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Cloning of the Genes for Degradation of the Herbicides EPTC (*S*-Ethyl Dipropylthiocarbamate) and Atrazine from *Rhodococcus* sp. Strain TE1†

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The degradation of the herbicides EPTC (*S*-ethyl dipropylthiocarbamate) and atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) is associated with an indigenous plasmid in *Rhodococcus* sp. strain TE1. Plasmid DNA libraries of *Rhodococcus* sp. strain TE1 were constructed in a *Rhodococcus-Escherichia coli* shuttle vector, pBS305, and transferred into *Rhodococcus* sp. strain TE3, a derivative of *Rhodococcus* sp. strain TE1 lacking herbicide degradation activity, to select transformants capable of growing on EPTC as the sole source of carbon ($EPTC^+$). Analysis of plasmids from the $EPTC^+$ transformants indicated that the *eptA* gene, which codes for the enzyme required for EPTC degradation, resides on a 6.2-kb *Kpn*I fragment. The cloned fragment also harbored the gene required for atrazine N dealkylation (*atrA*). The plasmid carrying the cloned fragment could be electroporated into a number of other *Rhodococcus* strains in which both *eptA* and *atrA* were fully expressed. No expression of the cloned genes was evident in *E. coli* strains. Subcloning of the 6.2-kb fragment to distinguish between EPTC- and atrazine-degrading genes was not successful.

Atrazine and thiocarbamate herbicides have been extensively used over the last 30 years for weed control in the production of a variety of agricultural crops. Atrazine is one of the most heavily used herbicides in North America (2, 20). The detection of atrazine in groundwater has prompted environmental concern about pollution (12). Genetic engineering of atrazine-metabolizing bacteria for the construction of stable and effective strains for bioremediation of *s*-triazine-contaminated wastes and spills has been suggested (8), and the usefulness of microorganisms for waste disposal has been documented (18). A number of bacterial strains, all belonging to the genus *Rhodococcus*, which can degrade the herbicide EPTC (*S*-ethyl dipropylthiocarbamate) (1, 24) and are also able to metabolize the *s*-triazine herbicides atrazine, simazine, and propazine (4, 5) have recently been isolated. The degradation of EPTC and the metabolism of the *s*-triazine herbicides were reported to be associated with a somewhat unstable 77-kb plasmid in *Rhodococcus* sp. strain TE1 (5), the most-studied representative of the isolates. This strain was, therefore, considered the most suitable to attempt the cloning of EPTC and atrazine degradation genes.

Although rhodococci are ubiquitous in the environment and possess diverse metabolic activities, including the degradation of a variety of alkanes, halogenated aliphatics, and aromatics and other xenobiotic pollutants (11, 29), progress in understanding the genetics of this gram-positive genus has been rather slow (11) mainly because of the lack of development of suitable methodology. Some important progress has, however, been made in the last few years. Two *Rhodococcus* bacteriophages, ϕEC and NJL, which may be potentially useful as cloning vectors have been characterized (7, 23). Recently, several cloning vectors have been constructed by cloning the origin of replication from indigenous plasmids in *Rhodococcus* strains. Almost all of these vectors are *Rhodococcus-Escherichia coli* shuttle vectors (11). We have recently improved one of these vectors, pMVS301 (28), to construct pBS305, which is relatively small and possesses multiple cloning sites (22). This vector, pBS305, was used in this study to clone the genes involved in EPTC and atrazine degradation from the plasmids in *Rhodococcus* sp. strain TE1.

