JOURNAL OF BACTERIOLOGY, Feb. 1995, p. 676–687
0021-9193/95/$04.00+0

Vol. 177, No. 3

Degradation of the Thiocarbamate Herbicide EPTC (*S*-Ethyl Dipropylcarbamothioate) and Biosafening by *Rhodococcus* sp. Strain NI86/21 Involve an Inducible Cytochrome P-450 System and Aldehyde Dehydrogenase

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Received 30 August 1994/Accepted 23 November 1994

Determination of the N-terminal sequences of two EPTC (*S*-ethyl dipropylcarbamothioate)-induced proteins from thiocarbamate-degrading *Rhodococcus* sp. strain NI86/21 resolved by two-dimensional electrophoresis enabled the localization of the respective structural genes on two distinct DNA fragments. One of these strongly induced proteins is a NAD^+-dependent dehydrogenase active on aliphatic aldehydes. The second protein was identified as a cytochrome P-450 enzyme. The cytochrome P-450 gene represents the first member of a new family, *CYP116*. Downstream of the cytochrome P-450 gene, two genes for a [2Fe-2S] ferredoxin (rhodocoxin) and a ferredoxin reductase are located. A putative regulatory gene encoding a new member of the AraC-XylS family of positive transcriptional regulators is divergently transcribed from the cytochrome P-450 gene. By hybridization, it was demonstrated that the aldehyde dehydrogenase gene is widespread in the *Rhodococcus* genus, but the components of the cytochrome P-450 system are unique to *Rhodococcus* sp. strain NI86/21. Overexpression in *Escherichia coli* was achieved for all of these proteins except for the regulatory protein. Evidence for the involvement of this cytochrome P-450 system in EPTC degradation and herbicide biosafening for maize was obtained by complementation experiments using EPTC-negative *Rhodococcus erythropolis* SQ1 and mutant FAJ2027 as acceptor strains. N dealkylation by cytochrome P-450 and conversion of the released aldehyde into the corresponding carboxylic acid by aldehyde dehydrogenase are proposed as the reactions initiating thiocarbamate catabolism in *Rhodococcus* sp. strain NI86/21. In addition to the major metabolite *N*-depropyl EPTC, another degradation product was identified, EPTC-sulfoxide.

The carbamates represent a major group of herbicides with an estimated market size of $750 million in 1992 (63). Thiocarbamates are generally used as graminicides and applied to soil before emergence of crops; for example, the indicated thiocarbamates are used for the following crops: maize, EPTC (*S*-ethyl dipropylcarbamothioate) and butylate [*S*-ethyl bis(2-methylpropyl)carbamothioate]; rice, molinate (*S*-ethyl hexahydro-1*H*-azepine-1-carbothioate) and thiobencarb {*S*-[(4-chlorophenyl)methyl] diethylcarbamothioate}; sugar beets, cycloate (*S*-ethyl cyclohexylethylcarbamothioate) and pebulate (*S*-propyl butylethylcarbamothioate); and soybeans, vernolate (*S*-propyl dipropylcarbamothioate).

Application of the soil herbicide EPTC to corn (*Zea mays* L.) against monocot weeds such as wild proso millet (*Panicum miliaceum* L. subsp. *ruderale* (Kitagawa) Tzevelev) and shattercane (*Sorghum bicolor* (L.) Moench) started in the late 1960s, when it was demonstrated that combined application of so-called "safeners" such as 1,2-naphthalic anhydride protected the crop plants against the phytotoxic effects arising at the herbicide levels required for efficient weed control (30). Such protection was also achieved by inoculation of the plants with selected bacterial strains acting as biosafeners (38). Early research showed that injury caused by the herbicide EPTC to sugar beets was reduced significantly with bacterial fertilizers such as Azobacterin (30). Nagy et al. (53) reported a biosafening effect for the nocardioform actinomycete *Rhodococcus* sp. strain NI86/21. This isolate protected maize plants from the harmful effects of EPTC and butylate at an inoculum density of 2.3×10^4 CFU/g of soil, whereas 2.3×10^6 CFU/g was required for biosafening against vernolate. This strain was able to utilize these herbicides as the sole carbon and nitrogen sources. *Rhodococcus* sp. strain NI86/21 was not active as a biosafener with molinate or cycloate. Molinate could not be degraded by this strain, whereas growth on cycloate was slow compared with growth on EPTC, butylate, or vernolate (50). In the biosafening experiments, the weed control remained excellent, possibly by selective enrichment of the thiocarbamate-degrading *Rhodococcus* sp. in the rhizosphere of the crop plants. Accelerated microbial degradation of several other agrochemicals in the root zone of various crops has been observed (2). Bacterial degradation of EPTC appears to be largely confined to *Rhodococcus* species. Other *Rhodococcus* strains with EPTC-degrading activity are *Rhodococcus* sp. strain TE1 (4) and *Rhodococcus* sp. strain JE1 (15). Dick et al. (15) proposed hydroxylation of the α-propyl carbon of the *N*,*N*-dialkyl moiety as the major route for EPTC metabolism by strain JE1. The unstable α-hydroxypropyl EPTC thus produced would decompose into propionaldehyde and N-depropyl EPTC. The latter compound was identified as a major metabolite by gas chromatography-mass spectrometry (GC-MS). Another important metabolite was only tentatively identified as EPTC-sulfoxide (15). Dipropylamine but not *N*-depropyl EPTC was generated during degradation of EPTC by

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Rhodococcus sp. strain TE1, suggesting the existence of another pathway in that strain (4). Dipropylamine, together with acetaldehyde and carbonyl sulfide, is also produced in a mouse liver microsome system by decomposition of unstable α-hydroxyethyl EPTC (8). Hydroxylation of the other carbons was also observed, including α-hydroxylation of the *N*-propyl side chain, generating *N*-depropyl EPTC and propionaldehyde. In these microsomes, EPTC-sulfoxide was the major metabolite. In rat liver microsomes, sulfoxidation of the disulfiram metabolite *S*-methyl *N*,*N*-diethylcarbamothioate appears to be mediated by a cytochrome P-450 (29, 45).

The mammalian enzymes involved in thiocarbamate degradation have not yet been purified. Also, biochemical or genetic evidence to substantiate the proposed pathways in EPTC-degrading *Rhodococcus* strains is lacking. Although the outstanding metabolic versatility of *Rhodococcus* species is well documented (21), understanding at molecular and genetic levels of the enzymatic processes involved is generally poor. Here, we report the identification and characterization of a unique gene cluster from *Rhodococcus* sp. strain NI86/21, containing a cytochrome P-450 system that confers both EPTC-degrading and biosafening abilities to *Rhodococcus erythropolis* SQ1 and to an EPTC-negative mutant of *Rhodococcus* sp. strain NI86/21. This cytochrome P-450 and an aldehyde dehydrogenase constitute the major thiocarbamate-induced enzymes in *Rhodococcus* sp. strain NI86/21.

MATERIALS AND METHODS

Materials. EPTC, butylate, vernolate, cycloate, molinate, and two commercial formulations of EPTC, Witox 72 EC (72% EPTC) and Alirox 80 EC (72% EPTC plus 8% chemical biosafener AD-67 [*N*-dichloroacetyl-1-oxa-4-aza-spiro-4,5-decane]), were obtained from North-Hungarian Chemical Works (Sajobábony, Hungary). In most experiments, technical-grade herbicides (98% purity) were used. For analytical purposes, 99.8% pure EPTC was used. In the bioassay, Alirox 80 EC and Witox 72 EC were added.

Bacterial strains, plasmids, phages, and growth conditions. The strains, plasmids, and phages used in this study are listed in Table 1. Both *Escherichia coli* and *Rhodococcus* species were routinely grown in Luria-Bertani (LB) medium. Ampicillin was used at 50 or 100 μg/ml, and chloramphenicol was used at 40 μg/ml. To monitor thiocarbamate decomposition, *Rhodococcus* cells were grown in LB medium, washed, and resuspended in basal salt medium (BSM) supplemented with 100 μg of the appropriate thiocarbamate per ml as described previously (51). *R. erythropolis* SQ1 and *Rhodococcus* sp. strain FAJ2027 competent cells were transformed by electroporation (14), using the *E. coli-Rhodococcus* shuttle vector pDA71 (66).

Chemical mutagenesis of *Rhodococcus* sp. strain NI86/21. *Rhodococcus* sp. strain NI86/21 cells were grown in LB medium to mid-logarithmic phase, washed and resuspended in BSM supplemented with 100 μg of EPTC per ml and 40 μg of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine per ml. Cells were exposed to the mutagenic agent for 30 min at 30°C, washed, and resuspended in BSM supplemented with EPTC and ampicillin (600 μg/ml) in order to kill cells growing on EPTC. The surviving cells were screened for loss of EPTC-degrading activity. One such mutant strain (FAJ2027) was selected for further study.

Biosafener assay. *Rhodococcus* cells grown in LB medium to late logarithmic phase were harvested by centrifugation, washed twice with BSM, and resuspended in plant medium (Martin-Prével-Charpentier-Lavigne [MPCL] medium) (42). Then, 40 ml of MPCL medium containing 1.25 mg of EPTC active ingredient (Witox 72 EC) and about 4×10^{10} cells was added to separate pots, each containing 10 maize seeds (Pioneer 3535) placed on top of 125 g of vermiculite. Subsequently, the seeds were covered with 25 g of vermiculite and placed in a plant growth chamber, with a 12-h day (28°C)–12-h night (22°C) cycle. In a control experiment, Alirox 80 EC was used. Results were evaluated after 10 days.

