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## Cloning, Sequencing, and Characterization of the Hexahydro-1,3,5-Trinitro-1,3,5-Triazine Degradation Gene Cluster from *Rhodococcus rhodochrous*

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**Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is a high explosive which presents an environmental hazard as a major land and groundwater contaminant. *Rhodococcus rhodochrous* strain 11Y was isolated from explosive contaminated land and is capable of degrading RDX when provided as the sole source of nitrogen for growth. Products of RDX degradation in resting-cell incubations were analyzed and found to include nitrite, formaldehyde, and formate. No ammonium was excreted into the medium, and no dead-end metabolites were observed. The gene responsible for the degradation of RDX in strain 11Y is a constitutively expressed cytochrome P450-like gene, *xplA*, which is found in a gene cluster with an adrenodoxin reductase homologue, *xplB*. The cytochrome P450 also has a flavodoxin domain at the N terminus. This study is the first to present a gene which has been identified as being responsible for RDX biodegradation. The mechanism of action of XplA on RDX is thought to involve initial denitration followed by spontaneous ring cleavage and mineralization.**

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is one of the most important and widely used military explosives. Due to its manufacture, decommissioning, and disposal it has become a serious pollutant at many sites, particularly across the United States and Germany, and its recalcitrance in the environment has led to its persistence in soil and groundwater. RDX is a potent convulsant due to its effects on the central nervous system and is also a class C, possible, carcinogen (7).

For a long time it was thought that RDX biodegradation could occur only under anaerobic conditions (15, 26); however, aerobic degradation of RDX has been more recently demonstrated by bacterial consortia and pure strains. Coryneform bacteria (45), *Stenotrophomonas maltophilia* strain PB1 (4), and a rhodococcal strain (9) have been shown to utilize RDX as a sole source of nitrogen, generally degrading RDX at higher rates than anaerobic systems do.

The first pathway for the aerobic degradation of RDX by a strain of *Rhodococcus* was proposed very recently (16) and involves denitration as the first step followed by spontaneous ring cleavage (Fig. 1). Positively identified products include nitrite (NO<sub>2</sub><sup>-</sup>), nitrous oxide (N<sub>2</sub>O), formaldehyde (HCHO), and carbon dioxide. A dead-end intermediate with the molecular formula C<sub>2</sub>H<sub>5</sub>N<sub>3</sub>O<sub>3</sub> was also found to accumulate, indicating that not all the components of RDX were mineralized.

This paper reports the isolation and identification of a *Rhodococcus rhodochrous* strain which can degrade RDX as a nitrogen source and the first cloning and expression of a gene responsible for RDX degradation. This gene is constitutively expressed, and its product has homology to a cytochrome P450.

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### MATERIALS AND METHODS

**Reagents.** RDX (>95% purity as determined by high-performance liquid chromatography HPLC) was kindly provided by the Defence Science and Technology Laboratory (Dstl), Fort Halstead. Other chemicals were of analytical grade and, unless otherwise stated, were obtained from Anachem (Luton, United Kingdom), Fisher (Loughborough, United Kingdom), Life Technologies (Paisley, United Kingdom), or Sigma (Poole, United Kingdom). RDX was routinely dissolved in dimethylformamide to a concentration of 1 M before further dilution.

**Selective enrichment cultures.** Soil samples were provided by Dstl from a site that had been heavily contaminated with RDX and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX). The enrichment used RDX as a sole nitrogen source, as described by Binks et al. (4).

**Identification of *R. rhodochrous* strain 11Y.** 16S ribosomal DNA sequences were amplified by PCR (44). Complete identification was performed by comparison with the databases by using BLAST ([www.ncbi.nlm.nih.gov/BLAST](http://www.ncbi.nlm.nih.gov/BLAST)). Strain 11Y was also classified by the National Collection of Industrial and Marine Bacteria (NCIMB) (Aberdeen, United Kingdom) and designated NCIMB 40820.

**Growth conditions.** Minimal medium used consisted of 40 mM potassium phosphate buffer (pH 7.2) containing 10 mM glycerol, 5 mM glucose, 5 mM succinate, trace elements (33), and, unless otherwise specified, 250 μM RDX as a sole nitrogen source. Cultures were grown at 30°C with shaking at 110 rpm.

**Determination of microbial growth and substrate utilization.** Bacterial growth was measured routinely by determination of culture optical density at 600 nm (OD<sub>600</sub>) with a UV-160A recording spectrophotometer (Shimadzu Corp., Kyoto, Japan).

Degradation of RDX was assayed by monitoring the decrease in concentration by HPLC. Samples were removed from the cultures at regular intervals, the cells were removed by centrifugation, and the supernatant (100 μl) was analyzed by HPLC.

**Resting-cell incubations.** Strain 11Y was grown to late-log phase, harvested, and rinsed in 40 mM potassium phosphate buffer (pH 7.5). Cells were used at a final concentration of 0.05 g (wet weight)/ml and incubated in 40 mM potassium phosphate buffer (pH 7.5) plus 0.25 mM RDX at 30°C with agitation every 5 min. When assays for nitrite, nitrate, and formate were performed, the supernatants of samples were assayed immediately. Glacial acetic acid was used to precipitate any protein present, and the resulting supernatant was analyzed by HPLC, by ion chromatography, and for formaldehyde.