The bacterial strains and plasmids used in this study are described in Table 1. Shuttle vector pMVS301 was kindly provided by William Finnerty (University of Georgia, Athens, Ga.), and *Rhodococcus corallinus* NRRL B-15444R was supplied by Walter Mulbry (Pesticide Biodegradation Laboratory, U.S. Department of Agriculture, Beltsville, Md.). *Rhodococcus* strains were grown in BMN medium (3) supplemented with 0.1% glycerol (BMNG) or in Luria-Bertani (LB) medium at 30°C with shaking. *E. coli* strains were grown in BMNG medium with the supplements required or in LB medium at 37°C with shaking. LB medium was supplemented with 1.5% (wt/vol) Bacto-Agar (Difco) for growth on solid medium, and BMN medium was solidified with 1% Bacteriological Agar (Oxoid) and supplemented with 60 to 75 μg of filter-sterilized EPTC per ml (this medium was designated BMN-EPTC-agar) to assess the ability of the transformants to grow on EPTC as the sole source of carbon. The antibiotic concentrations used were ampicillin at 100 μg/ml, kanamycin at 25 μg/ml, rifampin at 10 μg/ml, and thiostrepton at 10 μg/ml. Thiostrepton was kindly supplied by S. Lucania of Squibb-Bristol Myers, Princeton, N.J.

The ability of the cells to metabolize EPTC and atrazine was tested in batch cultures in BMNG medium supplemented with EPTC (60 μg/ml) and atrazine (10 μg/ml) by gas chromatography and by high-pressure liquid chromatography (HPLC), as described previously (5, 24). The herbicides were quantified by comparing the peak areas on the chromatograms with those of

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype	Origin or reference
Rhodococcus spp.		
TE1	*eptA*$^+$ *atrA*$^+$, isolated from EPTC-treated soil	24
TE3	*eptA* and *atrA* derivative of TE1	24
UP	*eptA atrA* Rif^r Km^r, from untreated soil	5
R. rhodochrous 13808	*eptA atrA*	ATCC
R. rhodochrous 14347	*eptA atrA*	ATCC
R. erythropolis 4277	*eptA atrA*	ATCC
R. maris 35013	*eptA atrA*	ATCC
R. luteus 35014	*eptA atrA*	ATCC
R. coprophilus 29080	*eptA atrA*	ATCC
R. sphaeroides 35035	*eptA atrA*	ATCC
R. corallinus NRRL B-15444R	*trzA*$^+$ *eptA atrA*	21
E. coli		
XL-1	*recA lac hsdR* (F′ *lacI*^{q} *lacZ*M15 Tn*10*)	Stratagene Co.
HB101	F$^-$ *recA hsdS supE*	6
Plasmids		
pBluescript (KS+)	Cloning vector in *E. coli*, Ap^r	Stratagene Co.
pMVS301	*E. coli-Rhodococcus* shuttle vector, Ap^r Thio^r	28
pBS305	*E. coli-Rhodococcus* shuttle vector, Ap^r Thio^r	22
pKL1	6.2-kb *Kpn*I fragment cloned on pBS305	This work
pKL10	pKL1 with insert in opposite orientation	This work
pKLB1	6.2-kb *Kpn*I fragment on pBluescript	This work
pKLB2	*Bgl*II fragment deleted from pKLB1	This work
pKLB3	*Mlu*I fragment deleted from pKLB1	This work
pKLB4	3.5-kb *Mlu*I fragment on pBluescript	This work
pKLB5	2.2-kb *Eco*RI fragment on pBluescript	This work
pKLB6	3.9-kb *Eco*RV fragment on pBluescript	This work
pEPT2-pEPT6	5-kb *Xba*I fragment from pBS305 inserted into pKLB2-pKLB6	This work

authentic standards (≥97% pure) run under identical gas chromatography and HPLC operating conditions.

Plasmids were isolated from *Rhodococcus* cultures grown overnight in LB medium, and ampicillin (50 μg/ml) was added 2 h before the cells were harvested. Cells were washed and resuspended in Tris-EDTA (10 mM Tris–1 mM EDTA, pH 8.0) buffer. Lysozyme was added to yield a concentration of 10 mg/ml, and the suspension was incubated at 37°C for 30 min. A modified alkaline lysis procedure (19) was then used to release the plasmids from the cells. Plasmid DNA from *Rhodococcus* sp. strain TE1 which was used for construction of gene libraries was further purified by centrifugation in a cesium chloride-ethidium bromide density gradient. The purified preparations were verified by agarose gel electrophoresis in Tris-borate–EDTA buffer (90 mM Tris-borate–2 mM EDTA).

