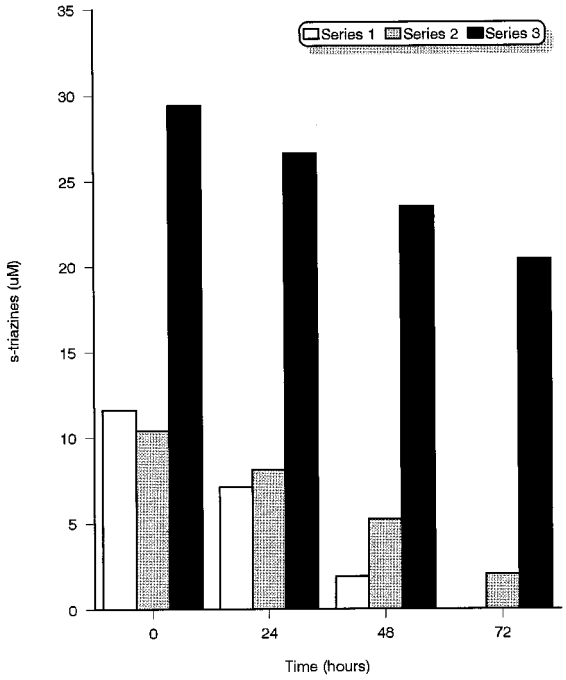


FIG. 7. Effect of the presence of CIAT on the dechlorination of CEAT. Series 1, dechlorination of CEAT by *Rhodococcus* sp. strain TE3/pP18; series 2, dechlorination of CEAT in the presence of CIAT by strain TE3/pP18; series 3, dechlorination of CIAT in the presence of CEAT by strain TE3/pP18.

CIAT formed from atrazine by *Rhodococcus* sp. strain TE1 in the initial 4 days. Similar results were reported with the hydrolase enzyme (23).

To further test the effect of CIAT on dechlorination of CEAT, we first grew *Rhodococcus* sp. strain TE1 in BMNG medium containing 10 mg of atrazine per liter for about 5 days, at which time atrazine was completely degraded to CEAT and CIAT. The cells were then aseptically removed by centrifugation, and the cell-free supernatant containing only CEAT and CIAT was inoculated with *Rhodococcus* sp. strain TE3 cells carrying pP18. Another portion of the cell-free supernatant was inoculated with strain TE3 without the plasmid and served as the control. Both suspensions were incubated, and the dechlorination of CEAT and CIAT was monitored over time and compared with the dechlorination of 2 mg of CEAT per liter alone (close to the amount of CEAT produced by strain TE1 from 10 mg of atrazine per liter) inoculated with strain TE3 cells carrying pP18. Results in Fig. 7 show that 2 mg of CEAT per liter was completely dechlorinated after 72 h of incubation and that the presence of CIAT in the medium inhibited CEAT dechlorination by the cloned *s*-triazine hydrolase. The results are consistent with the reported affinities of the two substrates with the purified enzyme (23).

Plasmid pKL13, which carries both the *atrA* gene and the *trzA* gene, was transformed into *Rhodococcus* sp. strain TE3, *Rhodococcus* sp. strain UP, and *R. erythropolis* ATCC 4277. The recombinant strains degraded atrazine similarly to the results obtained with strain TE1 carrying pP18 (Table 2). The cloning of the *atrA* gene from *Rhodococcus* sp. strain TE1 has been reported previously (26).

DISCUSSION

Since our report on the cloning of *atrA* genes (26), the need to obtain genes for the dechlorination of atrazine or the products of *atrA* activity have become increasingly important in the overall context of developing technology to accomplish atrazine detoxification. The results reported in this paper provide two such systems; one combines atrazine N-dealkylation activity in *Rhodococcus* sp. strain TE1 and the cloned *trzA* genes, while the other results from transformed *Rhodococcus* strains which harbor both *atrA* and *trzA* on the pBS305 vector. The combined catabolic capabilities of dealkylation and dechlorination reported here would result in the production of OEAT and *N*-isopropylammeline (OAIT) from atrazine, which undergo deamination to produce OOET and *N*-isopropylammelide (OOIT), the two nonphytotoxic products of atrazine.

