Thiocarbamate Herbicide-Inducible Nonheme Haloperoxidase of *Rhodococcus erythropolis* NI86/21

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During biodegradation of thiocarbamate herbicides by *Rhodococcus erythropolis* NI86/21, a protein with an M_r of 30,000 is induced (I. Nagy, G. Schoofs, F. Compennolle, P. Proost, J. Vanderleyden, and R. De Mot, J. Bacteriol. 177:676–687, 1995). Based on N-terminal sequence data for the protein purified by two-dimensional electrophoresis, the corresponding structural gene, *thcF*, was cloned and sequenced. The deduced protein sequence of ThcF is homologous to those of nonheme haloperoxidases. A particularly high level of sequence identity (72.6%) was observed for the chloroperoxidase from *Pseudomonas pyrocinia*. A polyclonal antibody against the latter enzyme cross-reacted with ThcF either produced by the original *Rhodococcus* cells or overexpressed heterologously in *Escherichia coli*. In both thiocarbamate-grown *Rhodococcus* cells and *E. coli* cells expressing *thcF*, the haloperoxidase activity of ThcF was demonstrated. The thiocarbamate-inducible *R. erythropolis* ThcF protein represents the first (nonheme) haloperoxidase to be identified in a nocardioform actinomycete.

Soil isolates capable of degrading thiocarbamate herbicides are apparently largely confined to the genus *Rhodococcus* (3, 9, 17). These nocardioform actinomycetes display a large potential for biodegradation and bioconversion of a wide variety of organic substances (34). Recently, we identified the genes for a cytochrome P450 system, *thcRBCD*, that is required for N-dealkylation of thiocarbamates by *Rhodococcus* sp. strain NI86/21 (19). Moreover, the same enzyme system is involved in the N-dealkylation of the *s*-triazine herbicide atrazine by this strain (18). Subsequently it was shown that a very similar but plasmid-encoded enzyme system is used for the degradation of both thiocarbamates and *s*-triazines by another *Rhodococcus* isolate, strain TE1 (29, 30). Along with cytochrome P450, the aldehyde dehydrogenase ThcA (18) and the alcohol oxidoreductase ThcE (20) are induced in thiocarbamate- and atrazine-grown cells of *Rhodococcus* sp. strain NI86/21, in keeping with the N-dealkylation degradation route, which generates alkyl aldehydes that are suitable substrates for these enzymes. Two-dimensional protein electrophoresis revealed an additional thiocarbamate-inducible protein with an M_r of 30,000 in strain NI86/21 (19), which was not present in atrazine-grown cells (18).

In order to further elucidate the biodegradation of thiocarbamates by this strain, now identified as *Rhodococcus erythropolis* NI86/21, we have cloned, sequenced, and overexpressed the gene encoding this protein, ThcF, showing that it represents a nonheme haloperoxidase.

MATERIALS AND METHODS

Media and growth conditions. *Escherichia coli* strains were routinely grown in Luria-Bertani medium at 37°C. *Rhodococcus* species were cultivated at 30°C in basal salt medium (BSM) with appropriate carbon sources (19, 20). In selective media, ampicillin was used at 100 µg/ml and chloramphenicol was used at 40 µg/ml. *S*-ethyl dipropylcarbamothioate (EPTC) and *S*-propyl dipropylcarbamothioate (vernolate) were obtained from North-Hungarian Chemical Works (Sajóbáony, Hungary). Identification of strain NI86/21 was carried out at the

Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Braunschweig, Germany.

Protein electrophoresis, N-terminal sequencing, and immunodetection. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and two-dimensional electrophoresis were carried out as described previously (7). Protein spots or bands of interest were electroblotted and then subjected to N-terminal sequence analysis (16) with an Applied Biosystems 477A sequencer. For immunodetection after Western blotting, rabbit antiserum raised against purified *Pseudomonas pyrocinia* chloroperoxidase (diluted 10,000-fold) was used (2), followed by incubation with goat anti-rabbit immunoglobulin G-conjugated alkaline phosphatase (12).