Transformations of *Rhodococcus* strains were carried out by electroporation with an Electro Cell Manipulator 600 electroporation system (BTX Corp., San Diego, Calif.) connected to a pulse controller (25-μF capacitor, external resistance of 400 Ω), as follows. An overnight culture of *Rhodococcus* strains was inoculated into LB medium and grown for 16 to 18 h to an optical density (600 nm) of about 1. Cells were chilled on ice for 30 min and were harvested by centrifugation at 4°C. Cells were washed once with ice-cold water prior to two washings with ice-cold 10% glycerol and were resuspended in 10% glycerol at 1/20 of the original culture volume. Electroporation was performed with either fresh competent cells or previously frozen and thawed cells. This process was accomplished by mixing 400 μl of cells with about 400 ng of plasmid DNA and chilling on ice for 30 min prior to transfer to a 2-mm gapped electrocuvette (BTX Corp.). Recipient cells were subjected to a single pulse at a field strength of 12 kV/cm with resistance timing set at R9 (480 Ω). Cells were then placed on ice for 10 min, and 1 ml of LB broth was added. The cuvette was gently shaken (50 to 60 rpm) for 4 h at 30°C. The suspension was then transferred to a sterile Microfuge tube, centrifuged, washed with BMN medium, and plated on selection plates.

Total plasmid DNA from *Rhodococcus* sp. strain TE1 was used for the construction of libraries and cloning of the *eptA* (EPTC-metabolizing) gene. It was digested with *Hin*dIII, *Pst*I, or *Kpn*I. The digested DNA fragments were mixed with the same restriction enzyme-digested and alkaline phosphatase-treated pBS305 vector. Ligation was carried out at 16°C overnight with T4 DNA ligase, and the mixture was electroporated into *Rhodococcus* sp. strain TE3 as described above. Cells were washed thoroughly with BMN and plated on BMN-EPTC-agar plates before incubation at 30°C.

Colonies were present on several BMN-EPTC-agar plates after 4 days. The corresponding library DNA used to transform the cells showing growth on the plates was constructed by cutting the source DNA and vector with either *Kpn*I or *Hin*dIII before ligation. No EPTC$^+$ colonies were obtained from the transformations with library DNA constructed with *Pst*I, suggesting that *Pst*I digestion likely destroyed the *eptA* gene. Only a few EPTC$^+$ transformants were obtained with the DNA library constructed with *Hin*dIII, and all contained larger DNA inserts than the transformants obtained with the DNA library constructed with *Kpn*I (results not shown). Plasmid DNA isolated from several transformants obtained with *Kpn*I was transformed into *E. coli* XL-1. Analysis of the plasmids isolated from transformed XL-1 showed that they all carried a 6.2-kb *Kpn*I fragment on vector pBS305. One of the plasmids was designated pKL1.

The presence of pKL1 does not affect the growth rate of *Rhodococcus* sp. strain TE3 (data not shown). The degradation efficiency of strain TE3 carrying pKL1 was almost identical to that of *Rhodococcus* sp. strain TE1 during incubation with EPTC (Fig. 1A). No EPTC-degrading activity was observed