Chemical analyses. To monitor the EPTC decomposition rate, an HP5890A gas chromatograph equipped with a Hewlett-Packard HP1 methyl silicone gum-coated column was used (length, 5 m; inner diameter, 0.53 mm; film thickness, 2.65 μm; N_2 gas flow, 15 ml/min). Samples were injected on-column at 60°C. The column temperature was then raised at 20°C/min to 200°C. The temperature of the flame ionizing detector was maintained at 250°C. Samples were prepared as previously described (77). For GC-MS analysis of EPTC and its biodegradation products, a Kratos MS50TC mass spectrometer (electron impact, 70 eV, 220°C) coupled to a DS90 data system was used. The gas chromatograph was equipped with a Chrompack CPSil5 silicone-coated capillary column (length, 25 m; inner diameter, 0.32 mm; film thickness, 1 μm; He gas flow, 2 ml/min). After on-column injection at 100°C, the temperature was raised at 10°C/min to 250°C. An EPTC-sulfoxide standard was prepared by reaction of EPTC (189 mg, 1 mmol) with 1.1 eq of *m*-chloroperbenzoic acid in chloroform (10 ml) at room temperature for 1 h (35). Organic acids were removed by washing the chloroform solution with aqueous sodium carbonate. The solution of EPTC-sulfoxide was stored at 0°C.

Spectrophotometric analysis of cytochrome P-450. *Rhodococcus* cells resuspended in 50 mM phosphate buffer (pH 7) containing 0.3 mM phenylmethylsulfonyl fluoride were broken by three passages through a French pressure cell (SLM-Aminco) at 25,000 lb/in^2. The supernatants obtained after centrifugation at 12,000 × *g* at 4°C (15 min) were used for recording difference spectra in the presence of CO or EPTC (48). For the A_{452} peak, an extinction coefficient of 91 mM^{-1} cm^{-1} was used to quantify cytochrome P-450 in crude extracts (49). The protein concentration was determined by the bicinchoninic acid method (73).

Two-dimensional electrophoresis and N-terminal sequencing. *Rhodococcus* sp. strain NI86/21 cells were grown aerobically (200 rpm) in BSM supplemented with 100 μg of sodium acetate per ml, EPTC, butylate, vernolate, or cycloate. Another 100 μg of carbon source and 600 μg of ampicillin per ml were added after the decomposition of the first load. When the herbicide decomposition was completed, cells were harvested by centrifugation, washed with BSM, and incubated for 30 min at 37°C with 2 mg of lysozyme per ml in 10 mM Tris · HCl–1 mM Na_2EDTA. The cells were washed again, and proteins were extracted with phenol and analyzed by two-dimensional polyacrylamide electrophoresis (13). Two EPTC-induced proteins with relative molecular weights (M_rs) of 56,000 and 49,000 were cut out from several gels, pooled by preparative sodium dodecyl sulfate (SDS) electrophoresis, and then electroblotted to a polyvinylidene difluoride membrane (Millipore) (44). The N-terminal amino acid sequences were determined by automated Edman degradation using an Applied Biosystems 477A sequencer: TXXARPGTADAIMSFQSRYDNWIGNEWVAPVKGQYF ENPTPVTGQNFXDVAR for the larger protein and TVDHAPEGVKSPTGX PVSGM for the smaller protein (with X denoting residues that could not be reliably identified).

Cloning and sequence analysis of genes encoding EPTC-induced proteins. Two oligonucleotide mixtures were synthesized as probes for the genes encoding the EPTC-induced proteins: 5′-TAYGAYAAYTGGATHGGIAAYGARTGG GT-3′ for the protein with an M_r of 56,000 and 5′-GAYCAYGCICCIGARGG IGTIAA-3′ for the protein with an M_r of 49,000 (with H representing A, C, or T). Total DNA cut with different restriction endonucleases and blotted on Hybond-N membranes (Amersham) was hybridized to these digoxigenin-labeled oligonucleotide probes at 42 and at 50°C, respectively. The membranes were then washed twice at room temperature with 300 mM NaCl–30 mM sodium citrate containing 0.1% SDS and twice with 150 mM NaCl–15 mM sodium citrate containing 0.1% SDS at 45 and 53°C, respectively. Total DNA of *Rhodococcus* species was extracted by the method of Verhasselt et al. (84), modified by including an ampicillin and lysozyme pretreatment of cells, as carried out for the protein isolation. DNA fragments of interest were purified from agarose gels and cloned in pUC18 or LambdaGEM-12. Double-stranded DNA sequencing of fragments subcloned in pUC18 or pUC19 was carried out with an automated sequencer (ALF; Pharmacia Biotech). Computer-assisted sequence analyses were performed by using the PCGENE software package (IntelliGenetics, Inc.). Potential coding regions were identified with the GCWIND program (72). For homology searches, the FASTA (60) and BLASTP (1) programs were used.

Heterologous expression in *E. coli*. A number of cloned *Rhodococcus* genes were overexpressed in *E. coli* HB101 by using the pCE30 system (19). The *Sma*I-*Eco*RI fragment of pFAJ2028 carrying *thcA* was cloned in the corresponding sites of pCE30, producing pFAJ2160. For *thcB*, the *Nde*I-*Pvu*II fragment from λFAJ2028 was ligated to *Sma*I-cut vector (after the *Nde*I site was filled in with Klenow polymerase), and the resulting clones were screened for the desired orientation of the fragment (pFAJ2277). Since no expression was initially obtained for pCE30 clones with *thcC* and *thcD*, their coding regions were amplified from genomic DNA by PCR using primers with 5′ tags for *Eco*RI and *Bam*HI in order to enable correct insertion of these genes in the expression vector. For *thcD*, the primer combination 5′-ATAGGATCCAGGAGACAGACATGCCT ACCGTCACC-3′ (forward) and 5′-ATAGAATTCCGATGATGACGATGC TCATACTTG-3′ (reverse) was used. In the mutagenic primer, nucleotides differing from the genomic DNA sequence to create an improved ribosome binding site and an AUG start are underlined. Amplification of the reductase gene was achieved with 5′-ATAGGATCCGGAGGAGCAAGTATGAGCATCGTC-3′ as the forward primer and 5′-ATAGAATTCCTCTCATGACGCCACCACCTGA CG-3′ as the reverse primer. For the PCRs, 25 to 30 cycles were used, with denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and elongation at 72°C for 3 min. After *Eco*RI and *Bam*HI digestion, the appropriate bands were purified by using GeneClean (Bio 101) before ligation to pCE30. The pCE30 clones carrying the PCR-amplified genes for *thcC* (pFAJ2255, complete insert) and *thcD* (pFAJ2321, C-terminal end of insert) were verified by DNA sequencing using the M13 universal primer. Bacteriophage λ promoter-mediated overexpression of the cloned genes was induced by shifting the incubation temperature from 30 to 42°C (19). N-terminal sequences of the overexpressed proteins were determined by microsequencing after electrophoretic separation and blotting onto a polyvinylidene difluoride membrane (44). Heterologously expressed aldehyde dehydrogenase activity in cell extracts was assayed spectrophotometrically (58). No activity was detectable in control cells. The staining procedure of

TABLE 1. Bacterial strains, plasmids, and phages used

Strain, plasmid, or phage	Relevant characteristics	Reference or source
E. coli		
DH5α	*hsdR17 endA1 thi-1 gyrA96 relA1 recA1 supE44* Δ*lacU169* (ϕ80*lacZ* ΔM15)	68
HB101	*recA13 proA2 lacY1 hsdS20 endA rpsL20 ara-14 galK2 xyl-5 mtl-1 supE44*	68
MM294-1	*endA1 hsdR17*	66
Rhodococcus sp.		
NI86/21	Thiocarbamate-degrading strain with biosafener activity	NCAIM[a]
FAJ2027	EPTC-negative mutant of strain NI86/21	This study
Rhodococcus aichiensis N938[Tb]		24
R. coprophilus N774[T]		24
R. erythropolis		
N11[T]		24
SQ1	Mutant of ATCC 4277-1 with increased transformability	66
R. fascians		
D188	Isolated from *Chrysanthemum morifolium*	14
D188-5	pD188-cured mutant of *R. fascians* D188	14
Rhodococcus globerulus R58[T]		24
Rhodococcus luteus LMG5360[T]		LMG[c]
R. maris N1015[T]		24
R. rhodochrous N54[T]		24
Rhodococcus ruber N361[T]		24
pUC18	*E. coli* cloning vector; Amp[r]	68
pUC19	*E. coli* cloning vector; Amp[r]	68
pCE30	Vector for temperature-dependent overexpression in *E. coli*	19
pDA71	*E. coli-Rhodococcus* shuttle vector; Amp[r] (*E. coli*) Cm[r] (*Rhodococcus*)	E. R. Dabbs
pFAJ2028	pUC18 with 4-kb *Eco*RI fragment of *Rhodococcus* sp. strain NI86/21 containing *thcA*	This work
pFAJ2098	pUC18 with 7-kb *Bam*HI fragment of *Rhodococcus* sp. strain NI86/21 containing the region upstream of *thcA*	This work
pFAJ2133	pUC18 with 5.7-kb *Sac*I-*Kpn*I fragment of λFAJ2028	This work
pFAJ2160	pCE30 with *Eco*RI-*Sma*I fragment of pFAJ2028 (*thcA*)	This work
pFAJ2277	pCE30 with *Nde*I-*Pvu*II fragment of λFAJ2028 (*thcB*) in *Sma*I site	This work
pFAJ2255	pCE30 with PCR-generated *Eco*RI-*Bam*HI fragment (*thcC*)	This work
pFAJ2321	pCE30 with PCR-generated *Eco*RI-*Bam*HI fragment (*thcD*)	This work
pFAJ2309	pDA71 with *Eco*RV-*Hin*dIII fragment of pFAJ2133 (region from *thcB* to *thcD*) in *Bgl*II site	This work
pFAJ2350	pDA71 with *Eco*RV-*Pvu*II fragment of pFAJ2133 (*thcB*) in *Bgl*II site	This work
pFAJ2352	pDA71 with *Bam*HI fragment of λFAJ2028 (region from *orf4* to *orf5*) in *Bgl*II site	This work
pFAJ2353	pDA71 with *Sca*I-*Kpn*I fragment of pFAJ2133 (region from *thcR* to *thcD*) in *Bgl*II site	This work
pFAJ2354	pDA71 with *Pvu*II-*Kpn*I fragment of pFAJ2133 (*thcC* and *thcD*) in *Bgl*II site	This work
LambdaGEM-12	Lambda replacement vector	Promega
λFAJ2028	LambdaGEM-12 with 14-kb *Sac*I fragment of *Rhodococcus* sp. strain NI86/21 containing *thcB*	This work

[a] NCAIM, National Collection of Agricultural and Industrial Microorganisms, Budapest, Hungary.
[b] Superscript T, type strain.
[c] LMG, culture collection of the Laboratory of Microbiology, University of Ghent.