**HPLC.** HPLC measurements were performed with a Waters system consisting of a 510 pump, a 7120 WISP 48-vial autosampler, and a 2487 dual λ wavelength detector. Integrations were performed using Waters Millennium software. Sam-

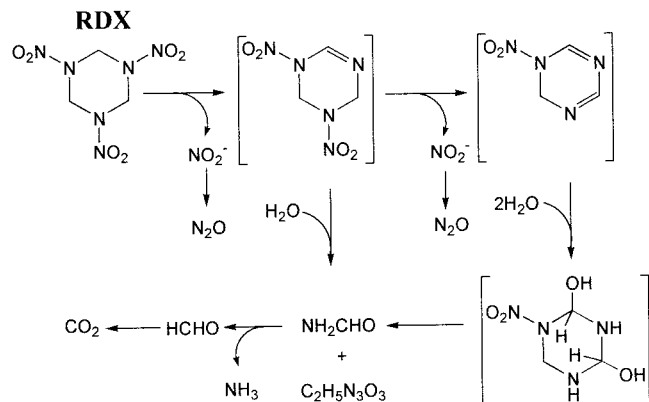


FIG. 1. Proposed pathway for the aerobic degradation of RDX by *Rhodococcus* sp. strain DN22 (16).

ples (100  $\mu$ l) were separated on a 5- $\mu$ m C<sub>18</sub> reversed-phase column (250 by 4.6 mm; HPLC Technology, Welwyn Garden City, United Kingdom). A reverse-phase isocratic mobile phase consisting of acetonitrile and water (50:50, vol/vol) was delivered at a flow rate of 1 ml/min. RDX elution was monitored at 205 nm.

**Analysis of metabolites.** Nitrite, formate, and ammonium were analyzed by ion chromatography performed on a Dionex system comprising an AS40 autosampler and a DX-120 ion chromatograph, with reference to authentic standards. Integrations were performed using Dionex PeakNet software. Samples (25  $\mu$ l) were assayed for anions by using an AS14A column (250 by 4 mm; Dionex) with 8 mM Na<sub>2</sub>CO<sub>3</sub>-1 mM NaHCO<sub>3</sub> as the eluent and were assayed for cations by using a CS12A column (250 by 4 mm; Dionex) with 20 mM methanesulfonic acid as the eluent, both at a flow rate of 1 ml/min. Formaldehyde was analyzed using the Hantzsch spectrophotometric assay (29).

**DNA manipulation.** Restriction endonucleases and other enzymes were purchased from New England Biolabs (Hitchin, United Kingdom) and used as recommended by the manufacturer. Plasmid DNA was prepared using the QIAprep spin Miniprep kit (Qiagen, Crawley, United Kingdom). Ligations were designed as described by Sambrook et al. (35).

**Preparation of *Rhodococcus* genomic DNA and library.** Genomic DNA was prepared from 50-ml cultures (3). The DNA was quantified and subjected to partial digestion with *Sau*3AI. Fragments of 5 to 10 kb were selected and ligated into the *Bgl*III site of pDA71 (12). One Shot *E. coli* cells were used as the hosts for the library (Invitrogen, Groningen, The Netherlands).

**Transformation of *R. rhodochrous* CW25.** *R. rhodochrous* CW25 was described by Quan and Dabbs (32). A polyethylene glycol (PEG)-mediated method, adapted from that of Dabbs and Sole (13), was used to transform *R. rhodochrous* CW25. Cells were grown in LBSG medium (1% [wt/vol] tryptone, 0.5% [wt/vol] yeast extract, 0.5% [wt/vol] NaCl, 10.3% [wt/vol] sucrose, 3% [wt/vol] glycine) to an OD<sub>600</sub> of 2 to 3 and stored for up to 1 month at 4°C. A 50- $\mu$ l aliquot of cells was used for each transformation. The cells were rinsed twice in autoclaved B buffer [0.3 M sucrose, 0.01 M MgCl<sub>2</sub>, 0.025 M *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) (pH 7.2)] and resuspended in the original volume of B buffer containing 5 mg of lysozyme per ml. After a 90-min incubation at 37°C, the cells were rinsed gently in freshly made P buffer (B buffer with KH<sub>2</sub>PO<sub>4</sub> added to a final concentration of 0.3 mM and CaCl<sub>2</sub> added to 0.02 M) and gently resuspended in the original volume of P buffer, and 2  $\mu$ l of plasmid DNA (0.7  $\mu$ g) was added. After 5 min, an equal volume of PPEG (P buffer with 50% [wt/vol] PEG6000) was gently mixed in, and the mixture was left for 5 min. The cells were spread onto cold regeneration plates (LBSG with glycine omitted and 1.5% [wt/vol] agar and 0.4% [wt/vol] MgCl<sub>2</sub> added; after autoclaving and immediately before pouring the plates, 2.4% [vol/vol] 1 M CaCl<sub>2</sub>, 1.2% [vol/vol] 0.03 M KH<sub>2</sub>PO<sub>4</sub>, and 2% [vol/vol] 0.25 M TES [pH 7.2] were added). After a 12-h recovery at 30°C, chloramphenicol to a final concentration of 40  $\mu$ g/ml in the agar was introduced, underneath the agar, using a sterile spatula. Cells were incubated further at 30°C until distinct colonies appeared.

**Screens for RDX-degrading activity in vivo.** A zone-of-clearance screen was performed to identify RDX-degrading strains, using RDX layered plates. These consist of a bottom layer of 0.6% (wt/vol) agarose with phosphate buffer and a top layer of 2% (wt/vol) agarose with phosphate buffer, carbon sources, and trace elements as described above and 5 to 10 mM RDX to form an opaque dispersion.

Bacteria were also grown in minimal medium containing RDX as the sole

nitrogen source, as described above, and the medium was assayed by thin-layer chromatography (TLC) 7 days later for RDX degradation.

**TLC.** Bacterial cultures were centrifuged, and 0.5 ml of the supernatant was extracted into 0.5 ml of ethyl acetate. This was evaporated off, and any residue was dissolved in 20  $\mu$ l of acetone. Polygram Sil G/UV plates (Machery-Nagel, Duren, Germany) were used in a solvent system of chloroform-acetone (2:1). Alkaline hydrolysis was used to liberate nitrite from RDX, which was detected using a colorimetric assay (4).