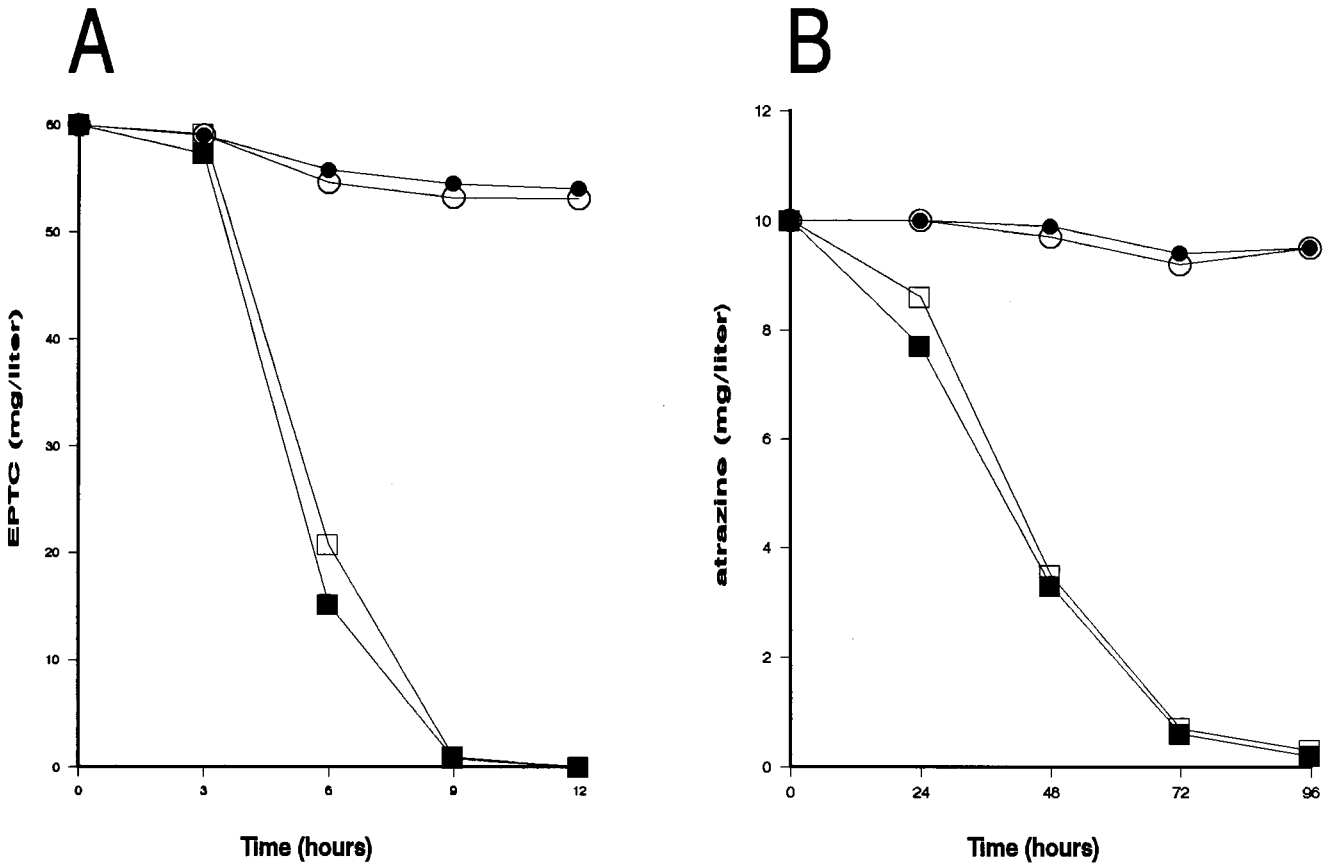

FIG. 1. (A) EPTC degradation by *Rhodococcus* sp. strain TE3 carrying the cloned *eptA* gene. ○, uninoculated control; ●, with strain TE3; □, with *Rhodococcus* sp. strain TE1; ■, with strain TE3 carrying pKL1. (B) Atrazine degradation by *Rhodococcus* sp. strain TE3 carrying the cloned *atrA* gene. ○, uninoculated control; ●, with strain TE3; □, with *Rhodococcus* sp. strain TE1; ■, with strain TE3 carrying pKL1.

with two *E. coli* strains, XL-1 and HB101, carrying plasmid pKL1.