Enzyme assay and protein determination. Cells collected by centrifugation (8,000 × g, 10 min) from 200 ml of culture broth were washed once with 100 mM potassium phosphate buffer (pH 6.8) and resuspended in the same buffer (10 ml per 100 mg of cells). A Fastprep shaking apparatus (Savant Inc., Farmingdale, N.Y.) was used to break cells of *E. coli* (six times for 20 s at speed 5) or *R. erythropolis* (three times for 40 s at speed 6), with intermittent cooling on ice. Cell debris was removed by centrifugation (12,000 × g, 10 min), and the supernatant was dialyzed overnight at 4°C against 100 mM ammonium acetate, pH 6.8. The dialyzed samples were used for enzyme assays and protein determination. Haloperoxidase activity was measured spectrophotometrically with monochlorodimedone as a substrate (26). One enzyme unit converted 1 µmol of substrate per min. The bicinchoninic acid method was used for protein measurements, with bovine serum albumin as a standard (32).

Cloning and sequence analysis of the *thcF* gene. Based on the sequence EIFYKDWG near the N terminus of the thiocarbamate-inducible protein with an apparent molecular weight of 30,000, the oligonucleotide mixture 5'-GARA THTTYTAYAARGAYTGGGG-3' (with R representing A or G; H representing A, C, or T; and Y representing C or T) was synthesized. These 23-mers were used as a digoxigenin-labeled probe in Southern and colony hybridizations as described previously (19). The probe revealed a single hybridizing band of about 3.5 kb in *SalI*-digested total DNA of *R. erythropolis* NI86/21. From an enriched pUC18 library consisting of size-selected *SalI* fragments, the corresponding cloned DNA fragment (pFAJ2282) was identified by colony hybridization. The hybridizing 1.5-kb *EcoRV-SalI* fragment was subcloned in *SmaI-SalI*-digested pUC18 to generate pFAJ2346. This plasmid was used to generate overlapping subclones which were subjected to double-stranded DNA sequence analysis with the automated A.L.F. sequencer (Pharmacia Biotech). The PCGENE software (IntelliGenetics) was used for computer-assisted sequence analyses. The programs GCWIND (31) and FRAME (4) facilitated identification of potential coding regions. FASTA (22) and BLAST (1) searches were used to trace homologous protein sequences in the databases.

Heterologous expression of *thcF*. The pCE30 system was used to drive overexpression of ThcF in *E. coli* (10). The 1.5-kb *EcoRI* fragment of pFAJ2346 (retaining the *EcoRI-SmaI* part of the pUC18 polylinker) was cloned in pCE30 in the appropriate orientation (to give pFAJ2384). Bacteriophage λ promoter-mediated overexpression was induced by shifting the incubation temperature from 30 to 42°C (10). The 2.4-kb *BglII-BamHI* fragment of pFAJ2282 was cloned into the *BglII* site of pDA71 (to give pFAJ2509) for expression in *R. erythropolis* SQ1. Following electroporation, chloramphenicol-resistant colonies of strain

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant characteristic(s)	Reference or source
<i>Dietzia maris</i> N1015 ^a		11
<i>E. coli</i>		
DH5 α	<i>hsdR17 endA1 thi-1 gyrA96 relA1 recA1 supE44 ΔlacU169 (ϕ80lacZΔM15)</i>	28
HB101	<i>recA13 proA2 lacY1 hsdS20 endA rpsL20 ara-14 galK2 xyl-5 mtl-1 supE44</i>	28
MM294-1	<i>endA1 hsdR17</i>	27
<i>Gordona aichiense</i> N938 ^a		11
<i>G. rubropertinctus</i> ATCC 25593 ^a		E. Dabbs
<i>Rhodococcus australis</i> CSIRA448		E. Dabbs
<i>Rhodococcus coprophilus</i> N774		11
<i>R. erythropolis</i>		
NI86/21	Thiocarbamate-degrading strain with biosafener activity	19
SQ1	Mutant of ATCC 4277-1 with increased transformability	27
DSM743		O. Asperger
DSM1069		E. Dabbs
DSM43066		O. Asperger
<i>Rhodococcus fascians</i>		
D188	Isolated from <i>Chrysanthemum morifolium</i>	8
D188-5	pD188-cured mutant of <i>R. fascians</i> D188	8
LMG5360	Previously classified as <i>Rhodococcus luteus</i>	LMG ^b
<i>Rhodococcus globerulus</i> R58		11
<i>Rhodococcus rhodochrous</i>		
N54		11
ATCC 12674		E. Dabbs
<i>Rhodococcus ruber</i> N361		11
Plasmids		
pUC18	<i>E. coli</i> cloning vector; Amp ^r	
pCE30	Vector for temperature-dependent overexpression in <i>E. coli</i>	10
pDA71	<i>E. coli</i> - <i>Rhodococcus</i> shuttle vector; Amp ^r (<i>E. coli</i>) Cm ^r (<i>Rhodococcus</i>)	5
pFAJ2282	pUC18 with 3.5-kb <i>SalI</i> fragment of <i>R. erythropolis</i> NI86/21 carrying <i>thcF</i>	This work
pFAJ2346	pUC18 with 1.5-kb <i>EcoRV</i> - <i>SalI</i> fragment of pFAJ2282 containing <i>thcF</i>	This work
pFAJ2384	pCE30 with 1.1-kb <i>EcoRI</i> fragment of pFAJ2346 containing <i>thcF</i>	This work
pFAJ2509	pDA71 with 2.4-kb <i>BglII</i> - <i>BamHI</i> fragment of pFAJ2282 carrying <i>thcF</i>	This work