**DNA sequencing and analysis.** Sequencing of pHSX1 was performed at the Department of Biochemistry, and the Department of Genetics, University of Cambridge. DNA primers were synthesized by Sigma-Genosys (Pampisford, United Kingdom).

All sequence data were analyzed using the BLASTN and BLASTP programs (<http://www.ncbi.nlm.nih.gov/BLAST>), (1) and the Wisconsin package version 10.2 (Genetics Computer Group, Madison, Wis.).

**RNA manipulation.** RNA was extracted using the second method described in reference 28. Contaminating DNA was removed using RQ1 RNase-free DNase (Promega U.K., Southampton, United Kingdom). Reverse transcription-PCR (RT-PCR) was performed with the Access RT-PCR system (Promega) on 1  $\mu$ g of RNA, using the concentrations, conditions, and controls recommended in the protocol.

**Nucleotide sequence accession numbers.** The nucleotide sequences of the strain 11Y 16S rRNA gene and the *xpl* gene fragment have been assigned accession numbers AF439261 and AF449421 respectively, by GenBank (<http://www.ncbi.nlm.nih.gov/Genbank/>).

## RESULTS

### Isolation and identification of *R. rhodochrous* strain 11Y.

Explosive-contaminated soil was used as the source of inocula for the isolation and identification of strains able to grow with RDX. Selective enrichments were performed under aerobic conditions, with RDX supplied as the sole source of nitrogen. One bacterial strain, designated strain 11Y, was purified and found to be able to grow using RDX as the sole nitrogen source with simultaneous loss of RDX from the growth medium as analyzed by HPLC (data not shown).

Strain 11Y was identified using the complete 16S ribosomal DNA gene sequence and comparative phylogenetic analysis and was identified as a species of *Rhodococcus*. Further characterization was carried out by NCIMB, which found strain 11Y to be a gram-positive, nonsporulating, nonmotile organism that is oxidase negative and catalase positive; cell wall and fatty acid analysis showed the presence of mycolic acids and confirmed that the isolate was a strain of *R. rhodochrous*.

**Growth of *R. rhodochrous* strain 11Y on RDX as a nitrogen source.** Cultures of *R. rhodochrous* strain 11Y were routinely grown in minimal medium with glucose, succinate, and glycerol as carbon sources and RDX as the sole nitrogen source at a concentration of 250  $\mu$ M, just below the aqueous solubility limit of 270  $\mu$ M. HPLC analysis of the culture medium showed complete disappearance of RDX within 21 h, with the cells reaching a final OD<sub>600</sub> of 1.8 (Fig. 2). Since the RDX is used as a source of nitrogen, it was thought that nitrogen-containing metabolites such as nitrite and ammonium might appear in the medium before being utilized by the bacteria. Such metabolites have been seen during microbial RDX breakdown in previous studies (5, 9), and strain 11Y can use both as sources of nitrogen for growth (data not shown). No accumulation of either was observed using ion chromatography, even when RDX was added at the higher concentration of 1 mM. Sterile control flasks showed no loss of RDX over the course of the experiment.

To give an indication of how many molecules of nitrogen

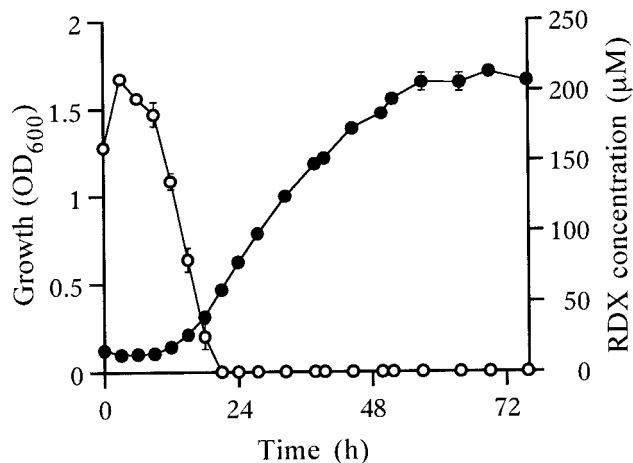


FIG. 2. Growth of *R. rhodochrous* strain 11Y with RDX as the sole nitrogen source. The data show OD<sub>600</sub> (●) and RDX concentration (○). Error bars indicate one standard deviation for triplicate samples.

from RDX are used by *R. rhodochrous* strain 11Y, bacterial growth on media containing RDX or NH<sub>4</sub>Cl was compared. Strain 11Y grown on RDX as the sole source of nitrogen produced half as much growth, as determined by OD<sub>600</sub>, as did cells grown on equivalent amounts of NH<sub>4</sub>Cl (Fig. 3), indicating that three of the six nitrogen atoms from RDX are used for growth.