Harada (28) was used to demonstrate aldehyde dehydrogenase activity after native electrophoresis on a 12.5% PhastGel (Pharmacia Biotech).

Heterologous hybridizations. Southern hybridizations of total DNA of nine *Rhodococcus* species were carried out with the following probes from *Rhodococcus* sp. strain NI86/21: the *Bst*EII fragment of *thcA*, the *Bgl*II fragment of *thcB*, the *Pvu*II-*Bbr*PI fragment containing *thcC*, the *Bbr*PI fragment of *thcD*, and the *Eco*RV-*Eco*RI fragment of *thcR*. After overnight hybridization at 60°C, the blots were washed twice at room temperature with 300 mM NaCl–30 mM sodium citrate containing 0.1% SDS and twice at 65°C with 75 mM NaCl–7.5 mM sodium citrate containing 0.1% SDS.

Nucleotide sequence accession numbers. The nucleotide sequences presented here have been assigned accession no. U17129 and U17130 by GenBank.

RESULTS

Evidence for EPTC-induced proteins determined by two-dimensional electrophoresis. Comparative two-dimensional electrophoresis of proteins extracted from *Rhodococcus* sp. strain NI86/21 cells grown in the presence of EPTC revealed that two major proteins with apparent molecular weights of 56,000 and 49,000 were induced in the presence of EPTC (Fig. 1). These proteins were not detectable in acetate- or glycerol-grown cells but were equally well induced in cells growing on butylate, vernolate, and cycloate (data not shown). With propionaldehyde, ethanolamine, or ethanol as a carbon source, the larger protein was also produced at a high level in *Rhodococcus* sp. strain NI86/21 cells, but the smaller protein appeared specific for EPTC-grown cells (data not shown). Two additional thiocarbamate-induced proteins with M_rs of 51,000 and 30,000 that were present in comparatively small amounts (Fig. 1) are under further investigation.

Cloning of genes encoding EPTC-induced proteins. By hybridization with oligonucleotide mixtures deduced from the N-terminal amino acid sequences of the two major EPTC-induced proteins (see Materials and Methods), genomic DNA

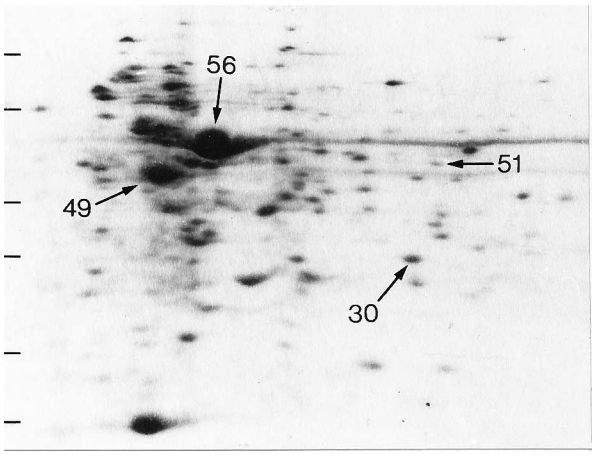

FIG. 1. Two-dimensional protein analysis of EPTC-grown cells of *Rhodococcus* sp. strain NI86/21. The EPTC-induced spots (arrows) with the estimated M_rs (in thousands) are indicated. The positions of the molecular weight markers (in thousands: 97.4, 66.2, 42.7, 31.0, 21.5, and 14.4, from top to bottom) are indicated on the left. The pH gradient extends from left (acidic) to right (basic).

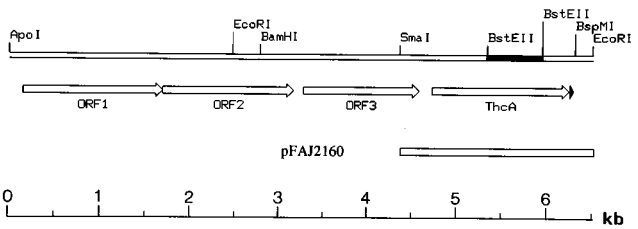

FIG. 2. Map of the 6,517-bp *Apo*I-*Eco*RI DNA region of *Rhodococcus* sp. strain NI86/21 containing the EPTC-inducible aldehyde dehydrogenase gene *thcA*. The positions of three other open reading frames upstream of *thcA* are also indicated. The location of a potential stem-loop structure (ΔG, −32.4 kcal [ca. −136 kJ]) (solid arrowhead), the DNA probe used in heterologous hybridizations (solid bar), and the DNA fragment inserted in pCE30 (pFAJ2160) for expression of *thcA* in *E. coli* (open bar) are shown. Only restriction sites that are mentioned in the text are shown. In the 5,173-bp *Bsp*MI-*Kpn*I region downstream of *thcA*, genes involved in ethanolamine metabolism are located (11).

fragments carrying the respective structural genes of *Rhodococcus* sp. strain NI86/21 were identified. For the larger protein, an *Eco*RI fragment of about 4 kb cloned in pUC18 (pFAJ2028) was selected by colony hybridization. The 309-bp *Eco*RI-*Bam*HI fragment of pFAJ2028 (Fig. 2) was used to clone the upstream DNA region as an overlapping 7-kb *Bam*HI fragment in pUC18 (pFAJ2098). Repeated attempts to clone different fragments hybridizing with the oligonucleotide probe for the second EPTC-induced protein (M_r, 49,000) in pUC18 all failed, because only inserts with apparent deletions were obtained. This problem was circumvented by cloning a 14-kb *Sac*I fragment in LambdaGEM-12 (λFAJ2028).

Identification of *thcA*, encoding an EPTC-induced aldehyde dehydrogenase. Sequence analysis of the 4-kb *Eco*RI fragment revealed the presence of an open reading frame (*thcA*) encoding a protein of 506 amino acids (Fig. 3). The deduced polypeptide sequence matches the N-terminal sequence obtained by microsequencing of the larger EPTC-induced protein. In addition, the calculated molecular mass of the mature protein (54,863 Da) is close to the M_r estimate of 56,000. This protein shows significant homology with NAD^+-dependent aldehyde dehydrogenases from several sources. The best alignments were obtained with the enzymes from *Alcaligenes eutrophus* (65) and *Vibrio cholerae* (58), revealing 73 and 60% identity, respectively (data not shown). Another putative homolog (66% identity) was recently identified in *E. coli* by genomic sequencing (74). The high level of sequence homology between these aldehyde dehydrogenases suggests similar substrate specificities. Although the enzymes from *V. cholerae* and *A. eutrophus* are known to oxidize acetaldehyde to acetic acid, the substrate range of these aldehyde dehydrogenases has not yet been determined. The *A. eutrophus* enzyme is involved in the catabolism of acetoin and ethanol (65). The aldehyde dehydrogenase of *V. cholerae* is part of the ToxR regulon, but its metabolic function has not yet been determined (58).

In aldehyde dehydrogenases, a glutamic acid and a cysteine residue have been implicated in the catalytic activity (31). For the mammalian enzymes, two consensus sequences containing these residues have been proposed (PROSITE release 12.0) (3). The *Rhodococcus* aldehyde dehydrogenase contains the [LIVMFGA]-E-[ILSTA]-[GS]-G-[KNM]-[SAN]-[TAPF] consensus sequence with glutamic acid between residues 262 and 269 (LELGGKSP), but the active site region with cysteine between residues 294 and 306 (FALNQGEVCTAPS) slightly deviates from the mammalian consensus sequence [FYV]-X_3-G-[QE]-X-C-[LIVMGSTNC]-[AGCN]-X-[GSTDNER]. In the *Rhodococcus* aldehyde dehydrogenase, a G-X-G-X_3-G motif is found between residues 219 and 225 (GFGVEAG). This glycine motif involved in NAD^+ binding is found in aldehyde dehydrogenases catalyzing irreversible reactions (33), whereas the G-X-G-X_2-G coenzyme-binding motif is typically present in dehydrogenases, such as alcohol dehydrogenase, catalyzing reversible reactions (71).

By using the internal *Bst*EII fragment (Fig. 2) of *thcA* as a probe, a strong hybridization signal was obtained with total DNA from nine other *Rhodococcus* species (listed in Table 1), indicating that the gene is widespread in this genus. Southern blots of plasmid preparations (separated by conventional electrophoresis) and of total DNA preparations (separated by pulsed-field electrophoresis) probed with *thcA* indicated that this gene is not carried on a circular or linear plasmid of *Rhodococcus* sp. strain NI86/21 (data not shown).