^a Formerly classified as a *Rhodococcus* species.

^b LMG, Culture Collection at the Laboratory of Microbiology, University of Ghent, Ghent, Belgium.

SQ1 were screened by colony hybridization for the presence of pFAJ2509. Gel filtration on Superdex 75, equilibrated with 100 mM ammonium acetate buffer (pH 6.8) containing 0.2 M potassium chloride, was used to estimate the relative molecular weight of the native enzyme expressed in *E. coli* HB101.

Heterologous hybridizations. Southern hybridizations of total DNAs from *Rhodococcus* and related species cut with *Bam*HI were carried out with the digoxigenin-labeled 675-bp *Pvu*II-*Hind*II internal fragment of *thcF* under conditions described previously (19).

Nucleotide sequence accession number. The nucleotide sequence presented here has been assigned accession no. U95170 by GenBank.

RESULTS AND DISCUSSION

Identification of strain NI86/21. The preliminarily identified strain NI86/21 (Table 1) was further characterized by chemotaxonomic analyses. The combination of *meso*-diaminopimelic acid as a diagnostic peptidoglycan amino acid, mycolic acids with chain lengths of C₃₄ to C₄₀, and a high proportion of tuberculostearic acid is consistent with the strain being a member of the genus *Rhodococcus*. Comparison of qualitative and quantitative chemotaxonomical data with the DSMZ database on *Rhodococcus* species enabled identification of strain

NI86/21 as *R. erythropolis*. This was confirmed by the partial 16S rDNA sequence revealing 100% identity with the *R. erythropolis* sequence.

Characterization of the gene encoding the thiocarbamate-inducible protein ThcF. The previously identified protein with an *M_r* of 30,000 that is induced in *R. erythropolis* NI86/21 when the strain is grown in the presence of various thiocarbamates (19) was purified by two-dimensional electrophoresis and subjected to N-terminal sequencing following electroblotting. For this protein, designated ThcF (for thiocarbamate-inducible protein F), the sequence PFVTASDGTIFYKDWGSGRPI was determined. By using an oligonucleotide mixture corresponding to the subsequence EIFYKDWG, a single hybridizing band of about 3.5 kb was revealed by Southern hybridization with *SalI*-digested genomic DNA of strain NI86/21. From a size-selected library in pUC18, the hybridizing genomic fragment was cloned, and subclones were subjected to DNA sequence analysis. The high GC content of the DNA fragment (about 67%) facilitated identification of the most likely coding