To determine whether RDX-degrading activity is induced by the presence of RDX, resting-cell incubations were performed comparing cells grown using NH<sub>4</sub>Cl as the sole nitrogen source with cells grown using RDX. Both were found to be capable of removing RDX, although RDX-grown cells removed approximately twice as much RDX as did the NH<sub>4</sub>Cl-grown cells over a 30-min period (data not shown). These data indicate that the RDX-degrading activity is present at significant levels in the absence of RDX but that some upregulation does occur in the

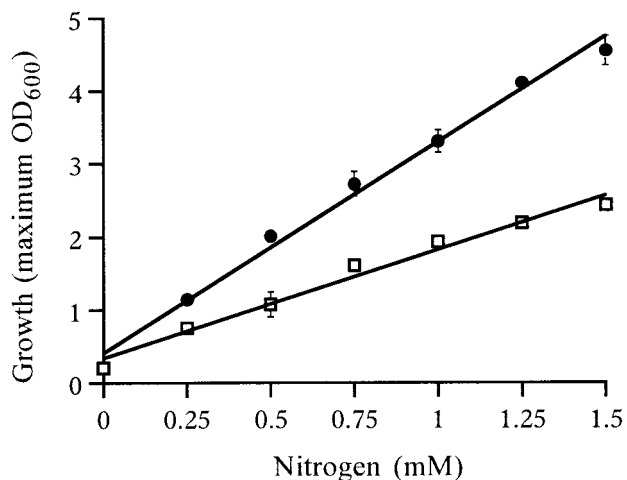


FIG. 3. Maximum growth of *R. rhodochrous* strain 11Y on RDX and ammonium. The final OD<sub>600</sub> on increasing concentrations of RDX (□) and ammonium (●) is shown. The RDX best-fit line has a gradient of 1.486 and  $r^2$  of 0.981. The ammonium best-fit line has a gradient of 2.894 and  $r^2$  of 0.991. Error bars indicate one standard deviation for triplicate samples.

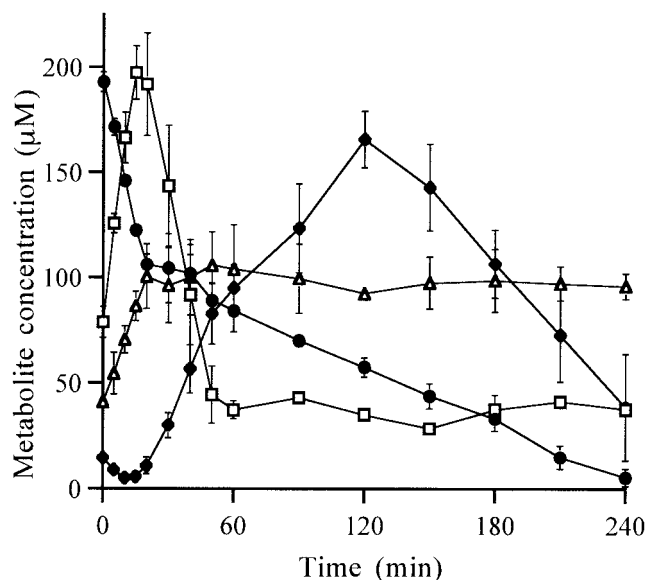


FIG. 4. Resting-cell biotransformations of RDX using strain 11Y. RDX concentration (●), nitrite concentration (□), formate concentration (◆), and formaldehyde concentration (△) are shown. Error bars indicate one standard deviation for triplicate samples.

presence of RDX. No growth was observed on the explosives HMX or TNT (2,4,6-trinitrotoluene). RDX cannot be used by strain 11Y as a source of carbon.

**Products of RDX degradation by whole cells of *R. rhodochrous* strain 11Y.** Analysis of metabolites produced during the metabolism of a given compound can give an indication of the mechanism by which the degradation occurs. Cells of *R. rhodochrous* strain 11Y were grown for 48 h with RDX provided as the sole source of nitrogen, harvested, resuspended, and incubated with 250 µM RDX in phosphate buffer.

RDX disappearance was observed over 4 h, and no other metabolites were seen to accumulate when the reaction mixture was analyzed by HPLC. Ion chromatography identified several metabolites in samples taken from the resting-cell supernatant (Fig. 4). Nitrite transiently appeared in almost equimolar concentrations with the original concentration of RDX within 15 min, and then the concentration decreased. Production of formaldehyde was slower, but it remained in the supernatant, and formate was produced over a longer period and showed a subsequent decline in concentration. The concentration of ammonium produced was not above that seen in controls, and no nitrate was produced. Controls consisted of incubations of RDX without cells or cells without RDX, and these showed very low levels of formate and formaldehyde production. No nitrite was observed and no RDX was degraded in the absence of strain 11Y.

**Cloning, sequencing and subcloning of a 7.5-kb region conferring RDX activity.** A genomic library from *R. rhodochrous* strain 11Y was constructed in *Escherichia coli* using the *E. coli*-*Rhodococcus* shuttle vector pDA71 (12) and genomic fragments of 5 to 10 kb. The library was transferred to the host *R. rhodochrous* strain CW25. Strain CW25 cannot utilize RDX as a source of nitrogen but can grow on nitrite, which was shown above to be a breakdown product of RDX. Any clones

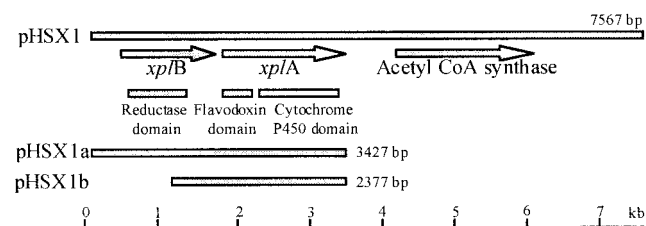


FIG. 5. Map of the 7,567-bp region found to be responsible for conferring the ability to degrade RDX. The open reading frames correspond to proteins with homology to a reductase (*xplB*), cytochrome P450 (*xplA*), and acetyl-CoA synthase. Areas identified as protein domains are indicated. The subcloned DNA fragments inserted into pDA71 are shown. Both conferred activity against RDX on strain CW25.

conferring upon strain CW25 the ability to degrade RDX should be able to grow on the nitrite as a source of nitrogen. The library in strain CW25 provided approximately threefold coverage of the genome of strain 11Y.

