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Degradation of Pentaerythritol Tetranitrate by *Enterobacter cloacae* PB2

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A mixed microbial culture capable of metabolizing the explosive pentaerythritol tetranitrate (PETN) was obtained from soil enrichments under aerobic and nitrogen-limiting conditions. A strain of *Enterobacter cloacae*, designated PB2, was isolated from this culture and was found to use PETN as a sole source of nitrogen for growth. Growth yields suggested that 2 to 3 mol of nitrogen was utilized per mol of PETN. The metabolites pentaerythritol dinitrate, 3-hydroxy-2,2-*bis*-[(nitrooxy)methyl]propanal, and 2,2-*bis*-[(nitrooxy)methyl]-propanedial were identified by mass spectrometry and ^{1}H-nuclear magnetic resonance. An NADPH-dependent PETN reductase was isolated from cell extracts and shown to liberate nitrite from PETN, producing pentaerythritol tri- and dinitrates which were identified by mass spectrometry. PETN reductase was purified to apparent homogeneity by ion-exchange and affinity chromatography. The purified enzyme was found to be a monomeric flavoprotein with a M_r of approximately 40,000, binding flavin mononucleotide noncovalently.

Pentaerythritol tetranitrate (PETN) (2,2-*bis*[(nitrooxy)methyl]-1,3-propanediol dinitrate [ester]), a nitrate ester produced by nitration of pentaerythritol, is a powerful explosive used in blasting caps and detonators (16, 22) and is also used as a coronary vasodilator for the control of angina. Production and use of PETN and other nitrate esters such as glycerol trinitrate (GTN; nitroglycerin), ethylene glycol dinitrate (EGDN), and cellulose nitrate (nitrocellulose) can lead to contamination of the environment (6, 20). Nitrate esters appear to be extremely rare in nature, although it has been reported that nitrate esters of long-chain unsaturated alcohols are produced by certain insects as sex pheromones (5), and low concentrations of alkyl nitrates are produced in the atmosphere through the reaction of nitrogen oxides with hydrocarbons (15). We are not aware of any reports of naturally occurring multiply nitrated polyols. The environmental fate of such compounds, produced in large quantities by the chemical industry, is therefore a matter of interest.

Studies of the metabolism of PETN in mammals, prompted by its pharmaceutical use, have shown that PETN is sequentially denitrated to produce the tri-, di-, and mononitrates in untreated or unpreserved urine and fecal matter, suggesting that microorganisms are responsible (8). To date, no further studies appear to have been made on the microbial degradation of PETN. Pentaerythritol itself is generally considered to be recalcitrant to microbial attack, although there has been a report of its degradation by a bacterium (12).

In this paper, we describe the isolation from explosive-contaminated soil of a strain of *Enterobacter cloacae*, designated PB2, which is capable of aerobic growth with PETN as a sole nitrogen source.

MATERIALS AND METHODS

Chemicals. PETN, GTN, and EGDN were kindly provided by the Defence Research Agency (Fort Halstead, United Kingdom). Other chemicals were of analytical grade and were obtained from BDH Ltd. (Poole, United Kingdom), Sigma Chemical Company Ltd. (Poole, United Kingdom), or Aldrich (Gillingham, United Kingdom).

Liquid chromatography. High-performance liquid chromatography (HPLC) measurements were performed with a Hewlett-Packard 1050 series component system consisting of a multiple wavelength detector, quaternary pump, and 21-vial autosampler. Integrations were performed by using Hewlett-Packard Chemstation software. Samples (15 μl) were separated with a 10-μm C_{18} reversed-phase column (length, 25 cm; diameter, 4.6 mm; HPLC Technology Ltd., Macclesfield, United Kingdom). A reverse-phase isocratic mobile phase consisting of methanol-water (65:35, vol/vol) was delivered at a flow rate of 1.5 ml/min. PETN elution was monitored at 205 nm.