1 GATATCGAAACCGCCCGGTACCCGGGCGAGACCACAAGGAGCAGACCATGCCGTTCTGTCACCGCCAGCGACGGAACCGAAATCTTCTACAAGGACTGGGG
ThcF > M P E F V T A S D G T E I F Y K D W G
101 CTCGGGCGCGCGCATGTTCCACCACGGCTGGCCGCTGAGCTCGGACGACTGGGACTCCAGCTGCTCTTCTCGTCCAGCGGGGCTACCGGTCATC
S G R E I M F H H G W P L S S D D W D S Q L L F L V Q R G Y R V I
201 GCCACGACCGCCCGCGGCGCGGCTCCGCCAGGTCCGGCCAGGGCAGACATGGACCACTACGACGCGGACCGCCCGCGTGGTGGCGCACCTCG
A H D R R R G H G R S A Q V G H G H D M D H Y A A D A A V V A H L G
301 GCCTGCGCGACGTCTCCACGTCCGCCACTCCACGGTGGTGGAGAGGTGCGCCGTTACGTTGCCAGGCACGGTTCGGGGCGGGTCCGAAGGCGCTCT
L R D V V H V G H S T G G G E V A R Y V A R H G A G R V A K A V L
401 CATCGGAGCGGTCCCGCGCTCATGGTCCAGACCGAATCGAACCCGGAAGGGTTCGCGGTTCGAGGCTCTCGACGGCTTCGCGAGGCGCGTGGTTACCAAC
I G A V P P L M V Q T E S N P E G L P V E V F D G F R E A V V T N
501 CGCTCCAGTTCTACCTCGACCTCGCTCCGGGCGGTTCTACGGGTTCACCGCCCGGGCGCGGACATCTCCAGGGCGTCATCCAGAACTGGTGGCGCC
R S Q F Y L D L A S G P F Y G F N R P G A D I S Q G V I Q N W W R Q
601 AGGGAATGACCGCGCAGCGCAGCTACGAGGGGATCAAGGCATTCTCGGAGACGACTTCACCGACGACCTCCGGGCGGATCGACGTGCCCGACGCT
G M T G S A Q A H Y E G I K A F S E T D F T D D L R A I D V P T L
701 CATCATGACCGGCGACGACGACAGATCGTCCGATCGCAACTCCGCCGAGACGGCGGTACGTTGGTCAAGAACGCGCGCTGAAGGTCTACCGGGG
I M H G D D D Q I V P I A N S A E T A V T L V K N A R L K V Y P G
801 CTGTGCGACGGCATGTGCACCGTGAACGCCGACACCGTCAACGCCGACCTCCTCAGCTTCATCGAGAGCTGACACCGGTCTGGCACCAGACTCAGCCG
L S H G M C T V N A D T V N A D L L S F I E S *
901 TCAGCACAAGACAGAAATCACCATGACCGCAACCTACCGGCTACGTGACCGTCTCTGGGACGCAAAGGCGAAGCCGGCAGGGAGGCGCGAC
1001 TCAAGGCGTTCATACCGCGCGCTCACCCCTCGGCAACGACCCCGCAACATCGACTACGAGGCCACGAAATCGAGGCGAGACCTCGCAATTCGT
1101 CATCTACGAACGCTGGGAGACACGCGCCACCTCGACGCCACCTCGTGCACCCCGGATGCGGGAATCTGTCGCGAGATGCTCGAACTGATCGACGGA
1201 TCC

FIG. 1. Nucleotide and deduced amino acid sequences of the *EcoRV*-*Bam*HI DNA fragment carrying the *thcF* gene of *R. erythropolis* NI86/21. The putative ribosome binding site is shown in italic. The stop codon is marked with an asterisk. The amino acid residues that were confirmed by N-terminal sequence analysis are underlined.

region by the GCWIND and FRAME programs, based on the biased codon usage in high-GC coding regions. The ATG start codon is preceded by a putative ribosome binding site (AAG GAG) with an appropriate spacing of 6 nucleotides (Fig. 1). The deduced amino acid sequence (274 residues) matches the N-terminal sequence data (Fig. 1). Also, the calculated value for the molecular mass (29,664 Da) is very close to the apparent molecular weight (19).

Identification of ThcF as a nonheme haloperoxidase. Database searches revealed a strong homology of *R. erythropolis* ThcF with the nonheme chloroperoxidases from *P. pyrocinia* (72.6% identity [36]) and *Streptomyces lividans* (65.3% identity [2]). A high level of amino acid sequence identity was also observed for a novel esterase of *Pseudomonas putida* (67.2% identity [21]), as shown in Fig. 2. In addition, ThcF displays significant, although substantially lower, sequence similarity with bromoperoxidase A1 of *Streptomyces aureofaciens* (50% identity [25]), the bifunctional esterase-bromoperoxidase of *Pseudomonas fluorescens* (47.5% [23]), a putative bromoperoxidase of a *Synechocystis* sp. identified by genomic sequencing (41.6% [14]), and bromoperoxidase A2 of *S. aureofaciens* (36.9% [26]). The residues of the conserved catalytic triad S, D,

and H, essential for halogenation (13, 24), are present in ThcF at positions 95, 225, and 254, respectively (Fig. 2).