Library clones containing a gene conferring RDX-degrading ability were initially selected for by screening for growth in minimal liquid medium with RDX supplied as the sole nitrogen source. A zone-of-clearance method was developed to screen the clones further. RDX forms a dispersion in agarose when added at a concentration above its solubility limit, which gives agarose plates an opaque appearance. Bacteria which can degrade RDX, when incubated on these plates, form a zone of clearance around the colonies. This second level of screening allowed the identification of four clones of strain CW25 which had gained the ability to degrade RDX. All the RDX-degrading clones were found to contain identical plasmids, as determined by restriction analysis (data not shown). This plasmid, designated pHSX1, has an insert of 7.5 kb.

Cells of *R. rhodochrous* strain CW25 were retransformed with pHSX1 and analyzed by zone-of-clearance and TLC assays, which confirmed that the plasmid confers the ability to degrade RDX, in contrast to cells containing the vector pDA71 (data not shown). This evidence strongly suggested that pHSX1 encodes an activity which enables bacteria to degrade RDX.

The entire insert of pHSX1 was sequenced; three open reading frames in the same orientation were identified, and deduced amino acid sequences were subject to BLAST searches. The first open reading frame encodes a polypeptide of 425 amino acids with homology to a reductase from *Citrobacter braakii* (GenBank accession no. AAL57615; BLAST E value of  $1 \times 10^{-75}$  [2]), the second consists of 552 amino acids with homology to a cytochrome P450-like protein from *Bacillus subtilis* (accession no. P53554; BLAST E value of  $4 \times 10^{-35}$ ) with a flavodoxin domain at the N terminus (PFAM accession no. PF00258; <http://www.sanger.ac.uk/Software/Pfam/>), and the third consists of 626 amino acids with homology to an acetyl coenzyme A (acetyl-CoA) synthase from *Sinorhizobium meliloti* (accession no. BAA86907; BLAST E value of 0) (Fig. 5).

**Growth characteristics of *R. rhodochrous* strain CW25 (pHSX1).** The ability of strain CW25(pHSX1) to degrade RDX was compared to that of strain 11Y (Fig. 6). Growth curves showed that strain CW25(pHSX1) can grow on RDX as the sole nitrogen source and degrade it over time. This confirmed that pHSX1 confers the ability to degrade RDX on strain CW25. Strain CW25(pHSX1) has a longer lag period

and a lower final OD<sub>600</sub> than strain 11Y. Nitrite and ammonium assays were performed on the supernatant as before and showed that neither accumulated during growth of either bacterium, and no RDX degradation was detected in an untransformed strain CW25 control.

**Subcloning and sequence comparisons of the *xpl* genes.** Subcloning experiments were performed on pHSX1 to ascertain which of the above genes are responsible for the degradation of RDX (Fig. 5). A 3.5-kb section of pHSX1 containing the putative reductase and P450 genes and the 426-bp upstream region was amplified by PCR and inserted into pDA71 (pHSX1a). This section conferred RDX-degrading ability on strain CW25. A 2.3-kb region containing the P450-like gene with a 700-bp upstream region was constructed in pDA71 (pHSX1b) and was also found to confer activity on strain CW25, indicating that strain CW25 can provide any other components required for activity. The P450-like and reductase-like genes were designated *xplA* and *xplB*, respectively (for "explosive-degrading").

Amino acid sequence comparisons with other cytochromes P450 provide information on conserved, and functionally important, residues. The highest similarity score of the deduced amino acid sequence of XplA (26% identity and 42% similarities) was found with a P450-like protein, BioI (CYP107H1), involved in biotin biosynthesis from *B. subtilis* (6), similar scores were found between XplA and putative cytochromes P450 from the *Mycobacterium tuberculosis* H37Rv genome. Areas of high conservation are indicated in an alignment of the amino acid sequences in Fig. 7. Generally, the C terminus is much more highly conserved than the N terminus. In particular, the residues Glu-430, Arg-433, and Cys-503 (numbers refer to XplA) are absolutely conserved, with the cysteine and surrounding area thought to be involved in binding the heme moiety (36) (<http://drnelson.utmem.edu/PIR.P450.description.html>). Arg-249 and Arg-443 are also conserved compared to

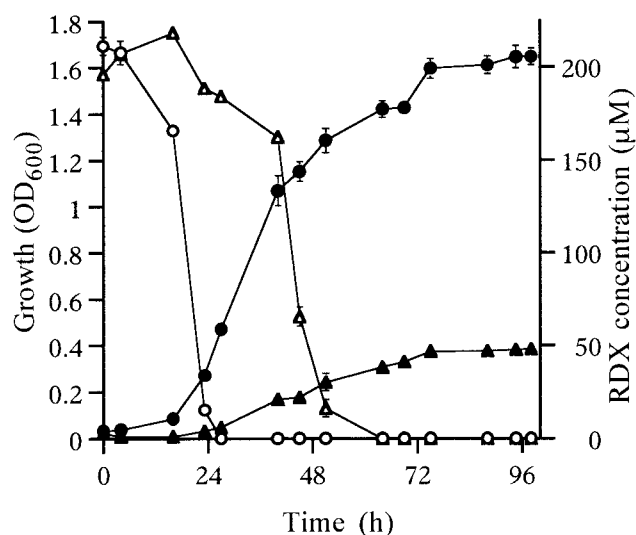


FIG. 6. Growth of *R. rhodochrous* strain 11Y and *R. rhodochrous* strain CW25(pHSX1). The OD<sub>600</sub> of strain 11Y (●) and strain CW25 (pHSX1) (▲) and the RDX concentration when supplied to strain 11Y (○), and strain CW25(pHSX1) (△) are shown. Error bars indicate one standard deviation for triplicate samples.