The 77-kb plasmid in *Rhodococcus* sp. strain TE1 has also been shown to be associated with atrazine metabolism, because *Rhodococcus* sp. strain TE3 which lacks this plasmid is unable to metabolize atrazine (5). However, unlike EPTC, which can serve as the sole source of carbon for growth, there is no direct selection system for cloning for the *atrA* (atrazine-metabolizing) gene. We attempted to obtain the *atrA* clone by screening the whole DNA library of *Rhodococcus* sp. strain TE1 plasmid DNA.

The first strain we screened was *Rhodococcus* sp. strain TE3 with plasmid pKL1, which surprisingly was able to metabolize atrazine as rapidly as *Rhodococcus* sp. strain TE1 (Fig. 1B). Also, like *Rhodococcus* sp. strain TE1, the degradation of atrazine by strain TE3 carrying pKL1 produced the two dealkylated metabolites deethylatrazine and deisopropylatrazine (5). Like the *eptA* gene, *atrA* (atrazine-metabolizing activity) was not expressed in the *E. coli* cells carrying pKL1.

To test for the expression of the *eptA* and *atrA* genes in other *Rhodococcus* strains, plasmid pKL1 was used to transform eight *Rhodococcus* strains from seven different species (American Type Culture Collection [ATCC] and Northern Regional Research Laboratory strains [Table 2]), as well as *Rhodococcus* sp. strain UP. Thiostrepton-resistant colonies were obtained from only *Rhodococcus* sp. strain UP, *Rhodococcus erythropolis* 4277, and *Rhodococcus sphaeroides* 35014. The transformed strain UP and *R. erythropolis* 4277 with pKL1 degraded both EPTC and atrazine. Thiostrepton-resistant transformants of *R. sphaeroides* 35014, however, did not degrade either of the herbicides (Table 2). Plasmids isolated from two of the transformants showed that they did not contain the plasmid pKL1 (data not shown).

Unlike *Rhodococcus* sp. strain TE3 with pKL1, *Rhodococcus* sp. strains UP and *R. erythropolis* 4277 carrying pKL1, although capable of degrading EPTC, did not grow on EPTC (Table 2).

TABLE 2. Transformation of plasmid pKL1 with *Rhodococcus* strains and expression of *eptA* and *atrA*

Strain	Transformation results		
	Transformation by pKL1	EptA and AtrA activities	Growth on EPTC
Rhodococcus sp. strain TE3	+	+	+
Rhodococcus sp. strain UP	+	+	−
R. erythropolis ATCC 4277	+	+	−
R. luteus ATCC 35014	+	−	−
R. rhodochrous			
ATCC 14347	−	NT[a]	NT
ATCC 13808	−	NT	NT
R. maris ATCC 35013	−	NT	NT
R. sphaeroides ATCC 35035	−	NT	NT
R. coprophilus ATCC 29080	−	NT	NT
R. corallinus NRRL B-15444R	−	NT	NT

[a] NT, no transformants obtained.