No haloperoxidase activity was detected in cells of strain NI86/21 grown with fructose as a carbon source (Table 2). In the presence of thiocarbamates, haloperoxidase activity was induced. A higher enzyme level was obtained with vernolate (11.2 mU/mg) than with EPTC (5.9 mU/mg) as a carbon source. An antiserum raised against the *P. pyrocinia* chloroperoxidase revealed a protein band with an M_r of 30,000 (same position as for the purified *Pseudomonas* enzyme) on a Western blot for EPTC-induced *Rhodococcus* cells but not for uninduced cells (Fig. 3). No enzymatic activity was detectable in the presence of phenylmethylsulfonyl fluoride. Inhibition of other nonheme haloperoxidases by phenylmethylsulfonyl fluoride was reported previously (23, 24). These data further substantiate the identification of ThcF as a putative haloperoxidase based on sequence analysis of *thcF*.

Distribution of *thcF* among *Rhodococcus* species. Several representative *Rhodococcus* species and some species formerly classified in the *Rhodococcus* genus were screened for the presence of *thcF*-homologous genes by Southern hybridization with an internal fragment of *thcF* (data not shown). Among the

<i>R. ery</i>	MPFVTASDGTEIFYKDWG--SGRPIMFHHGWPLSSDDWDSQLFLVQGRYVIAHRRGHGRSAQVGHGHDMDHYAADAADVVAHLGLRDVHVHGHSTGG	98
<i>P. pyr</i>	MPYVTTKDNVEIFYKDWGPKDAQPIVFFHHGWPLSGDDWDAQMLFFVQKGYRVIAHRRGHGRSAQVSDGHGDMHYAADAFAVVEALDLRNVHGHSTGG	100
<i>S. liv</i>	MGTVTTSDGTNIFYKDWGPRDGLPVVFFHHGWPLSADDWDNQLMFLFLSHGYRVIAHRRGHGRSDQPSGTGHMDTYAADVAALTEALDLRGAVHGHSTGG	100
<i>P. put</i>	MSYVTTKDGVIIFYKDWGPRDAPVIHFFHHGWPLSADDWDAQMLFFLAHGYRVVAHRRGHGRSSQVWDGHGDMHYADDVAADVVAHLGIQGAHVHGHSTGG	100
	= == = = =	
<i>R. ery</i>	GEVARYVARHG--AGRVAKAVLIGAVPPLMVQTESNPEGLPVEVDFGFAEVVNTNRSQFYLDLASGPFYGFNRPAGADISQGVQINWWRQGMTGSAQAHYE	196
<i>P. pyr</i>	GEVARYVANDGQPAVRVAKAVLVSAPVPLMLKTESNPEGLPIEVDFGFRKALADNRAQFFLDVPTGPFFYGFNRAQATVHQGVIRNWRQGMESAKAHYD	200
<i>S. liv</i>	GEVARYVARAE--PGRVAKAVLVSAPVPMVKSDTNPDLPLEVDFEFRAALANRAQFYIDVPSGPFYGFNREGATVSGQLIDHWLQGMMAANAHYE	198
<i>P. put</i>	GEVVRVMARHP--ADKAVAKAVLIAAPVPLMVQTPDNPGLPKSVDFGFAQVANSRAQFYRDVDPAGPFYGFNRPVGDASEGIIGNWRRGMIGSAKAHYD	198
	== == = =	
<i>R. ery</i>	GIKAFSETDFTDDLRAIDVPTLIMHGDDQIVPIANSAETAATVTLVKNARLKVYPGLSHGMCTVNADTVNADLLSFIES	274
<i>P. pyr</i>	GIKAFSETDQTEDLKSIIVPTLVHGEDDQIVPIADAALKSIKLLQNGTLKTYPGYSHGMLTVNADVLNADLLAFVQA	278
<i>S. liv</i>	CIAAFSETDFTDDLKRIDVPTLVHAGTDDQVVPYADAAPKSAELLANATLKSVEGLPHGMLSTHPEVLNPDLLAFVKS	276
<i>P. put</i>	GIVAFSQDFTEDLKGITQPVLMVHGGDDQIVPYENSGLLSAKLLPNTLKTQGYPHGMPPTTHADVINADLLAFIRS	276
	= == = =	

FIG. 2. Multiple sequence alignment of ThcF from *R. erythropolis* NI86/21 (*R. ery*) with the nonheme chloroperoxidases from *P. pyrocinia* (*P. pyr*) and *S. lividans* (*S. liv*) and with the thermostable esterase from *P. putida* (*P. put*). Residues that are perfectly conserved (=) or similar (—) are marked. The SDH catalytic triad is in boldface.