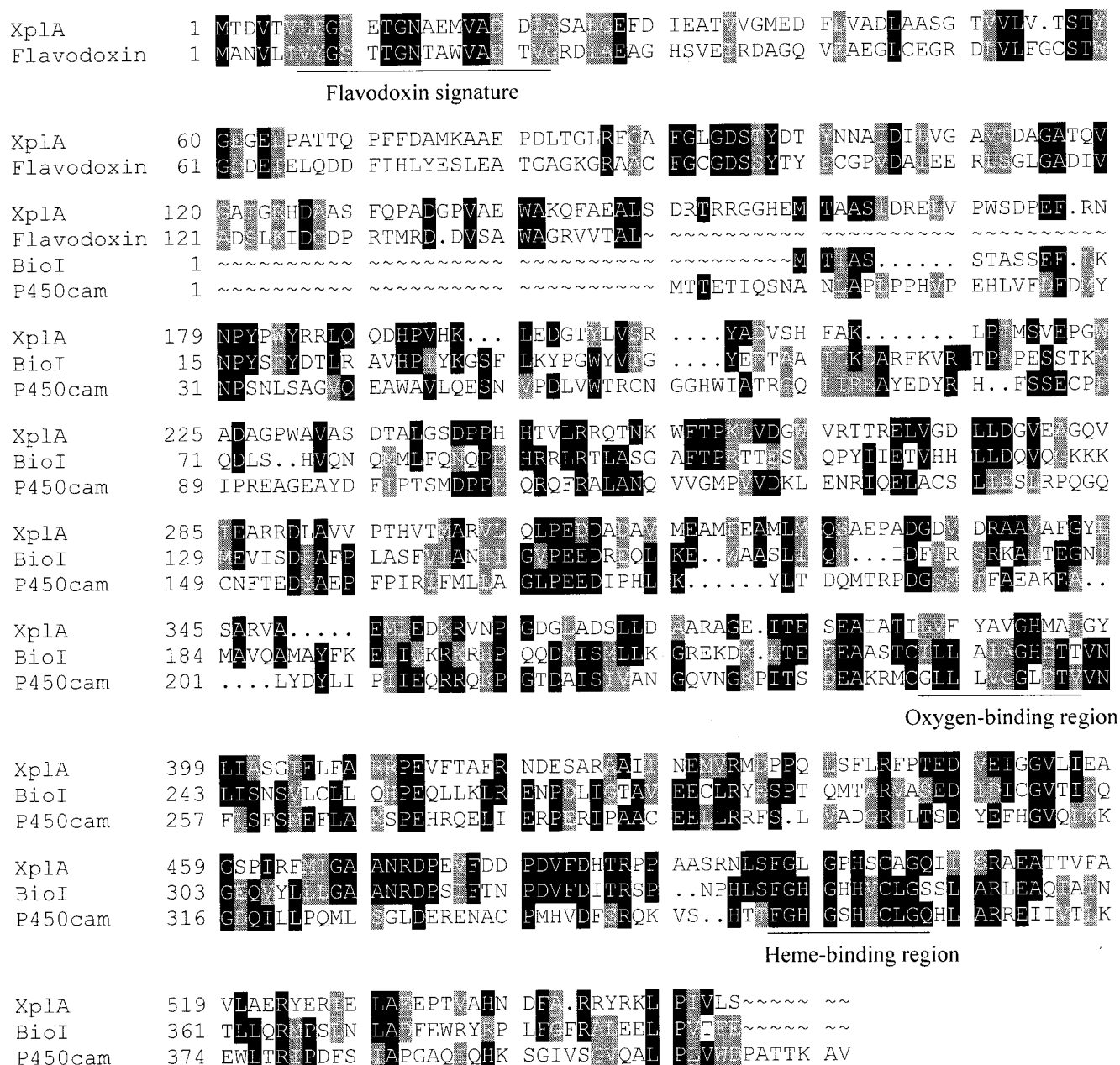


FIG. 7. Alignment of protein encoded by *xplA* with *Desulfovibrio vulgaris* flavodoxin (22) (Genbank accession no. P71165) at the N terminus, *B. subtilis* P450-like BioI (CYP107H1; accession no. P53554) involved in biotin biosynthesis (6), and P450<sub>cam</sub> (CYP101; accession no. P00183) from *P. putida* (41). Identical residues are shaded black, and similar residues are shaded grey. Conserved regions are underlined.

P450<sub>cam</sub> and are thought to be involved in hydrogen bonding to the heme (31). The oxygen binding region consensus present in many cytochromes P450 is not found in XplA, in particular the Thr-252 residue of P450<sub>cam</sub>, which was thought to play a crucial role in the monooxygenation reaction (20) but appears not to be highly conserved (<http://www.icgeb.trieste.it/~p450srv/>).

The flavodoxin domain comprising approximately the N-terminal 158 residues of XplA contains most of the elements of the flavodoxin signature [LIV]-[LIVFY]-[FY]-x-[ST]-x<sub>2</sub>-[AGC]-x-T-x<sub>3</sub>-A-x<sub>2</sub>-[LIV] (<http://ca.expasy.org/prosite/> accession no. PDOC00178) (residues 6 to 22), which appears to be

involved in the binding of the phosphate group of flavin mononucleotide (FMN) (8). The only exception is the Thr which should fall at position 15 (bold type in signature above) (43) but is replaced by Ala in XplA.