^{1}H-nuclear magnetic resonance (NMR) and mass spectrometry. ^{1}H-NMR spectra were recorded on a Bruker 400-MHz spectrometer with deuterated acetone as solvent. Electron impact (EI) mass spectrometry was performed on a Kratos model MF890 mass spectrometer. ^{1}H-NMR and mass spectrometry were performed by the Department of Chemistry, University of Cambridge.

Enrichment cultures and growth of bacteria. Selective enrichment cultures were used to obtain bacterial populations that were capable of growth on PETN as a sole nitrogen source. Soil and water samples were collected from a site that had been heavily contaminated with munition compounds. Eight samples were taken at random locations and stored at 4°C.

The enrichment medium consisted of 10 mM potassium phosphate buffer (pH 7.3), containing 0.25 mM $MgSO_4$, 5 mM glucose, 5 mM succinate, 10 mM glycerol, 1 mM nitrogen (as NH_4NO_3, NH_4Cl, or PETN), and trace elements (14). The nitrogen source was either PETN or NH_4NO_3. Inocula were prepared from soil as described by Cook and Hutter (2). A 1-ml aliquot of soil washings or water sample was inoculated into 24 ml of sterile medium with PETN as sole nitrogen source in 100-ml conical flasks and incubated at 30°C on a rotary shaker at 170 rpm.

The first enrichment cultures were incubated for 1 week. Those cultures which gave more growth (scored visually) than the control without a nitrogen source were subcultured into the same medium. After three PETN-limited subcultures, positive enrichments were streaked onto enrichment medium solidified with 1.5% (wt/vol) powdered agar (Difco Laboratories, Detroit, Mich.). Representatives of each colony type were picked from the agar plates and tested for the ability to utilize PETN for growth in liquid medium. The same medium was used for growth studies as for enrichments.

For the preparation of PETN reductase, *E. cloacae* PB2 was grown in SOB medium (17) at 30°C with rotary shaking at 170 rpm.

Determination of microbial growth and substrate utilization. Bacterial growth was measured routinely by monitoring the A_{600}. Growth was also measured as protein content of the cultures. Bacteria were precipitated with 0.5 M trichloroacetic acid (final concentration) and collected by centrifugation (13,000 × *g* for 20 min), after which the supernatant was discarded. The bacteria were suspended in 0.66 M NaOH at 30°C for 24 h and assayed for protein by the method of Bradford (1).

The degradation of PETN by the mixed culture and *E. cloacae* PB2 was assayed by monitoring the decrease in concentration by HPLC. Samples (1 ml)

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were removed from the cultures at regular intervals and diluted with 1 volume of acetone to solubilize PETN and metabolites. Cells and precipitated protein were removed by centrifugation, and the supernatant was analyzed by HPLC.

Isolation of metabolites. After cultures had entered stationary phase (140 h), metabolites were extracted into ethyl acetate which was then removed by rotary evaporation. Extracted metabolites were resuspended into acetone, and their presence was confirmed by HPLC. The metabolites were separated on SIL G/UV_{254} (Aldrich) thin-layer chromatography plates with the solvent system toluene–ethyl acetate–*n*-butanol–water (10/5/2/2, by volume) and visualized with a modification of Griess reagent (16). The plates were eluted, and the solvent was evaporated. The plates were then sprayed with 1 M sodium hydroxide solution, heated to 150°C for 5 min, and sprayed with a solution of 8% (wt/vol) sulfanilamide and 0.4% (wt/vol) *N*-1-naphthylethylenediamine hydrochloride in 8% (vol/vol) orthophosphoric acid. Nitrite-containing compounds developed a red coloration at room temperature.