TABLE 2. Induction of haloperoxidase activity in thiocarbamate-grown *R. erythropolis* NI86/21 compared with levels of heterologous expression of ThcF in *R. erythropolis* SQ1 and *E. coli* HB101

Strain and growth conditions	Haloperoxidase activity (mU/mg)
<i>R. erythropolis</i> NI86/21 ^a	
Fructose.....	ND ^d
EPTC.....	5.9
Vernolate.....	11.2
<i>R. erythropolis</i> SQ1 ^b	
Control.....	2.3
With pFAJ2509.....	3.0
<i>E. coli</i> HB101(pFAJ2384) ^c	
Control.....	ND
Induced.....	57.9

^a Cells were grown in BSM with the indicated carbon source.

^b Cells were grown in BSM with fructose.

^c Cells grown in Luria-Bertani medium were induced by a temperature upshift (10).

^d ND, no detectable activity.

17 strains tested (belonging to 10 species [Table 1]), no putative homolog was detected except in the case of *Gordona* (*Rhodococcus*) *rubropertinctus* ATCC 25593, where the presence of a faint band may indicate the presence of a related haloperoxidase gene.

Our hybridization data indicate that ThcF homologs are absent from several other *Rhodococcus* species and from several other strains of *R. erythropolis*. Such a narrow distribution is also the case for the cytochrome P450 system, encoded by *thcRBCD*, which catalyzes the N dealkylation of thiocarbamates and atrazine (18, 19). The aldehydes thus generated induce the aldehyde dehydrogenase ThcA (18) and the alcohol oxidoreductase ThcE, both of which are widely distributed among rhodococci, but not ThcF (20). The observation that ThcF is not induced during atrazine degradation (18) further suggests that the thiocarbamates, or some of their metabolites, are responsible for the induction. At present we have not been able to identify compounds other than thiocarbamates capable of inducing ThcF in *R. erythropolis* NI86/21. It cannot be excluded that the thiocarbamates mimic the inducer activity of a naturally occurring, possibly structurally related, substance. All other nonheme haloperoxidases isolated so far are produced constitutively.

FIG. 3. Immunodetection of ThcF by Western blotting with a *P. pyrocinia* antiserum. Lane 1, control cells of *R. erythropolis* NI86/21; lane 2, EPTC-induced cells of strain NI86/21; lane 3, *R. erythropolis* SQ1 containing pDA71; lane 4, *R. erythropolis* containing pFAJ2509; lane 5, *E. coli* containing pFAJ2384 (induced); lane 6, *E. coli* containing pCE30 (induced); lane 7, *E. coli* containing pCE30 (uninduced); lane 8, purified *P. pyrocinia* chloroperoxidase.

FIG. 4. Heterologous expression of *thcF* in *E. coli* analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Lane 1, molecular weight markers (in thousands); lane 2, control cells (not induced); lanes 3 to 5, cells induced for 4, 16, and 24 h, respectively. The position of the overexpressed protein is marked with an arrow.

Heterologous expression of ThcF. Overexpression of the *thcF* gene in *E. coli* HB101 was achieved by using the pCE30 system (Fig. 4). A protein of the appropriate size accumulated in the induced cells and was identified as ThcF by N-terminal sequence analysis (PFVTA). This shows that in *E. coli* the formylmethionine residue is also cleaved off. Compared to the case for thiocarbamate-induced cells of *R. erythropolis* NI86/21, the specific activity was increased about 5- to 10-fold (to 57.9 mU/mg) upon heterologous expression in *E. coli* (Table 2).