The deduced reductase amino acid sequence, XplB, has the highest similarity to bovine mitochondrial adrenodoxin reductase (27% identities and 42% similarities) (34) of the proteins of known function (GenBank accession no. JT0751). Several putative mycobacterial reductases also have high amino acid similarity. Most of the elements of the flavin adenine dinucleotide (FAD) and NADP binding domains are present, with the

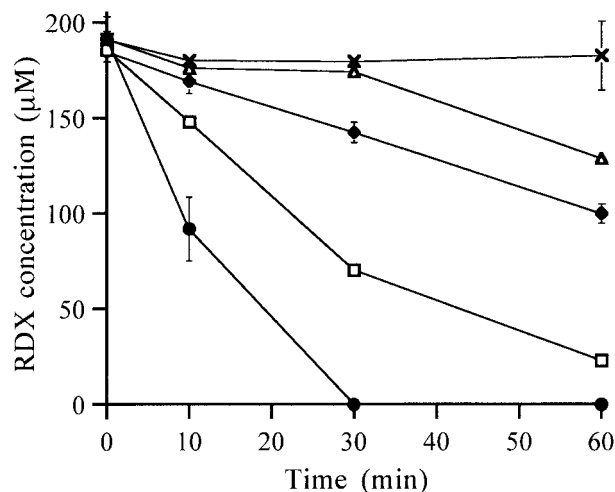


FIG. 8. Resting-cell incubations using metyrapone. RDX concentrations in incubations with strain 11Y, containing metyrapone at 0 mM (●), 0.1 mM (□), 0.5 mM (◆), 1 mM (△), and 10 mM (×), are shown. Error bars indicate one standard deviation for triplicate samples.

G-X-G-X-X-(G/A) motif allowing for a  $\beta\alpha\beta$  fold around the dinucleotides (37) as well as the presence of other residues characteristic of adrenodoxin reductases (46).

**RT-PCR confirms that transcription is constitutive.** To confirm that *xplA* and *xplB* are expressed in *R. rhodochrous* strain 11Y, RNA was harvested from cells grown overnight. RT-PCR, using primers designed to sequences within both genes, gave products of the appropriate sizes (data not shown). These fragments were cloned and sequenced, which confirmed that they were the correct products when compared to the original sequence. RT-PCR controls were performed in the absence of reverse transcriptase to confirm that the products were a result of amplification from RNA and not from any contaminating DNA. Since the RNA was extracted from cells which had been grown in Luria-Bertani broth it can be concluded that *xplA* and *xplB* are both transcribed constitutively and do not require the presence of RDX for induction.

RT-PCR was also performed to confirm that the two genes are not expressed as an operon, using forward primers designed to sequences within *xplB* and reverse primers designed to sequences within *xplA*. RT-PCR products were obtained using forward primers up to 649 nucleotides upstream of the initiation codon of *xplA*, but forward primers designed to be complementary to sequences further upstream (1,033 nucleotides from ATG of *xplA*) produced no RT-PCR products, indicating that the genes are transcribed independently.

**Metyrapone inhibits RDX degradation.** Metyrapone, a specific inhibitor of cytochromes P450, was added to resting-cell incubations of strain 11Y with RDX. The cells were grown to late log stage with RDX provided as the sole source of nitrogen, harvested, resuspended, and incubated with 250  $\mu$ M RDX and metyrapone at concentrations from 0.1 to 10 mM.

RDX removal was complete within 30 min in the absence of metyrapone, and increasing concentrations of the inhibitor resulted in slower disappearance (Fig. 8). At a metyrapone concentration of 10 mM, no RDX removal was observed, compared to a control incubated in the absence of strain 11Y.

Similarly, nitrite release was reduced in incubations containing increasing amounts of metyrapone (data not shown).

## DISCUSSION

*R. rhodochrous* strain 11Y has been found to degrade RDX as the sole source of nitrogen. Although rhodococcal species with this activity have previously been identified, no isolation of genes responsible for the activity has previously been described. Strain 11Y removes 250  $\mu$ M RDX from culture within 21 h, a rate comparable to that seen using *Rhodococcus* sp. strain DN22, which eliminated 160  $\mu$ M within 20 h (9). Bacterial utilization of RDX as a carbon source has never been documented and was not observed with strain 11Y; this activity would not be expected unless an RDX-degrading methylo-troph were isolated, since RDX is a one-carbon substrate (25).

In contrast to several studies of bacterial degradation of RDX, the activity seen in *R. rhodochrous* strain 11Y is not inducible but is upregulated in the presence of RDX. Inhibition of RDX degradation in the presence of ammonium has been observed by Yang et al. (45) and Coleman et al. (9); and Binks et al. (4) observed diauxic growth of a mixed culture containing *S. maltophilia* strain PB1 on two nitrogen sources. Strain 11Y appears to use 3 mol of nitrogen per mol of RDX; the same has been observed with other bacterial systems (4, 9).  $N_2O$  has been proposed as a significant product of RDX breakdown (39) and may account for some or all of the other nitrogens. One of the products of RDX degradation which appears to be used as a nitrogen source by bacteria is nitrite, which was identified in *Rhodococcus* sp. strain DN22 growth culture but was not seen in this study. This may be due to a more efficient nitrite reductase in strain 11Y, leading to a more rapid turnover.

Metabolites identified during cell growth or in resting-cell incubations can give clues to the mechanism of RDX degradation. Anaerobic biodegradation seems to occur through sequential reduction of the nitro groups to nitroso groups followed by possible, unstable, hydroxylamino intermediates and ring cleavage. This mechanism was postulated by McCormick et al. using anaerobic sludge (26), and some or all of the nitroso intermediates have been seen in many other anaerobic studies (18, 23). End products of this route appear to include formaldehyde or methanol and potentially toxic hydrazine derivatives, and it has been suggested that a type I nitroreductase is responsible for this activity (23). However, aerobic bacterial degradation would seem not to follow this route since no nitroso intermediates have been observed during the aerobic degradation of RDX.