Detection of PETN reductase activity. Late-exponential-phase cultures of the PETN-degrading single isolate, grown on the same medium used for enrichments, were harvested by centrifugation (9,000 × *g* for 20 min), washed, and suspended in 50 mM bis-Tris propane {1,3-bis[tris(hydroxymethyl)-methylamino]propane} buffer (pH 7). Cells were disrupted by sonication in an MSE Soniprep (Fisons Instruments, FSA Ltd.) with three 15-s bursts alternated with 30 s of cooling in melting ice. Cell debris and unbroken cells were removed by centrifugation at 20,000 × *g* for 20 min at 4°C (Sorvall RC5C).

PETN-degrading activity was determined by monitoring the disappearance of PETN by HPLC in 50 mM bis-Tris propane buffer (pH 7), containing PETN (47 μM, final concentration), 40 μl of crude extract, and NADPH (0.2 mM, final concentration) in a final volume of 1 ml.

Nitrite production was monitored with Griess reagent (16). The assay was carried out as described above and terminated by addition of ferricyanide (0.5 mM, final concentration) and phenazine methosulfate (0.2 mM, final concentration), which removed any remaining NADPH which would interfere with the assay (18). Sulfanilamide (15 mM, final concentration) and hydrochloric acid (0.17 M, final concentration) were added and left to stand for 15 min. *N*-1-Naphthylethylenediamine (0.9 mM, final concentration) was then added, and after 5 min, the color which developed was measured spectrophotometrically at 540 nm.

NADPH oxidation in the presence of crude extract was measured by monitoring the decrease in A_{340}.

Enzyme assays. PETN reductase activity was assayed by monitoring the oxidation of NADPH at 340 nm in a 1-ml reaction mixture consisting of 50 mM potassium phosphate buffer (pH 7.0), 0.2 mM NADPH, and 25 μM PETN, added as 1 μl of 25 mM PETN in dimethylformamide. One unit of enzyme activity was defined as that amount of activity oxidizing 1 μmol of NADPH per min.

Protein in extracts used in enzyme assays was measured by the method of Bradford (1).

Kinetic measurements. The pH optimum was determined by the standard assay except that the buffer was replaced by 50 mM bis-Tris propane (pH 6.5, 7, 7.5, and 8), 50 mM 2-[*N*-morpholino]ethanesulfonic acid (MES) (pH 5.5, 6, and 6.5), and 50 mM bis-Tris (pH 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, and 6.8).

Purification of PETN reductase. Cells from a 500-ml saturated culture of *E. cloacae* PB2 grown in a rich medium were harvested by centrifugation (6,500 × *g*, 10 min) and resuspended in 25 ml of buffer A (10 mM potassium phosphate, pH 7.0). The cell suspension was disrupted by sonication with an MSE Soniprep sonicator. The cells were held in an ice-water bath and sonicated for 10 bursts of 10 s at an amplitude of 12 μm, with 40-s pauses for cooling. Cell debris was removed by centrifugation (31,000 × *g*, 20 min).

Subsequent steps were performed at 4°C. The crude extract was loaded onto a column (1.6 cm, inner diameter) packed with 12 ml of Q-Sepharose Fastflow (Pharmacia, Uppsala, Sweden) (packed height, 6.0 cm) which had been equilibrated with buffer A. A flow rate of approximately 50 ml/cm^2/h was used. The column was washed with 80 ml of buffer A to remove unbound protein. PETN reductase activity was then eluted with 50 mM NaCl in buffer A. Active fractions (40 ml) were pooled. This product was diluted with 2 volumes of buffer A and 2 volumes of water (final composition, 6 mM phosphate, 10 mM NaCl) to reduce the ionic strength. The diluted product was loaded onto a column (5.0 cm, inner diameter) packed with 50 ml of Mimetic Orange 2 (Affinity Chromatography Ltd., Freeport, Ballasalla, Isle of Man, United Kingdom) (packed height, 2.6 cm) which had been equilibrated with buffer A. A flow rate of approximately 15 ml/cm^2/h was used. The column was washed with 150 ml of buffer A. The direction of flow was then reversed, and the PETN reductase activity was eluted with 50 mM NaCl in buffer A. Active fractions (60 ml) were pooled and diluted as described above. The diluted product was concentrated by ultrafiltration with an Amicon 8050 ultrafiltration cell (Amicon, Stonehouse, Gloucestershire, United Kingdom) with a membrane with a nominal M_r cutoff of 10,000 (Diaflo YM10; Amicon). Dilution prior to ultrafiltration was necessary as in the presence of high salt concentrations a significant amount of activity was lost to the permeate. The concentrated product (approximately 5 ml) was stored at −20°C. No significant loss of activity occurred over several weeks.