The *trzA* gene was initially located on a 3-kb *Pst*I-*Mlu*I fragment (Fig. 2B). A 1,434-bp ORF on this fragment showed the corresponding sequence to the N-terminal residues of *s*-triazine hydrolase, suggesting that this ORF codes for the *trzA* gene (Fig. 4). A FastDB search (IntelliGenetics, Inc., Mountain View, Calif.) of the GenBank and EMBL databases as well as the PIR and Swis-Pro protein databases revealed no significant similarities to *trzA* at either the nucleotide or amino acid level. This is not surprising, since *s*-triazine dechlorination genes are generally uncommon and only three other *s*-triazine degradation genes have been isolated (10, 11).

The enzymatic assays showed higher activity of the *s*-triazine hydrolase in different transformed *Rhodococcus* strains compared with the activity present in the parental *R. corallinus* strain. The cloned gene was apparently fully expressed in transformed *Rhodococcus* sp. strain TE3 without the inducer, while the hydrolase activity of the parent strain was greatly augmented in the presence of the inducer. This indicates that the expression of *s*-triazine hydrolase activity is regulated differently in the two systems by OOOT, the inducer used in this study. It is not clear if this observation is related to the fact that, unlike *R. corallinus*, *Rhodococcus* strain TE3 cannot utilize OOOT as a source of nitrogen for growth. The reported deaminase activity toward AAAT associated with *s*-triazine hydrolase (8, 23) was not detectable in the *Rhodococcus* recombinants, which did not utilize AAAT as a source of nitrogen for growth.

Microorganisms degrade atrazine in the environment by N-dealkylation, dechlorination, and deamination followed by cleavage of the *s*-triazine ring (12). Several bacterial isolates belonging to the genus *Rhodococcus* dealkylate atrazine but are unable to cleave the *s*-triazine ring (3, 4). Recently, two *Pseudomonas* isolates capable of mineralizing atrazine and utilizing the herbicide as the sole source of carbon and/or nitrogen for growth have been reported (18, 33). These bacteria degrade atrazine by a pathway that is as yet unelucidated but is different from that reported for rhodococci (3, 4, 23, 26). The initial product formed from atrazine by the *Pseudomonas* species appeared to be hydroxyatrazine, and the formation of dealkylated metabolites of atrazine was not evident.

The amount of atrazine dealkylated by *Rhodococcus* sp. strain TE1 or the recombinant strains carrying plasmid pKL1 (*atrA*) (26) (almost 10 mg of the herbicide per liter dealkylated in about 72 h) is much greater than the amount dealkylated by the only other bacteria, *Pseudomonas* species, reported to dealkylate atrazine (2). It took much longer to completely dealkylate and dechlorinate atrazine by strains carrying both *atrA* and *trzA*. The amount of atrazine directly dechlorinated by the two *Pseudomonas* strains is far greater than the amount dechlorinated by the recombinant *Rhodococcus* sp. strain TE1 carrying plasmid pP18 (*trzA*) (18, 33). Although the dechlorinating gene or its product has not been identified, the *Pseudomonas* strains may provide yet another system for atrazine detoxification.

Rhodococci are ubiquitous and numerous in soil, possess a wide spectrum of catabolic activities, show no catabolic repression, and are able to survive under extremely harsh conditions (13, 31). This makes them ideal candidates for manipulation for potential exploitation in commercial and bioremediation technology. The members of this genus have been successfully used in industry (31). The results of this study indicate that these bacteria may be used for the development of bioremediation technology for atrazine-contaminated wastes and spills. Atrazine degradation by the recombinant strains may be slow, but additional research into genetic regulatory mechanisms may provide ways to obtain bacterial strains with enhanced atrazine-catabolic functions. Manipulation of the cloned genes may also produce enzymes with higher activities. The cloned genes could be further engineered for introduction into plant protoplasts such that atrazine-resistant or -tolerant plants are generated for use when atrazine residues in soil may pose a phytotoxicity problem.

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