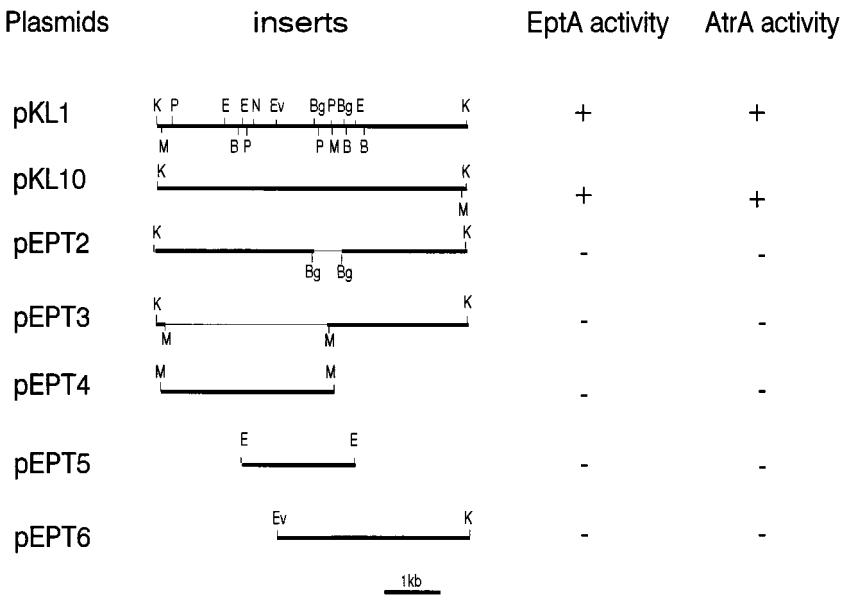

FIG. 2. Restriction map of the 6.2-kb *Kpn*I fragment and subclones. K, *Kpn*I; E, *Eco*RI; Ev, *Eco*RV; B, *Bam*HI; Bg, *Bgl*II; M, *Mlu*I; N, *Not*I; P, *Pst*I. The deleted fragments are indicated by thin lines. EPTC degradation and atrazine degradation in *Rhodococcus* sp. strain TE3 carrying the plasmids were tested.

This result suggests that the *eptA* gene on the 6.2-kb DNA fragment is involved in the initial step of EPTC degradation. Subsequent degradation (to provide carbon for growth) is carried out by other enzymes present in *Rhodococcus* sp. strain TE3 but missing in strains UP and *R. erythropolis* 4277.

The origin of replication in *Rhodococcus* spp. on pKL1 was originally derived from a 3.8-kb *Hin*dIII fragment on plasmid pMVS301. This plasmid has previously been shown to transform protoplasts of *R. erythropolis* 4277 in a manner similar to the electroporation-mediated transformation with pKL1 found here. Also as might be expected, we obtained no transformants with *Rhodococcus rhodochrous* 14347 and 13808. The protoplasts of these strains were also not transformed by pMVS301 (28). A few thiostrepton-resistant colonies were obtained with *Rhodococcus luteus* 35014 but were found not to contain pKL1. They were simply thiostrepton-resistant mutants, or they may have been transformants with pKL1 or part of pKL1 integrated into the chromosomal DNA or large plasmid in the cell. We did not obtain any transformants with *Rhodococcus maris* (ATCC 35013), *Rhodococcus coprophilus* (ATCC 29080), and *R. sphaeroides* (ATCC 35053). We also did not obtain any thiostrepton-resistant colonies with *R. corallinus* NRRL B-15444R (Table 2), a strain with unique *s*-triazine hydrolase activity, the product of the *trzA* gene (21). This enzyme dechlorinates deethylatrazine and deisopropylatrazine, the products of degradation of atrazine by AtrA. A successful transformation of *R. corallinus* NRRL B-15444R with pKL1 would produce a recombinant strain capable of dealkylating and dechlorinating atrazine.

The restriction map of the 6.2-kb *Kpn*I fragment cloned on pKL1, which carries both the *eptA* gene and the *atrA* gene, is shown in Fig. 2. Plasmid pKL10, which is the same as pKL1 except that the 6.2-kb fragment is inserted in the opposite direction on the vector, also exhibited the same catabolic activities as pKL1 when introduced into *Rhodococcus* sp. strain TE3, suggesting that the 6.2-kb *Kpn*I fragment carries the entire *eptA* and *atrA* genes, including their promoter(s).

In order to subclone the *eptA* and *atrA* genes, the 6.2-kb *Kpn*I fragment on pKL1 was cloned into pBluescript, forming pKLB1. This plasmid was digested with *Bgl*II, and the digested fragment was religated to form pKLB2, which carries a 500-bp deletion in the 6.2-kb insert. Plasmid pKLB1 was digested with *Mlu*I and religated, resulting in pKLB3. The 3.5-kb *Mlu*I fragment was isolated from an agarose gel with Geneclean (Bio 101 Ltd., La Jolla, Calif.) (27) and treated with Klenow enzyme to make blunt ends before being ligated with pBluescript digested with *Eco*RV, forming pKLB4. A 2.2-kb *Eco*RI fragment and a 3.9-kb *Eco*RV fragment from pKLB1 were isolated and inserted into the *Eco*RI site and *Eco*RV site of pBluescript, respectively, forming pKLB5 and pKLB6.