Polyacrylamide gel electrophoresis (PAGE). Sodium dodecyl sulfate (SDS)-PAGE was performed according to the method of Laemmli (9) with the Bio-Rad Mini Protean II electrophoresis system (Bio-Rad, Hemel Hempstead, United Kingdom). Vertical slab gels of 0.7 mm in thickness, containing 12% (wt/vol) acrylamide in the resolving gel, were run at 200 V for 45 min. Sigmamarkers (Sigma) were used as M_r standards. Protein was detected by staining the gel for 1 h with Coomassie Brilliant Blue R-250 dissolved in methanol-water-acetic acid (4:5:1, by volume). Gels were destained by repeated washing in the above solvent mixture.

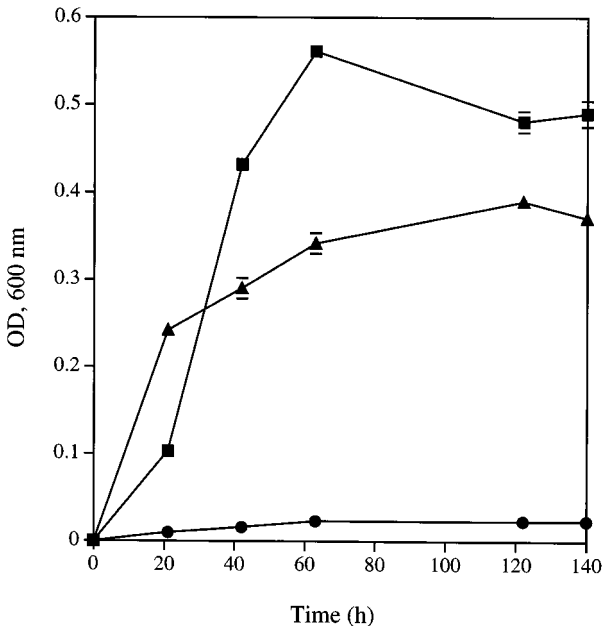

FIG. 1. Growth of a mixed bacterial culture with no nitrogen source (solid circles) or ammonium nitrate (solid squares) or PETN (solid triangles) as the sole source of nitrogen. A 1-ml aliquot of a late-exponential-phase mixed culture was inoculated into 24 ml of medium containing a mixed carbon source and a single, growth-limiting supply of nitrogen. Samples were taken at intervals and analyzed for growth by the increase in optical density at 600 nm. Error bars indicate one standard error.

Native M_r determination. The fast protein liquid chromatography system (Pharmacia) was used in combination with a Superose 6HR 10/30 column (1 by 30 cm) to determine the molecular weight of the native PETN reductase. Purified PETN reductase (10 μg) was applied to the column, which had previously been equilibrated with 50 mM bis-Tris propane (pH 7) at room temperature. The column was eluted with 50 mM bis-Tris propane (pH 7) at a flow rate of 0.4 ml/min, and 0.4-ml fractions were collected. The column was calibrated with the following enzymes as molecular weight markers: bovine serum albumin, ovalbumin, chymotrypsin, and RNase A (M_r, 67,000, 43,000, 25,000, and 17,500, respectively).