For expression in another *Rhodococcus* strain, the pDA71 plasmid carrying *thcF* (including about 1.2 kb of the upstream region to accommodate potential regulatory sequences) was introduced into *R. erythropolis* SQ1, which lacks a *thcF* homolog. However, compared to the specific activity in control cells with pDA71 (2.3 mU/mg), only weakly increased expression of *thcF* (3.0 mU/mg) was apparent (Table 2). The significant basal level of haloperoxidase activity detected in strain SQ1 suggests the presence of another haloperoxidase, different from ThcF, in this strain. This was confirmed by activity staining after native electrophoresis, which revealed a single band in strain SQ1 and an additional but much weaker band when this strain was transformed with the *thcF* gene (data not shown). The endogenous enzyme of strain SQ1 was not detectable on a Western blot (Fig. 3). The low level of expression of ThcF in strain SQ1 probably explains the failure to detect this protein on a Western blot (Fig. 3). The cross-reactivity of the *P. pyrocinia* antiserum with ThcF from *R. erythropolis* NI86/21 cells was also observed for the protein expressed in *E. coli* (Fig. 3). The second band with a slightly higher apparent molecular weight is a cross-reacting protein of *E. coli*, which is also present in induced cells not transformed with *thcF* (Fig. 3). Since no haloperoxidase activity was detectable in the latter cells, this band probably does not represent a nonheme haloperoxidase. Clearly, the pCE30-*E. coli* system, giving a higher expression level and lacking detectable background activity, is preferable for overproducing ThcF. Based on gel filtration, the apparent molecular weight was estimated to be 67,000. Given the subunit molecular mass of about 30 kDa, this value suggests a homodimeric structure rather than a homotrimeric structure. The related chloroperoxidases from *P. pyrocinia* and *S. lividans* are homodimers with M_r of 64,000 (2, 35).

Possible role of ThcF in thiocarbamate degradation. The thiocarbamate-inducible protein ThcF of *R. erythropolis* NI86/21

represents a new member of the nonheme haloperoxidase family, distinct from other halogenating enzymes, which are either heme or vanadium dependent (reviewed in reference 33). All haloperoxidases of bacterial origin currently known lack a heme moiety. They have been isolated mainly from *Streptomyces* and *Pseudomonas* species but were not previously reported for nocardioform actinomycetes. Although several of these species are known to produce halogenated metabolites, there is now evidence that the haloperoxidases are not involved in the biosynthesis of halogenated metabolites (33). A chloroperoxidase-negative mutant of *P. fluorescens* was not affected in production of its chlorinated secondary metabolite pyrrolnitrin (15), and the *Streptomyces* enzyme chlorinating tetracycline is not related to bacterial nonheme haloperoxidases (6). Thus, the biological role of bacterial nonheme haloperoxidases is not yet clear.

Given the low affinity for the cosubstrates (hydrogen peroxide and halides) and low specificity, it is likely that these enzymes catalyze reactions other than halogenation in vivo (33). The crystal structure of the cofactor-free bromoperoxidase A2 of *S. aureofaciens* revealed an α/β -hydrolase fold, with an S-D-H catalytic triad (13). It was shown that this triad, which is conserved among the cofactor-independent haloperoxidases, is essential for both halogenation and esterase activities of an apparently bifunctional enzyme of *P. fluorescens* with high sequence homology to nonheme haloperoxidases (23, 24). The high sequence homology of a stereoselective esterase from *P. putida* (21) with nonheme haloperoxidases, in particular those from *P. pyrocinia*, *S. lividans*, and also *R. erythropolis*, supports the notion that the halogenation reactions catalyzed by these enzymes may not reflect their main biological function. It should be pointed out that thiocarbamates consist of esters of the (theoretical) thiocarbamic acid. Purification of the overproduced recombinant ThcF enzyme will allow investigation of the possible action of ThcF on the thioester moiety of these herbicides. One should also bear in mind that the strongly oxidizing agent peracetic acid is formed from acetate and hydrogen peroxide at the serine residue of the catalytic triad in nonheme haloperoxidases (33). By using purified ThcF, it will be possible to investigate whether formation of this peroxy acid intermediate can give rise to oxidation of thiocarbamates. Notably, the sulfoxide of EPTC was detected as an important metabolite during degradation of this thiocarbamate by *R. erythropolis* NI86/21 (19). These further investigations on the catalytic activity of ThcF, along with the construction and characterization of a defined ThcF-negative mutant will not only contribute to our knowledge of bacterial thiocarbamate degradation but also provide insight into the biological role of nonheme haloperoxidases in bacteria.

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