Expression of *thcA* in *E. coli*. The *Eco*RI-*Sma*I fragment of pFAJ2028 carrying the coding region of *thcA* was cloned in the expression vector pCE30 to generate pFAJ2160. Upon temperature-induced expression, a protein of the expected size accumulated in *E. coli*. The N-terminal sequence of this heterologously produced protein (TKYARPG) was identical to the one deduced from the DNA sequence, except with methionine cleaved off. In induced *E. coli* cells, the presence of a single aldehyde dehydrogenase was evidenced by staining for this enzyme activity after native electrophoresis (data not shown). Such crude *E. coli* extracts were used to determine the relative activity of the cloned aldehyde dehydrogenase by using different aldehydes with propionaldehyde as a reference substrate (100% activity): acetaldehyde (24%), butyraldehyde (67%), isobutyraldehyde (105%), valeraldehyde (83%), *n*-hexanal (114%), *n*-octanal (145%), *n*-decanal (124%), and benzaldehyde (28%). Low activities (<10%) were detected with glyceraldehyde, pivaldehyde, and *p*-anisaldehyde, whereas formaldehyde oxidation was not detectable under the assay conditions used. These data indicate a clear substrate preference for long-chain aliphatic aldehydes, which is reminiscent of the NAD^+-dependent aldehyde dehydrogenase of the actinomycete *Amycolatopsis methanolica* (83). The subunit M_r of the latter enzyme (55,000) is quite close to the calculated molecular mass of ThcA.

DNA regions flanking *thcA*. In order to investigate whether the aldehyde dehydrogenase gene was part of an operon-like

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ORF3 > P  G  I  E  E  V  R  E  E  L  R  T  R  V  Q  A  A  L  L  R  G  G  D  A  N  L  L  A  R  W  T  T  S  V  H  G  R  E  D  S   404
4366 CCCGGGAATCGAAGAGGTGCGGGAAGAACTACGCACCAGGGTTCAGGCGGCTCTGCTGCGTGGAGGTGACGCCAACCTTCTGGCCCGGTGGACCACGTCCGTACACGGGCGCGAAGACTC
      V  V  W  E  A  Y  L  S  T  L  D  P  Q  S  P  L  Y  S  Q  V  Q  A  R  I  D  L  L  D  R  Q  L  G  I  *                    437
4486 CGTCGTGTGGGAGGCCTACCTGTCCACGTTGGATCCTCAATCTCCGCTGTATTCGCAGGTCCAGGCAAGAATTGATCTCCTCGACCGCCAATTGGGCATCTGAGAATCATCTGCGTTTCT
4606 CGCACGCAACGTACTTGCAACGTTGCAACTCCTAGTGTTGTGAATCACACCCCACCGGGGGGTGGGATTGCAGTCACCGATTTGGTGGGTGCGCCCAGGAAGATCACGTTTACATAGGAG
     ThcA > M  T  K  Y  A  R  P  G  T  A  D  A  I  M  S  F  Q  S  R  Y  D  N  W  I  G  N  E  W  V  A  P  V  K  G  Q  Y  F  E    38
4726 CTTGCAATGACAAAGTACGCCCGTCCAGGTACCGCCGACGCGATCATGTCCTTCCAGTCTCGATACGACAACTGGATCGGCAACGAATGGGTTGCTCCGGTCAAGGGTCAGTACTTCGAG
      N  P  T  P  V  T  G  Q  N  F  C  D  V  A  R  S  T  A  E  D  I  E  L  A  L  D  A  A  H  A  A  A  P  A  W  G  K  T  S  V    78
4846 AACCCGACACCGGTGACCGGACAGAATTTCTGTGATGTGGCTCGTTCCACCGCAGAAGACATCGAGCTCGCTCTCGACGCAGCGCACGCAGCAGCTCCGGCCTGGGGCAAGACCTCGGTC
      A  E  R  A  I  I  L  N  K  I  A  D  R  M  E  E  N  L  E  S  I  A  L  A  E  S  W  D  N  G  K  P  I  R  E  T  L  N  A  D   118
4966 GCCGAGCGCGCGATCATCTTGAACAAGATCGCTGATCGTATGGAGGAGAACCTCGAATCCATCGCACTCGCCGAGTCGTGGGACAACGGCAAGCCGATCCGCGAGACCCTCAACGCCGAC
      I  P  L  A  I  D  H  F  R  Y  F  A  G  A  I  R  A  Q  E  G  S  L  S  E  I  N  S  D  T  V  A  Y  H  F  H  E  P  L  G  V   158
5086 ATTCCGCTCGCCATCGATCACTTCCGTTACTTCGCGGGAGCTATTCGCGCACAGGAAGGTTCGCTTTCGGAGATCAACTCCGACACCGTGGCGTACCACTTCCACGAGCCGCTCGGCGTA
      V  G  Q  I  I  P  W  N  F  P  I  L  M  A  V  W  K  L  A  P  A  L  A  A  G  N  A  I  V  L  K  P  A  E  Q  T  P  V  S  I   198
5206 GTCGGCCAGATCATTCCGTGGAACTTCCCGATCCTCATGGCCGTGTGGAAGCTCGCTCCCGCCCTTGCTGCCGGCAATGCCATCGTGCTCAAGCCTGCCGAGCAGACACCCGTCTCGATC
      L  H  L  I  G  I  I  G  D  L  L  P  A  G  V  L  N  I  V  N  G  F  G  V  E  A  G  K  P  L  A  S  S  P  R  I  K  K  I  A   238
5326 CTGCACCTCATCGGCATCATCGGTGACCTGCTGCCTGCCGGTGTGCTCAACATCGTCAACGGCTTCGGCGTCGAGGCCGGAAAGCCGCTCGCGTCGAGCCCGCGTATCAAGAAGATCGCG
      F  T  G  E  T  T  T  G  R  L  I  M  Q  Y  A  S  Q  N  L  I  P  V  T  L  E  L  G  G  K  S  P  N  V  F  F  S  D  V  L  A   278
5446 TTCACCGGTGAAACCACCACAGGTCGCCTGATCATGCAGTACGCGTCGCAGAACCTCATCCCCGTGACCCTCGAACTCGGTGGCAAGAGCCCCAACGTCTTCTTCTCCGACGTTCTCGCC
      S  N  D  D  Y  Q  D  K  A  L  E  G  F  T  M  F  A  L  N  Q  G  E  V  C  T  A  P  S  R  A  L  I  Q  E  D  I  F  D  E  F   318
5566 TCCAATGACGATTACCAGGACAAGGCACTCGAGGGCTTCACGATGTTCGCCCTCAACCAGGGCGAGGTCTGCACAGCTCCGTCGCGTGCGCTGATTCAGGAAGACATCTTCGACGAGTTC
      L  A  M  A  A  I  R  T  K  A  V  R  Q  G  D  P  L  D  T  D  T  M  I  G  A  Q  A  S  N  D  Q  L  E  K  I  L  S  Y  I  E   358
5686 CTGGCGATGGCCGCCATTCGCACCAAGGCTGTGCGCCAAGGGGATCCGCTCGACACCGACACCATGATCGGTGCCCAGGCCAGCAACGATCAGCTCGAGAAGATCCTCTCGTACATCGAG
      I  G  K  A  E  G  A  K  V  I  T  G  G  E  R  A  E  L  G  G  D  L  S  G  G  Y  Y  V  Q  P  T  V  F  T  G  N  N  K  M  R   398
5806 ATCGGCAAGGCGGAAGGCGCCAAGGTCATCACCGGTGGTGAGCGGGCCGAACTCGGCGGAGACCTGTCCGGCGGCTACTACGTCCAGCCGACGGTGTTCACCGGCAACAACAAGATGCGC
      I  F  Q  E  I  F  G  P  V  V  S  V  T  S  F  K  D  Y  D  E  A  I  E  I  A  N  D  T  L  Y  G  L  G  A  G  V  W  S  R  D   438
5926 ATCTTCCAGGAGATCTTCGGACCCGTCGTCTCGGTCACCTCGTTCAAGGACTACGATGAGGCTATCGAAATCGCCAACGACACGCTCTACGGTTTGGGCGCCGGTGTCTGGTCCCGCGAC
      G  G  V  A  Y  R  A  G  R  D  I  Q  A  G  R  V  W  T  N  T  Y  H  Q  Y  P  A  H  A  A  F  G  G  Y  K  Q  S  G  I  G  R   478
6046 GGCGGAGTCGCGTACCGCGCAGGCCGCGACATCCAGGCCGGTCGCGTCTGGACCAACACGTACCACCAGTACCCGGCACACGCCGCTTTCGGTGGATACAAGCAGTCCGGCATCGGCCGC
      E  N  H  L  M  M  L  S  H  Y  Q  Q  T  K  N  L  L  V  S  Y  A  Q  K  A  Q  G  F  F  *                                   506
6166 GAGAACCACCTGATGATGCTCTCGCACTACCAGCAGACCAAGAACCTCCTGGTCAGCTACGCCCAGAAGGCTCAGGGCTTCTTCTGATCGACCCACCGGCACCCGTGAGCCCCTCGCTGC
6286 GGGTGCCGGTGCGAGGGACTGCAACACGCGAAACCTGCACAA
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FIG. 3. DNA sequence of the *Sma*I-*Bsp*MI region encoding the EPTC-inducible aldehyde dehydrogenase ThcA of *Rhodococcus* sp. strain NI86/21. Nucleotides potentially involved in formation of a stem-loop structure and amino acid residues confirmed by microsequencing (underlines) and stop codons (asterisks) are indicated. The nucleotide sequence of the complete 6,517-bp *Apo*I-*Eco*RI region shown in Fig. 2 has been submitted to GenBank (accession no. U17129).

structure, the DNA sequences of both the upstream and the downstream regions were determined. Sequence analysis of the downstream 5,173-bp *Bsp*MI-*Kpn*I region showed that it contains the structural genes for ethanolamine ammonia-lyase subunits (homologous to the *Salmonella typhimurium* enzyme) and a permease of the APC (amino acids-polyamines-choline) family (11). Ethanolamine ammonia-lyase catalyzes the deamination of ethanolamine and other aminoalcohols to the corresponding aldehydes. In *S. typhimurium*, the region upstream of the genes encoding the subunits of ethanolamine ammonia-lyase contains an aldehyde dehydrogenase which is involved in the further assimilation of acetaldehyde generated from ethanolamine (67). The similar gene organization of aldehyde dehydrogenase in *Rhodococcus* sp. strain NI86/21 and its induction by ethanolamine (see above) suggest that this enzyme may also participate in aminoalcohol metabolism.