Flavin identification. The flavin prosthetic group was dissociated from PETN reductase by boiling 80 μg of PETN reductase in 50 μl of buffer A for 3 min. Denatured protein was removed by centrifugation (13,000 × *g*, 5 min). Thin-layer chromatography of flavins was performed on silica plates with solvent systems A (2% [wt/vol] Na_2HPO_4 in water) and B (*n*-butanol–water–acetic acid–methanol, 14:14:1:6, by volume) (4). Flavins were visualized by their fluorescence when illuminated at 366 nm.

RESULTS

Enrichments and isolation of organisms. Enrichment cultures from explosive-contaminated soil led to the isolation of a mixed bacterial culture which was able to grow on PETN as a sole and limiting source of nitrogen to the same extent as when grown on NH_4NO_3, at the same concentration of combined nitrogen (Fig. 1). Control cultures containing no added nitrogen source did not support growth, indicating that growth of the mixed culture was not due to nitrogen fixation. Plating of bacteria onto PETN-containing solid medium resulted in the isolation of 10 or more different colony types. However, only one colony type, designated PB2, grew when inoculated into liquid media containing PETN as sole nitrogen source.

Organism PB2 was found to be a gram-negative rod and to

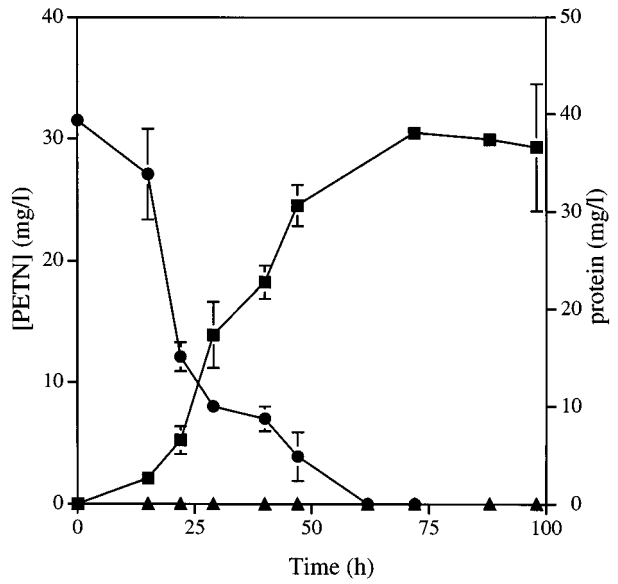

FIG. 2. Disappearance of PETN during growth of *E. cloacae* PB2. A 1-ml aliquot of late-exponential-phase culture of *E. cloacae* PB2 was inoculated into 24 ml of medium containing a mixed carbon source with or without PETN as the sole nitrogen source. Samples were taken at intervals and analyzed. ●, PETN; ■, protein in the culture with PETN; ▲, protein in the culture lacking PETN. Error bars indicate one standard error.

be motile, oxidase negative, and capable of fermenting glucose with the production of acid and gas. The methyl red test was negative, the Voges-Proskauer test was positive, indole was not produced from tryptophan, and citrate was utilized as a sole carbon source. Lysine decarboxylase was negative, ornithine decarboxylase and arginine dihyrolase were positive, and acid was produced from glycerol. On the basis of these and other biochemical tests, organism PB2 was identified by the National Collection of Industrial and Marine Bacteria (Aberdeen, United Kingdom) as a strain of *E. cloacae*, a common soil organism. *E. cloacae* PB2 has been deposited with the National Collection of Industrial and Marine Bacteria with the deposit number NCIMB 40718.

During growth of *E. cloacae* PB2 with PETN, the concentration of PETN in the medium decreased (Fig. 2). Growth on medium containing both NH_4NO_3 and PETN as nitrogen sources resulted in a growth curve characteristic of cometabolism (not shown). *E. cloacae* PB2 was unable to use pentaerythritol as a source of carbon.