The release of nitrite as the first metabolite in RDX degradation by strain 11Y indicates a mechanism similar to that of alkaline hydrolysis (19), and the production of the metabolites seen indicates that the RDX has been completely degraded. Denitration of the molecule as a first, important step in the breakdown of RDX has been suggested in a scheme put forward by Fournier et al. (16) working on *Rhodococcus* sp. strain DN22 (Fig. 1). This would destabilize the RDX to the extent that a spontaneous ring cleavage would occur (17). Nitrite, formaldehyde, and formate are seen in resting-cell incubations of *R. rhodochrous* strain 11Y with RDX, which would be explained by a denitration mechanism, although the products

formed from RDX degradation by strain 11Y do not appear to be the same as those seen using *Rhodococcus* sp. strain DN22. This may be due to different competing reactions within the bacteria after the initial denitration. Whether the formate is produced from formaldehyde is not clear, since the formaldehyde concentration does not decrease throughout the incubation time. Previous work on RDX degradation using anaerobic sludge requires the presence of acetogens to convert formaldehyde to formate (17), but both are produced in the alkaline hydrolysis and thermal decomposition of RDX (10, 19). The decrease in the rate of RDX disappearance after 20 min of incubation could be due to the metabolites affecting the cells deleteriously. In particular, a large amount of nitrite is produced at that time and may have toxic effects on the bacteria. Nitrous oxide was not assayed for, and no other intermediates or dead-end metabolites were observed using the HPLC parameters described.

Cytochromes P450 are heme-containing proteins that are able to catalyze a wide range of reactions. Eukaryotic cytochromes P450, such as those used to detoxify substances in the liver, fall into a group called type II, whereas bacterial and mitochondrial cytochromes P450 are type I, usually requiring ferredoxin and ferredoxin reductase to complete the multicomponent enzyme system (14). P450<sub>cam</sub> is the best characterized of the bacterial cytochromes P450, being responsible for the hydroxylation of camphor in *Pseudomonas putida*. Other examples of the range of reactions which can be catalyzed by cytochromes P450 include biotransformations of xenobiotics in rhodococci and related species, such as the dechlorination of pentachlorophenol and the degradation of ethoxyphenol and methoxybenzoate by P450<sub>RR1</sub> and P450<sub>RR2</sub> and of the herbicide EPTC by *thcB* (21, 27, 42). Commonly, rhodococcal cytochromes P450 involved in xenobiotic degradation are found in operons with genes coding for ferredoxin reductases and ferredoxins. In some cases, regulatory genes and other open reading frames, of unknown function to date, are found in the operon (6, 11, 27). The involvement of a P450 in the biodegradation of RDX by *Phanerochaete chrysosporium* has been speculated upon (39), and inhibition studies have also implied the involvement of a P450 in work on rhodococcal RDX degraders (N. Coleman and T. Duxbury, Abstr. 2nd Int. Symp. Biodegrad. Nitroaromatic Compounds Explosives, abstr. 15, 1999; Y. Teokoah, A. Nejidat and A. Abeliovich, Abstr. 2nd Int. Symp. Biodegrad. Nitroaromatic Compounds Explosives, abstr. 4, 1999).

A gene conferring RDX-degradative ability has been cloned from *R. rhodochrous* strain 11Y and has been identified as encoding a cytochrome P450-like enzyme. The *xplA* gene product has homology to a P450 with a flavodoxin domain at the N terminus. Bacterial cytochromes P450 are usually about 400 amino acids, but this N-terminal extension adds a further approximately 140 residues, which is in the size range of the short-chain bacterial flavodoxins. In some situations, flavodoxins are able to perform the same physiological roles as ferredoxins, being involved in electron transport, but using a flavin moiety as the redox center instead of an iron-sulfur center. Flavodoxins have no amino acid sequence homology to ferredoxins (40). In this case, the flavodoxin appears to be part of a compound protein with the putative cytochrome P450, possibly dispensing with the necessity for a separate ferredoxin. Met-158 could be postulated as being a start codon for the original

P450 since it falls between the two domains. Fused genes containing components of the P450 system are not uncommon. The best-known example is that of P450<sub>BM-3</sub> from *Bacillus megaterium*, in which the reductase is fused to the P450 in one gene. Crespi et al. (11) also reported the presence of a ferredoxin-like domain at the N terminus of a protein otherwise dissimilar to any protein involved in P450 reactions. A two-component bacterial cytochrome P450 system was reported by Serizawa and Matsuoka (38), with the reductase component containing both FAD and FMN groups and no ferredoxin being required for activity. A gene, *xplB*, with homology to adrenodoxin reductase, is found 62 bp upstream of *xplA*. Adrenodoxin reductases are components of a mitochondrial P450 pathway involved in steroid biosynthesis. They generally transfer electrons between NADP and adrenodoxin, a 2Fe2S ferredoxin. Although several mycobacterial proteins with homology have been identified, none have a proven function. The function of the flavodoxin domain in XplA as a redox agent and the possible use of XplB as a reductase in the mechanism have not yet been demonstrated. The functional role of a cytochrome P450 in the degradation of RDX by strain 11Y was strongly inferred by the use of the inhibitor metyrapone, which greatly reduced the rate at which RDX was removed.

Cytochromes P450 involved in xenobiotic metabolism are commonly induced by their substrate, including P450<sub>cam</sub> from *P. putida*, involved in the hydroxylation of camphor (24); P450<sub>SU1</sub> and P450<sub>SU2</sub>, involved in herbicide metabolism in *Streptomyces griseolus* (31); and a rhodococcal P450 involved in the degradation of the herbicide *S*-ethylpropylcarbanthioate (EPTC) (27). This appears not to be the case with *xplA*, and there are reports of other constitutive bacterial cytochromes P450 such as P450<sub>CON</sub> in *Streptomyces griseolus* (30).

The identification of the gene responsible for RDX degradation provides a new area for investigation in explosive bioremediation. Effort is now being directed to investigating the heterologous expression of the bacterial protein.

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