As shown in Fig. 2, three large open reading frames are present in the region upstream of the aldehyde dehydrogenase gene. The GUG start of ORF2 overlaps by 2 bases with the UGA stop codon for ORF1, which suggests a translational coupling (data not shown). For ORF1 and ORF3, no significant sequence homology with any known protein could be detected. On the basis of the presence of two PROSITE motifs, ORF2 could be assigned to the GMC (glucose-methanol-choline) oxidoreductase family of flavoproteins, which contains both oxidases and dehydrogenases (7, 32). Several members of this family catalyze the oxidation of alcohols to the corresponding aldehydes: formaldehyde, betaine aldehyde, and aliphatic aldehydes are produced by *Hansenula polymorpha* methanol oxidase (43), *E. coli* choline dehydrogenase (5, 41), and *Pseudomonas oleovorans* alcohol dehydrogenase (81), respectively. The genes for the last two enzymes are both part of an operon with an aldehyde dehydrogenase gene, which is reminiscent of the gene organization reported here for *Rhodococcus* sp. strain NI86/21. However, the extent of overall homology (31% identity with the closest relatives, *E. coli* choline dehydrogenase and *Drosophila* glucose dehydrogenase [40]) did not allow the assignment of a specific dehydrogenase or oxidase activity to the *Rhodococcus* protein (data not shown).

A novel type of cytochrome P-450 induced in EPTC-grown cells. On the *Sac*I fragment of λFAJ2028, the coding region for the EPTC-induced protein with an M_r of 49,000 was located by sequence analysis (Fig. 4). The N-terminal sequence of this protein (ThcB) was identical to the one deduced from the

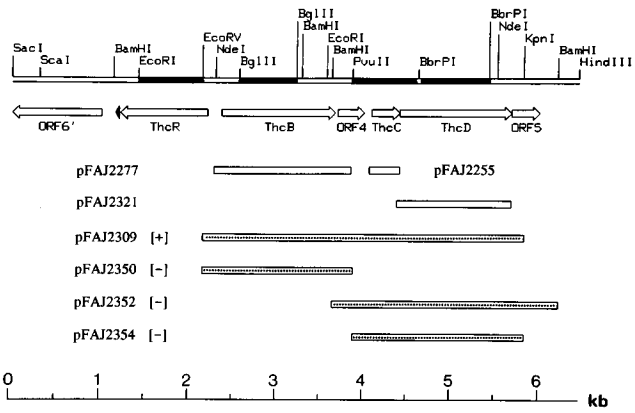

FIG. 4. Map of the 6,458-bp *Sac*I-*Hin*dIII DNA region of *Rhodococcus* sp. strain NI86/21 containing the EPTC-inducible cytochrome P-450 gene *thcB*. The other open reading frames shown correspond to a putative regulatory protein (ThcR), ferredoxin (ThcC), ferredoxin reductase (ThcD), and three unidentified polypeptides (ORF4, ORF5, and truncated ORF6′). The location of a potential stem-loop structure (Δ*G*, −38.2 kcal [ca. −160 kJ]) (solid arrowhead), the DNA probes used in heterologous hybridizations (solid bars), and the DNA fragments inserted in pCE30 for expression of *thcB* (pFAJ2277), *thcC* (pFAJ2255), and *thcD* (pFAJ2321) in *E. coli* (open bars) are shown. In addition, the DNA regions cloned in pDA71 that were introduced into *R. erythropolis* SQ1 to monitor acquisition of EPTC-degrading activity (shaded boxes) are indicated. The performances (+ or −) of the respective SQ1 transformants in the biosafener assay are indicated. Only restriction sites that are mentioned in the text are shown. In the 5,173-bp region downstream of *orf5*, cobalamin biosynthetic enzymes are encoded by the opposite strand (12).

DNA sequence (Fig. 5). The calculated molecular mass of 48,796 Da (without the first methionine) is in good agreement with the estimated molecular weight. The sequence (FGIGSH TCLG) in the C-terminal region matches the PROSITE consensus motif F-[SGNH]-X-[GD]-X-[RHPT]-X-C-[LIMVFAP]-[GAD] for cytochrome P-450 enzymes, spanning the hydrophobic cysteine-containing heme pocket (3). The essential threonine 252 identified in the oxygen-binding pocket of the camphor-hydroxylating cytochrome P-450 from *Pseudomonas putida* (36, 47) has an equivalent in the *Rhodococcus* enzyme (Thr-268) and in most other bacterial cytochrome P-450s (data not shown). A multiple alignment of available sequences for bacterial cytochrome P450s revealed that the *Rhodococcus* gene represents the first member of a new family, *CYP116* (54). The cytochrome P-450 from the *fas* operon of *Rhodococcus fascians*, which is required for efficient fasciation of host plants (9), bears little homology with the EPTC-induced cytochrome P-450 from *Rhodococcus* sp. strain NI86/21. On the basis of the lack of N-terminal sequence homology, the 2-ethoxyphenol-induced cytochrome $P\text{-}450_{RR1}$ from *Rhodococcus rhodochrous* (18) and the enzyme of *Rhodococcus* sp. strain NI86/21 also appear not to be related.

Spectrophotometric evidence for induction of a cytochrome P-450 enzyme was obtained from CO- and substrate-induced difference spectra. In the spectra of CO-treated versus non-treated reduced crude extracts of EPTC- and acetate-grown *Rhodococcus* sp. strain NI86/21 cells, a peak at 452 nm, characteristic for cytochrome P-450, was observed only for the thiocarbamate-grown cells (Fig. 6A). In these cells, the cytochrome P-450 content was about 180 pmol (8.8 μg) per mg of protein. Addition of thiocarbamates to a crude extract from the EPTC-grown cells generated a distinct peak in the substrate difference spectrum at 390 (EPTC; Fig. 6B), 391 (vernolate), or 392 (butylate) nm. The appearance of these peaks reflects the shift of cytochrome P-450 to the high-spin form concomitant with substrate binding (64). Under the conditions used, such a shift was not detectable with added cycloate. This may reflect the structural difference of the latter thiocarbamate with EPTC, butylate, and vernolate, in which an alkyl chain (propyl or isobutyryl) replaces the cyclohexyl substituent of cycloate.

With the internal *Sal*I fragment of *thcB* as a probe, a very weak signal was obtained for total DNA of *Rhodococcus maris* N1015, *R. rhodochrous* N54, and *Rhodococcus coprophilus* N774, whereas the other species tested (Table 1) were all negative (data not shown). These results indicate that in some *Rhodococcus* species, distantly related cytochrome P-450 genes may be present. Like the aldehyde dehydrogenase, the cytochrome P-450 was not plasmid encoded (data not shown).

Clustered genes for cytochrome P-450, a [2Fe-2S] ferredoxin, and a ferredoxin reductase. Immediately downstream of the cytochrome P-450 gene, the small ORF4 (103 amino acids) is located (Fig. 4), but no homologous protein was found in the databases. Further downstream, three additional open reading frames in which the start (AUG) and stop (UGA) codons of adjacent genes overlap, possibly reflecting their translational coupling, were identified (Fig. 5). No homolog for ORF5 (106 amino acids) could be traced by database searches, but the products of *thcC* and *thcD* were identified as a [2Fe-2S] ferredoxin (107 amino acids) and a ferredoxin reductase (427 amino acids), respectively.

The small protein (ThcC) from *Rhodococcus* sp. strain NI86/21, for which we propose the trivial name rhodocoxin, is highly homologous (43.4% identity) with the iron-sulfur protein putidaredoxin of *P. putida* (39, 61). Terpredoxin from a *Pseudomonas* sp. (34.9% identity) appeared to be less related (62). The cysteines required for coordinating the [2Fe-2S] cluster (6) are perfectly conserved among the three proteins (alignments not shown). For four of five carboxylate-containing residues of putidaredoxin which are involved in the reaction with putidaredoxin reductase (23), there are carboxylate-containing residues at the equivalent positions in rhodocoxin. In putidaredoxin, the C-terminal residue is important for the interaction with cytochrome $P\text{-}450_{cam}$ (10). Enzymatic removal of this tryptophan residue caused a much-reduced rate of enzymatic activity in a reconstituted system. Replacement of the tryptophan residue with different nonaromatic amino acids (including valine) affected the relatively high affinity of cytochrome $P\text{-}450_{cam}$ for the reduced putidaredoxin compared with the oxidized protein. However, the C-terminal residues of rhodocoxin (valine) and terpredoxin (alanine) are different, indicating that their roles in interaction with the respective cytochrome P-450 enzymes may differ. For ThcC, significant homology (31.8% identity) with the [2Fe-2S] ferredoxin of unknown function present in *E. coli* (75) was also found, but gaps were needed to optimize alignment (not shown).

Assignment of *thcD* as a ferredoxin (rhodocoxin) reductase gene is based on the strong homology of the deduced protein with a number of ferredoxin reductases located in various degradative operons from *Pseudomonas* species (17, 20, 39, 61, 62, 85). Sequence conservation was particularly obvious in the regions involved in adenine nucleotide binding (alignment not shown). The greater extent of homology of the putative rhodocoxin reductase with the putidaredoxin reductase (41.1% identity) than with the other ferredoxin reductases, ranging from 37.7% for rubredoxin reductase from *P. oleovorans* (17) to 30.3% for the BphG enzyme from *Pseudomonas* sp. strain LB400 (20), probably reflects in part the greater sequence similarity between their iron-sulfur protein substrates (see above).