Kinetics of PETN degradation. The growth yield of *E. cloacae* PB2 grown on NH_4Cl (68.5 ± 11.9 g of protein mol of N^{-1}) was similar to those reported in a review of bacterial cell composition (10). This suggests that the medium was adequate to support normal growth, which allowed the growth rate and thus the specific degradation rate of the organism to be examined meaningfully.

In cultures containing different initial concentrations of PETN, it was observed that PETN degradation was associated with growth (data not shown). The specific growth rate of *E. cloacae* PB2 in PETN-containing liquid culture was 0.112 h^{-1}. The growth yield of *E. cloacae* PB2 when grown on PETN as the sole nitrogen source (43.6 ± 2.4 g of protein mol of N as $PETN^{-1}$) was 0.64 times the yield of the same strain when grown with NH_4Cl (68.5 ± 11.9 g of protein mol of N^{-1}). PETN was stable in sterile control experiments, and cultures free of combined nitrogen showed no significant growth. This result implied that *E. cloacae* PB2 was using 2.5 nitrogen atoms from the PETN molecule.

The fact that PETN utilization was concomitant with growth allowed the specific degradation rate to be calculated from the specific growth rate ($hour^{-1}$) and growth yield (gram of protein mole of N^{-1}). This gave a value of 2.57 mmol of N g of $protein^{-1}$ h^{-1}. The specific degradation rate was also calculated from the differential plot of mmoles of PETN per second divided by protein concentration. This second method of calculating specific degradation rate gave a value of 1.03 mmol of PETN g of $protein^{-1}$ h^{-1}. This result implied that *E. cloacae* PB2 was using 2.5 nitrogen atoms from the PETN molecule. Comparison of the results of these two different ways of calculating specific degradation rate suggests that two to three of the four possible nitrogen atoms in PETN are being used for growth by *E. cloacae* PB2. The errors in specific degradation rate calculations result from the poor solubility of PETN, which makes concentration determination difficult.

Identification of whole-cell degradation products. Ethyl acetate extracts of supernatants from cultures grown with PETN contained PETN and three metabolites (as judged by HPLC). The metabolites were separated from PETN by thin-layer chromatography and found to have lower R_f values (0.64, 0.53, and 0.23) than PETN (R_f, 0.81), indicating that the unknown metabolites were more polar than PETN.

The identity of the unknown metabolites was investigated by EI mass spectrometry. The EI mass spectrum of metabolite 1 revealed a molecular ion with an *m/z* of 227 which is consistent with the empirical formula $C_5H_{10}N_2O_8$. The mass spectrum contained a fragment ion at *m/z* 209. We propose that this metabolite is pentaerythritol dinitrate (2,2-*bis*[(nitrooxy)methyl]-1,3-propanediol), a denitration product of PETN. The EI mass spectrum of metabolite 2 revealed a molecular ion with an *m/z* of 224 which corresponds to the empirical formula $C_5H_8N_2O_8$. The mass spectrum contained fragment ions at *m/z* 207, 170, 141, 99, 94, 76, 74, 158, 46, and 44. This is believed to represent 3-hydroxy-2,2-*bis*[(nitrooxy)methyl]propanal. The (EI) mass spectrum of metabolite 3 revealed a molecular ion with an *m/z* of 222 which corresponds to the empirical formula $C_5H_6N_2O_8$. The mass spectrum contained fragment ions at *m/z* 208, 177, and 91. This is believed to represent 2,2-*bis*[(nitrooxy)methyl]propanedial. The proposed structures of these metabolites are shown in Fig. 3.