Since neither *eptA* nor *atrA* was expressed in *E. coli* cells, all of the subclones had to be transformed into *Rhodococcus* strains to test for their degradative activities. For this transformation, 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. This fragment was inserted into plasmids pKLB2, pKLB3, pKLB4, pKLB5, and pKLB6, each digested with *Xba*I. Five new plasmids, pEPT2, pEPT3, pEPT4, pEPT5, and pEPT6, were isolated and electroporated into *Rhodococcus* sp. strain TE3. The transformants were tested for their EPTC and atrazine degradation activities. None of five DNA fragments carried functional *eptA* or *atrA* (Fig. 2).

The close link between the *eptA* gene and the *atrA* genes and the fact that we were unable to separate the two genes on the 6.2-kb fragment may indicate that *eptA* and *atrA* are actually the same gene. It is also possible that the two genes are organized in a complex structure. It is not unusual that degradative genes are clustered in one or more large operons in bacteria (16). It has also been suggested that a few common self-transmissible ancestor replicons were involved in the acquisition and spread of different catabolic modules in the environment (26).

The possibility that *eptA* and *atrA* code for regulatory functions for EPTC and atrazine degradation cannot be completely excluded. Under this scenario, it is assumed that genes coding for the degradation of the herbicides may be present but not activated in *Rhodococcus* sp. strain TE3, *R. erythropolis* 4277,

and *Rhodococcus* sp. strain UP. We suggest that it is more likely that *eptA* and *atrA* are structural genes coding for the enzymes involved in EPTC degradation and N dealkylation of atrazine. Sequencing of the 6.2-kb DNA fragment is currently under way in our laboratory. Detailed analysis of the genes would be helpful to understand and/or modify the metabolism of the herbicides. Manipulation of the genes to increase their level of expression may be useful for detoxification of herbicide wastes and contaminated soils.

Rhodococci exhibit a wide variety of metabolic capabilities, can persist under harsh environmental conditions, and apparently lack catabolic repression (11, 29). They can potentially be exploited in bioremediation as demonstrated with *Rhodococcus chlorophenolicus* (25). However, cloning genes from *Rhodococcus* species is still rare because of the lack of an effective system for introducing exogenous DNA into *Rhodococcus* cells. Several genes cloned so far from *Rhodococcus* spp. were initially cloned into an *E. coli* vector (9, 13–15). Evidence either that *Rhodococcus* genes are not expressed or that the *Rhodococcus* proteins are not active in *E. coli* cells has been presented (9, 14). It is desirable, therefore, to clone the genes directly into a *Rhodococcus* vector and select the clones directly in *Rhodococcus* strains. The newly constructed *Rhodococcus-E. coli* vector, pBS305, and the demonstrated efficient electroporation-mediated transformation system (22) fulfill this need, as shown in this study. The application of pBS305 as a cloning vector for the transformation of the *eptA* and *atrA* genes into some *Rhodococcus* species showed limitations (Table 2). These limitations may be due to the inability of pBS305 to replicate in these strains, restriction in the recipients to prevent introduction of the plasmid into the cell, or the fact that the thiostrepton resistance gene (*tsr*) on the plasmid is not expressed at a detectable level. It is also possible that the conditions used in our study to transform these strains were not optimal. Besides pBS305, which is derived from pMVS301 (28), several other *Rhodococcus* vectors for particular species, such as *R. rhodochrous* and *Rhodococcus fascians*, have been described (10, 13, 17). These plasmids, although not used for gene cloning directly, may prove useful for genetic studies of these *Rhodococcus* species.

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