The use of the less efficient GUG start codon has been proposed to be involved in posttranscriptional control of relative abundance of the putidaredoxin reductase in order to compensate for the lower turnover of the cytochrome P-450 (79). The rubredoxin reductase gene *alkT* of *P. oleovorans* is also poorly expressed compared with the other components of the alkane hydroxylase complex (17). Similarly, the GUG start codon of the rhodocoxin gene may be involved in maintaining a low intracellular level of this ferredoxin. Apparently, the levels of both rhodocoxin and rhodocoxin reductase in thiocarbamate-grown cells of *Rhodococcus* sp. strain NI86/21 are too low to be detectable by two-dimensional electrophoresis.

By hybridization with DNA probes for *thcC* (*Pvu*II-*Bbr*PI fragment) and *thcD* (*Bbr*PI fragment), no homologs could be detected in total DNA of the nine other *Rhodococcus* species listed in Table 1 (data not shown).

Heterologous expression of cytochrome P-450, rhodocoxin, and rhodocoxin reductase. By using the pCE30 vector system, *thcB*, *thcC*, and *thcD* were overexpressed in *E. coli*. For the rhodocoxin gene, both the start codon (AUG instead of GUG) and the putative ribosome binding site (AGGAGA instead of AGAGAGA) were modified by PCR to enable efficient expression in *E. coli*. Increased heterologous expression of putidaredoxin reductase when the GUG start codon was changed into an AUG start codon was reported by Peterson et al. (61). The N-terminal sequences of the overexpressed proteins of appropriate sizes were verified by microsequencing: SIVIIGS GQA for the reductase, (M)PTVTYVH for rhodocoxin, and (M)TVDHA for cytochrome P-450. In the last two cases, an additional protein starting with methionine (in parentheses) was produced. The percentages of fully methionine-processed

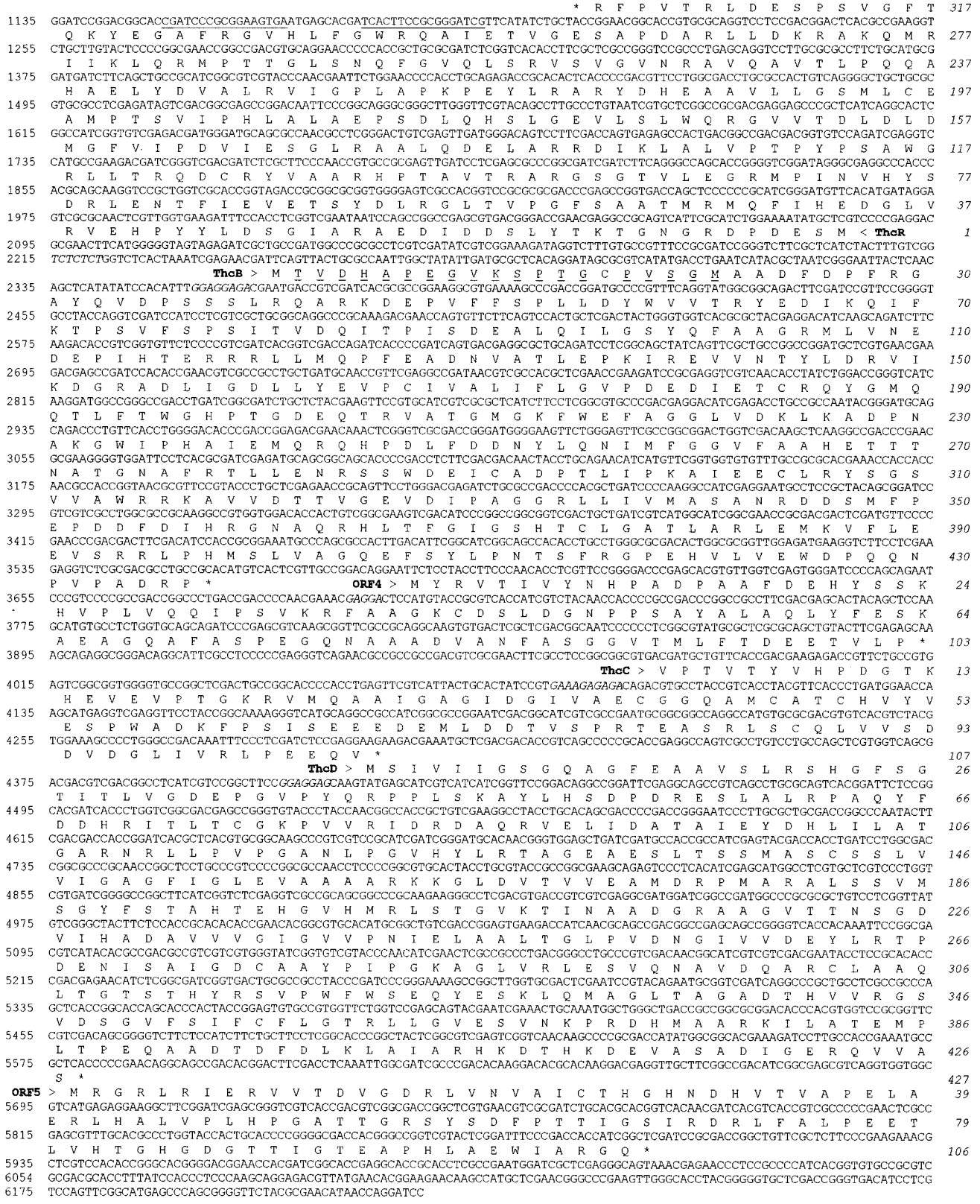

FIG. 5. Sequence of the *Rhodococcus* sp. strain NI86/21 DNA region encoding the regulatory protein ThcR, the EPTC-inducible cytochrome P-450 ThcB, the [2Fe-2S] ferredoxin ThcC, the ferredoxin reductase ThcD, ORF4, and ORF5. Nucleotides potentially involved in formation of a hairpin and amino acids confirmed by N-terminal sequence analysis (underlines) and stop codons (asterisks) are indicated. The sequence shown encompasses three *Bam*HI fragments. The nucleotide sequence of the entire 6,458-bp *Sac*I-*Hin*dIII region shown in Fig. 4 has been submitted to GenBank (accession no. U17130).

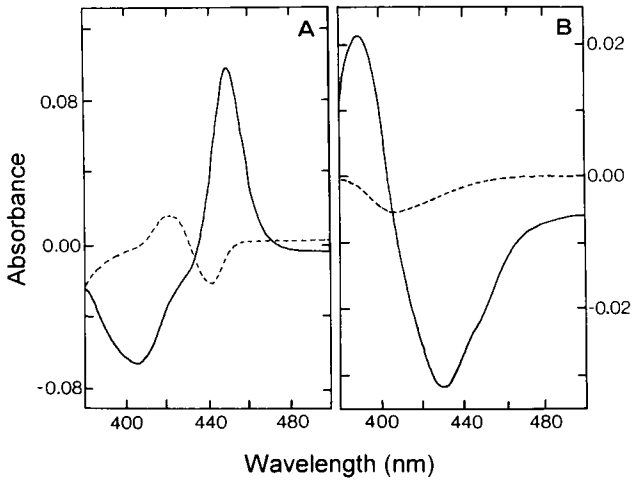

FIG. 6. Difference spectra in the presence of CO (A) and EPTC (B) for crude extracts of acetate-grown (– – –) and EPTC-grown (——) cells of *Rhodococcus* sp. strain NI86/21.

protein were about 60% for rhodocoxin and 55% for cytochrome P-450.

Gene for a regulatory protein of the AraC-XylS family upstream of cytochrome P-450. Upstream of the cytochrome P-450 from *Rhodococcus* sp. strain NI86/21, an open reading frame of 332 amino acids was located on the opposite strand (Fig. 4). This divergently transcribed gene (*thcR*) is followed by a sequence potentially forming a stable hairpin structure which probably aids in rho-independent transcription termination (Fig. 5). The sequence VTEIAQRWGFLHVGRFAGEYKQT FGVSP in the C-terminal part of this protein matches the PROSITE consensus sequence for bacterial regulatory proteins of the AraC-XylS family ([LIV]-X_2-[LIVMTA]-[GSA]-X_3-[GNQ]-[IFY]-X_5-[LF]-X_3-[FY]-X_7-P). In most members of this family, this signature pattern is located some 30 residues after the DNA-binding region (3). By the method of Dodd and Egan (16), such a putative DNA-binding motif was indeed found in this *Rhodococcus* protein between residues 241 and 263, with a spacing of 31 residues between the two regions. Consequently, this *Rhodococcus* protein belongs to the AraC-XylS family, most members of which represent positive transcriptional regulators (22). A dendrogram based on the multiple alignment of these proteins (data not shown) indicated that ThcR is quite distant from the other proteins of this family, including the only other member from a gram-positive bacterium, the methylphosphotriester-DNA alkyltransferase AdaA from *Bacillus subtilis* (22). The most closely related member, XylS from the TOL plasmid of *P. putida*, has only 21% identical and 14% similar residues. Local homology between these two proteins is most pronounced in the C-terminal region (36% identity and 17% similarity). Within the PROSITE fingerprint motif, identity amounts to 59%.

No homolog of this regulatory gene could be detected in several other *Rhodococcus* species by hybridization with the internal *Eco*RV-*Eco*RI fragment (data not shown). Repeated attempts to express the regulatory protein with the pCE30 system starting from a genomic or PCR-amplified fragment (including a modified ribosome binding site) have all failed, possibly because of lethal leaky expression in uninduced cells.