The identity of metabolite 1 was further investigated with ^{1}H-NMR at 400 MHz. The ^{1}H-NMR spectrum of pure PETN gave a single peak at 4.77 ppm which corresponds to the four equivalent methylene groups in PETN, while the ^{1}H-NMR

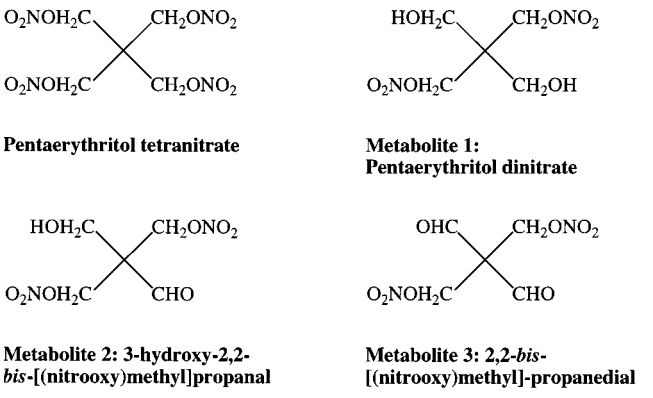

FIG. 3. Proposed structures of metabolites formed during growth of *E. cloacae* PB2 with PETN as nitrogen source.

TABLE 1. Purification of PETN reductase[a]

Step	Vol (ml)	Protein (mg)	Activity (U)	Yield (%)	Sp act (U/mg)	Purification factor
Crude extract	22	200 ± 10	63 ± 3	100	0.32 ± 0.03	1
Ion exchange	40	14.5 ± 0.7	52 ± 1	83	3.6 ± 0.2	11
Mimetic Orange 2 and ultrafiltration	6.0	4.4 ± 0.2	41 ± 1	64	9.3 ± 0.8	29

[a] Errors shown are one standard error.

spectrum of the unknown metabolite gave two separate peaks at 4.77 and 2.08 ppm. It is believed that the peak at 2.08 ppm corresponds to the methylene groups attached to the hydroxyl group in the proposed structural formula.

The (EI) mass spectroscopy and ^{1}H-NMR analysis of the unknown metabolites formed during PETN degradation by the bacterium suggest that at least two nitrogen atoms were used per PETN molecule; these metabolites provide no evidence for the removal of a third nitrate group.

Isolation and purification of PETN reductase. An NADPH-dependent PETN reductase activity was detected in crude cell extracts from PETN-grown PB2 cells. Oxidation of NADPH in the presence of PETN was concurrent with stoichiometric production of nitrite. The disappearance of PETN, liberation of nitrite, and the oxidation of NADPH were prevented completely by boiling the cell extracts for 1 min or by the addition of 6 M urea. PETN was stable in the absence of crude extract, and crude extract contained no compounds that interfered with the determination of PETN. PETN reductase activity was found to be produced at similar levels with PETN or NH_4NO_3 as nitrogen source or in rich media.

PETN reductase was purified from cell extracts of *E. cloacae* PB2 by ion-exchange and affinity chromatography. Results of one purification are shown in Table 1. PETN reductase was purified approximately 30-fold, and the product appeared to be homogeneous by SDS-PAGE (Fig. 4).

In one batch of purified PETN reductase, the specific rate of nitrite release was 4.6 μmol · min^{-1} mg^{-1}. The specific rate of NADPH oxidation was 4.2 μmol · min^{-1} mg^{-1}. This suggests that 1 mol of NADPH is oxidized per mol of nitrite released.

Characterization of PETN reductase. The native M_r of PETN reductase was estimated by gel filtration to be approximately 40,000. The subunit M_r, as estimated by SDS-PAGE, was 40,000 (Fig. 4). These results suggest that PETN reductase is a monomer with an M_r of approximately 40,000.

Kinetic experiments revealed that purified PETN reductase had an apparent K_m for PETN above the maximum solubility of PETN (6.6 μM) and a pH optimum of 6.5.

Purified PETN reductase was visibly yellow and showed a visible absorption spectrum characteristic of an oxidized flavoprotein (Fig. 5). The flavin was liberated from PETN reductase by boiling followed by removal of denatured protein by centrifugation, indicating that the flavin is not covalently bound. In two thin-layer chromatography systems, the liberated flavin comigrated with authentic flavin mononucleotide (FMN) and not with flavin adenine dinucleotide. Flavin standards subjected to the protocol used to liberate the flavin from PETN reductase showed no change; in particular, flavin adenine dinucleotide was not hydrolyzed to FMN. These results indicate that PETN reductase is a flavoprotein, binding FMN noncovalently.