Key role of cytochrome P-450 in EPTC degradation. Apparently, the *Rhodococcus* sp. strain NI86/21 dehydrogenase acting on long-chain aldehydes is not the key enzyme in EPTC degradation. It is widely present in EPTC-negative species of this genus and is most likely involved in, among other functions such as ethanolamine metabolism, the further assimilation of degradation products generated by the thiocarbamate-induced cytochrome P-450 of *Rhodococcus* sp. strain NI86/21 (see Discussion). Evidence for the direct involvement of the P-450 gene in EPTC degradation was obtained by identification of the genes required to confer EPTC-degrading ability and biosafener activity on the EPTC-negative strain *R. erythropolis* SQ1. By using the *E. coli-Rhodococcus* shuttle vector pDA71, selected fragments of the *Sca*I-*Hin*dIII region of λFAJ2028 were electroporated into this strain (Fig. 4). Chloramphenicol-resistant colonies were screened by hybridization for the presence of both vector and appropriate inserts. The biosafener activity of the transformants on inoculated maize seedlings growing in the presence of EPTC was then evaluated (Fig. 7). Only the DNA fragment spanning the genes from cytochrome P-450 through rhodocoxin reductase (pFAJ2309) conferred biosafener activity on *R. erythropolis* SQ1. Degradation in liquid culture, however, was slow compared with that by strain NI86/21. Whereas degradation of EPTC (100 μg/ml in BSM) by the latter strain was completed after 2 days, the SQ1 transformant containing pFAJ2309 required 12 days to completely decompose the EPTC. Residual amounts of EPTC after 2, 4, and 8 days were 93, 79, and 54%, respectively. This slow decomposition is probably due to the low concentration of cytochrome P-450 (9.7 pmol/mg of protein) in EPTC-grown cells of SQ1 with pFAJ2309, which was about 18-fold lower than that in strain NI86/21. The constructs lacking the cytochrome P-450 gene (pFAJ2352 and pFAJ2354) did not confer EPTC-degrading activity. The cytochrome P-450 gene alone (pFAJ2350) introduced into SQ1 was also not able to do so, indicating that rhodocoxin and rhodocoxin reductase are required for electron transfer to cytochrome P-450, thus providing the necessary regeneration of the redox system. On the other hand, EPTC-degrading activity of mutant FAJ2027 was fully restored (complete decomposition after 2 days) solely by introducing the gene for cytochrome P-450 (pFAJ2350). EPTC-grown cells of FAJ2027 containing pFAJ2350 accumulated almost wild-type levels of cytochrome P-450 (117 pmol/mg of protein). This indicates that *thcB* was inactivated by chemical mutagenesis, leaving the rhodocoxin-rhodocoxin reductase system intact in FAJ2027. pDA71 derivatives containing the regulatory gene, such as pFAJ2353, appeared unstable in strain SQ1, since only vectors with apparent deletions in the insert were detected. The above data also indicate that ORF5 is not required for expression of EPTC degradation in strain SQ1, but no definite conclusions can be drawn regarding the role, if any, of ORF4. The positioning of ORF4 is reminiscent of that of the transcribed but nonessential open reading frame of 139 amino acids located between genes for subunits of the multifunctional biphenyl dioxygenase and its associated ferredoxin-ferredoxin reductase components from *Pseudomonas* strains KF707 (76) and LB400 (20). However, there is no sequence homology with ORF4.

Identification of metabolites produced from EPTC by *Rhodococcus* sp. strain NI86/21. The GC-MS analysis resulted in detection and identification of two metabolites showing retention times of 6.6 and 12.1 min (EPTC, 9.5 min). The structure of the major compound which eluted first, the N-dealkylated product PrNHCOSEt (*N*-depropyl EPTC), was apparent from comparison of its mass spectrum with that of EPTC (Fig. 8). A mass shift of 42 Da was observed for the ions M^+, $[M\text{-}Et]^+$, $[M\text{-}Et\text{-}ethene]^+$, and $[M\text{-}SEt]^+$ detected at *m/z* ratios of 147 (189), 118 (160), 90 (132), and 86 (118), respectively. The ion $[EtSCO]^+$ at an *m/z* of 89 is common to both spectra, whereas the NH-hydrogen-retaining species $[EtSH]^+$ at an *m/z*

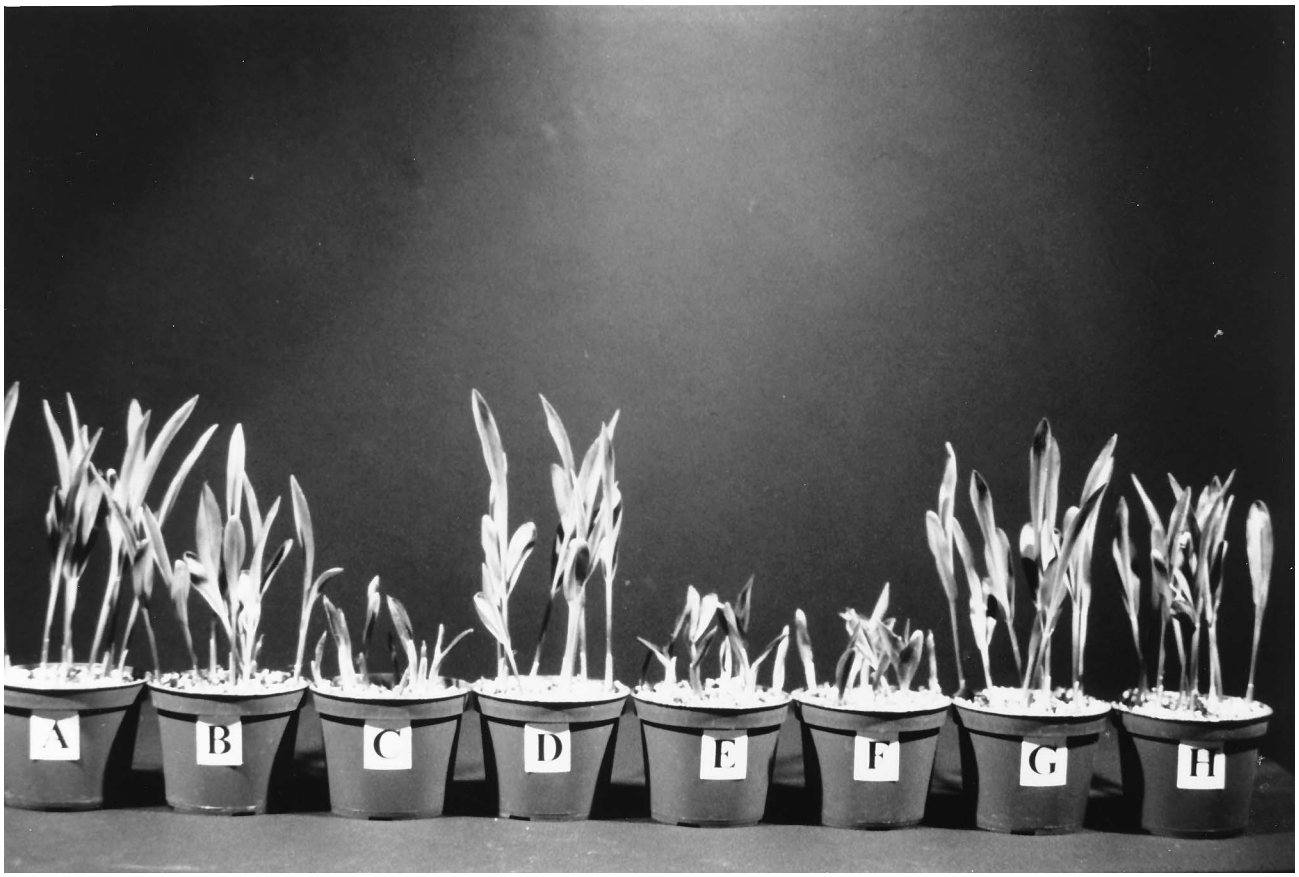

FIG. 7. Biosafener activity of *Rhodococcus* strains on EPTC-treated maize. Controls consisted of nontreated maize (A), a combined application of EPTC plus chemical safener AD-67 (Alirox 80 EC) (B), and application of EPTC without safener (Witox 72 EC) (C). The effects of different inoculations on maize plants growing in the presence of EPTC after 10 days are shown for *Rhodococcus* sp. strain NI86/21 (D), *R. erythropolis* SQ1 (E), EPTC-negative mutant FAJ2027 (F), *R. erythropolis* SQ1 carrying pFAJ2309 (G), and FAJ2027 carrying pFAJ2350 (H).

of 62 appears specific for the N-dealkylated product. The GC-MS properties, i.e., retention time and mass spectrum (*m*/*z* ratios of 128, 86, and 43), observed for the other metabolite were consistent with the sulfoxide structure, $Pr_2NCOS(O)Et$ (data not shown). Dick et al. (15) reported that GC-MS analysis of this compound was not successful because of thermal decomposition. However, degradation of the metabolite and the synthetic standard could be largely avoided by using on-column injection at a low temperature.

DISCUSSION

By transfer of the structural gene for the EPTC-inducible cytochrome P-450 of *Rhodococcus* sp. strain NI86/21 to an EPTC-negative mutant, the EPTC-degrading ability and biosafener activity were restored. In order to confer thiocarbamate-degrading ability and biosafener activity on *R. erythropolis* SQ1, a species which normally does not decompose EPTC, the cytochrome P-450 gene together with the closely linked genes for rhodocoxin and rhodocoxin reductase was required. Thus, a type II cytochrome P-450 system is involved in EPTC degradation and maize protection by *Rhodococcus* sp. strain NI86/21. On the basis of hybridization studies, no equivalent system appears to exist in other *Rhodococcus* species. Type II systems, which use a small iron-sulfur protein (ferredoxin) and a flavin adenine dinucleotide-containing reductase for transfer of electrons to the terminal cytochrome P-450, are typically found in bacteria and display a much narrower substrate specificity than the eukaryotic type I cytochrome P-450 systems, in which electrons are shuttled through an NADPH-cytochrome P-450 reductase (69).

The S-oxygenated and N-dealkylated metabolites of EPTC generated during degradation by *Rhodococcus* sp. strain NI86/21 are consistent with the involvement of a cytochrome P-450 system. Indeed, conversions catalyzed by various cytochrome P-450s include carbon hydroxylation, heteroatom oxygenation, and heteroatom dealkylation (26, 27, 34). Spectrophotometric analysis showed that cytochrome P-450 was able to bind EPTC, vernolate, and butylate.

Supporting evidence for the N dealkylation of EPTC to *N*-depropyl EPTC and propionaldehyde during biodegradation is the concomitant induction at a high level of an aldehyde dehydrogenase which is preferentially active on aliphatic aldehydes. All aldehydes potentially generated by N dealkylation of thiocarbamates known to be degraded by *Rhodococcus* sp. strain NI86/21 were suitable substrates for this NAD^+-dependent dehydrogenase: acetaldehyde (cycloate), propionaldehyde (EPTC and vernolate), and isobutyraldehyde (butylate). The fact that molinate is not metabolized by *Rhodococcus* sp. strain NI86/21 (50) is probably due to its basic structural difference from the former thiocarbamates. In molinate, nitrogen is part of a hexahydro-1*H*-azepine heterocycle. N dealkylation of molinate, if any, would therefore not generate a readily metabolizable free aldehyde. Although we have no evidence

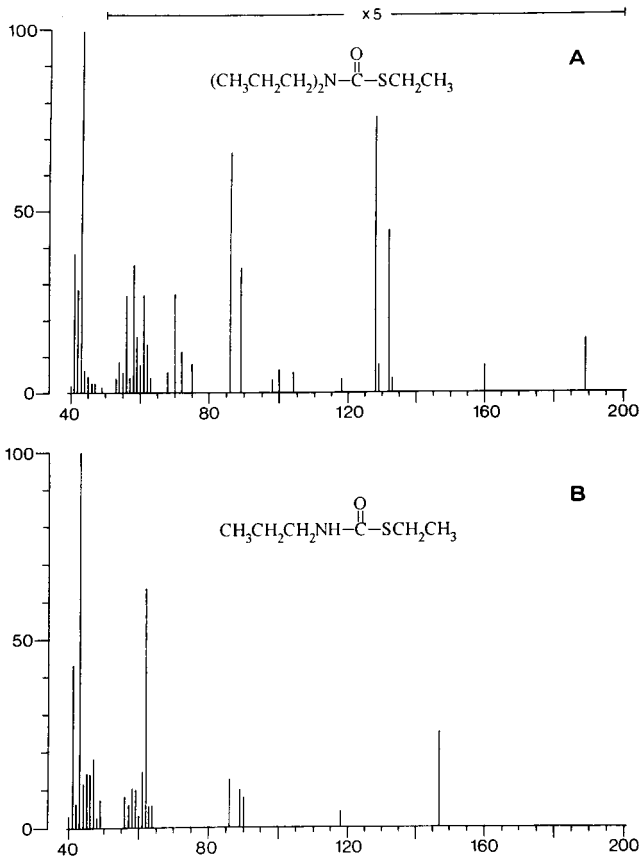

FIG. 8. Electron impact mass spectra of EPTC (A) and the N-dealkylation product of EPTC (B).

for α-carbon hydroxylation occurring in the *S*-alkyl side chain of EPTC during biodegradation by *Rhodococcus* sp. strain NI86/21, this reaction may produce acetaldehyde by decomposition of the unstable hydroxylated thiocarbamate, as observed with EPTC in mouse microsomes (8). For the gene encoding aldehyde dehydrogenase, which was not linked with the cytochrome P-450 DNA region, homologs were present in all *Rhodococcus* species tested, indicating that the enzyme most probably participates in other metabolic pathways as well. In view of the high reactivity and potential toxicity of aldehydes generated during thiocarbamate decomposition, the induction of an aldehyde dehydrogenase catalyzing an irreversible oxidation reaction could protect the *Rhodococcus* cells by efficient conversion of the aldehydes to the respective carboxylic acids. We have previously identified the rhodococcal homolog of the β subunit of the eukaryotic propionyl coenzyme A-carboxylase gene (52). This carboxylase may be involved in the further metabolism of EPTC-derived propionic acid. At present, it is not clear whether EPTC-sulfoxide constitutes a dead-end metabolite for *Rhodococcus* sp. strain NI86/21. In mammals and plants, thiocarbamate sulfoxides are further metabolized after coupling with glutathione (25, 35).

The biodegradation of EPTC by *Rhodococcus* sp. strain NI86/21 represents yet another example of the involvement of a cytochrome P-450 enzyme in an early step of xenobiotic biodegradation by bacteria (56, 69, 70). The actinomycetes in particular have been found to be a rich source of such enzymes. At present, involvement of a microbial cytochrome P-450 in herbicide degradation has been demonstrated only for the sulfonylurea-metabolizing *Streptomyces griseolus* (57). This system, which consists of two related cytochrome P-450s performing various types of oxidative reactions on sulfonylureas, seems not to be related to the EPTC-induced system of *Rhodococcus* sp. strain NI86/21, on the basis of the lack of primary sequence homology and basic differences in the respective electron supply systems. In *S. griseolus*, the structural genes for the ferredoxins of the [3Fe-4S] type are located downstream of their corresponding cytochrome P-450 genes, but there is no evidence for linkage to a ferredoxin reductase gene (55, 59). A similar gene organization has been described for the cytochrome P-450 induced in *Streptomyces griseus* growing in medium with soybean flour (78) and for the fasciation-related cytochrome P-450 with unknown function located on the pFiD188 plasmid of *R. fascians* (9). The cytochrome P-450 systems induced in *R. rhodochrous* strains by *n*-octane (70) or alkoxyphenols and alkoxybenzoates (18, 37) and in *Rhodococcus chlorophenolicus* by pentachlorophenol (80) have not yet been genetically characterized. Interestingly, the organization of the thiocarbamate-induced cytochrome P-450 system of *Rhodococcus* sp. strain NI86/21 is actually more similar to certain systems reported for *Pseudomonas* species (reviewed in reference 82) than to those currently characterized in other actinomycetes. The operons for biodegradation of camphor by *P. putida* (39, 61) and of α-terpineol by a *Pseudomonas* sp. (62) also contain a cytochrome P-450 gene linked with the downstream genes for the corresponding ferredoxin reductases and [2Fe-2S] ferredoxins. Unlike the cytochrome P-450s, the reductases and especially the iron-sulfur proteins of these *Pseudomonas* strains display remarkable sequence homology with the corresponding proteins from *Rhodococcus* sp. strain NI86/21. For both the reductase and the ferredoxin of the latter strain, the highest degree of homology (>40% identity) was found with components of the camphor-hydroxylating system from *P. putida.*

The putative regulatory protein ThcR belongs to the AraC-XylS family of regulators (22). Several of its members are involved in positive regulation of carbon source catabolism. ThcR shows significant homology with the positive regulator of alkylbenzoate metabolism, XylS from *P. putida*, in the C-terminal DNA-binding domain, but the level of homology is much lower in the N-terminal effector-binding domain (46). The divergent transcription of the regulatory genes observed with most members of the AraC-XylS family (22) is also found for ThcR. This suggests that ThcR, for which no homologous gene was detected in other rhodococci, may be involved in positive regulation of the cytochrome P-450 system for EPTC decomposition. A DNA fragment lacking *thcR* conferred EPTC-degrading ability on *R. erythropolis* SQ1, but the decomposition rate was quite low. Possibly, transcription of the EPTC-degrading genes in SQ1 was directed by vector promoter activity. Studies of DNA binding and possible effector interactions need to be carried out with heterologously expressed ThcR in order to confirm the proposed function of this protein.

The identification of a cytochrome P-450 system involved in EPTC degradation by *Rhodococcus* sp. strain NI86/21 provides new insights into the microbial pathway of thiocarbamate degradation. The availability of its heterologously overexpressed components will enable the use of a reconstituted system for the analysis of thiocarbamate substrate specificity and for the characterization of the degradation products generated from different thiocarbamates. An important matter that will be addressed is the regulation of EPTC biodegradation, including the role of the putative regulatory protein. We are continuing our effort to characterize additional thiocarbamate-induced proteins from *Rhodococcus* sp. strain NI86/21 in order to elu-

cidate the fate of the accumulating dealkylated and sulfoxidized products. The fact that *Rhodococcus* sp. strain NI86/21 can grow on thiocarbamates in otherwise nitrogen-free medium indicates that further degradation is possible, probably at a lower rate than the initial dealkylation or sulfoxidation.

ACKNOWLEDGMENTS

We thank P. Marijnen (Center for Human Genetics, Catholic University of Leuven, Heverlee, Belgium) for supplying synthetic oligonucleotides, M. Goodfellow (Medical School, Newcastle upon Tyne, United Kingdom) for providing *Rhodococcus* strains, D. Shields (Trinity College, Dublin, Ireland) for the GCWIND program, J. De Somer and K. Goethals (Laboratory of Genetics, University of Ghent, Ghent, Belgium) for sharing data prior to publication, and J. Nagy (North-Hungarian Chemical Works) for providing thiocarbamate herbicides. Plasmids pDA71 and pCE30, together with suitable host strains, were kindly provided by E. Dabbs (University of Witwatersrand, Johannesburg, South Africa) and N. Dixon (Australian National University, Canberra, Australia), respectively. I.N. and R.D.M. contributed equally to this work.

We are indebted to J. Van Damme for the use of the protein sequencing facilities at the Rega Institute of Medical Research (supported by the National Fund for Scientific Research). This research was supported in part by the National Fund for Scientific Research (Belgium) and by postdoctoral fellowships from the Research Council of the Catholic University of Leuven (F/9/21) and from the Commission of the European Community (ERB-CIPA-CT-92-2007) to I.N. R.D.M. is a Senior Research Associate with the National Fund for Scientific Research (Belgium).

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