Identification of the reaction products of PETN reductase. Following treatment of PETN with purified PETN reductase and NADPH, an ethyl acetate extract of the reaction mixture contained PETN and two metabolites (as judged by HPLC). The metabolites were separated from PETN by thin-layer chromatography and found to have lower R_f values (0.78 and 0.64) than PETN (R_f, 0.81).

The identity of the unknown metabolites was investigated by (EI) mass spectrometry. The EI mass spectrum of metabolite A revealed a molecular ion with a m/z of 271 which corresponds to the empirical formula $C_5H_9N_3O_{10}$. The mass spectrum contained fragment ions at m/z 239 and 207. This is believed to represent pentaerythritol trinitrate. The EI mass spectrum and R_f value of metabolite B were identical to those of metabolite 1 isolated from culture supernatant. This metabolite was identified as pentaerythritol dinitrate. It therefore appears that PETN reductase reductively liberates nitrite from PETN to form pentaerythritol trinitrate and then pentaerythritol dinitrate (Fig. 6), which is not a substrate for this enzyme. The presence of aldehydes formed from these alcohols in culture supernatants suggests subsequent dehydrogenase activity by another enzyme.

The specific activity of PETN reductase in crude extract was approximately 0.32 U/mg, equivalent to 9.7 mmol of PETN g of soluble protein^{-1} h^{-1}, assuming complete conversion of PETN to pentaerythritol dinitrate. This is probably sufficient to account for the observed specific degradation rate of PETN by growing cells of 1.03 mmol of PETN g of protein^{-1} h^{-1}.

Specificity of PETN reductase. Initial investigation has shown that PETN reductase liberates nitrite from GTN and EGDN in addition to PETN. The apparent K_m for GTN in 50 mM phosphate buffer, pH 7.0, in the presence of 0.2 mM NADPH, was measured by using GTN concentrations between 5 and 200 μM and was found to be 22 ± 2 μM (one standard error). The initial product of GTN denitration has not yet been determined. The apparent K_m for EGDN was measured with EGDN concentrations between 0.5 and 2.5 mM and was found to be approximately 2 mM. In conjunction with the observation that pentaerythritol dinitrate does not appear to be a substrate for this enzyme, this suggests that two nitrate groups in addition to that being reduced may be required for good binding or activity. The value of K_m for PETN could not be determined because of the poor solubility of this substrate.

DISCUSSION

In this study, we sought to isolate soil bacteria capable of degrading PETN. Because of the poor solubility of PETN in

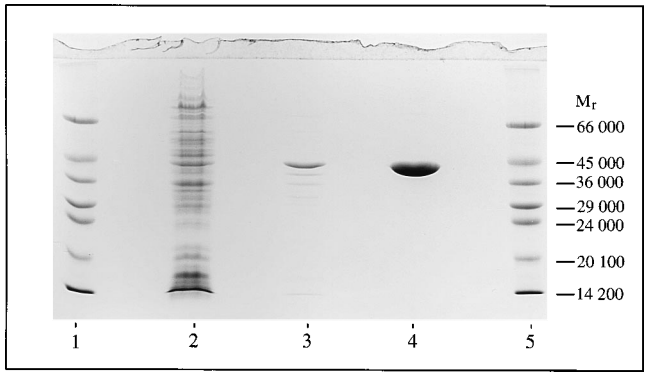

FIG. 4. SDS-PAGE gel showing purification of PETN reductase. Lanes 1 and 5, M_r markers; lane 2, crude extract, 36 μg of protein; lane 3, product from ion exchange, 3.6 μg of protein; lane 4, purified PETN reductase, 7.8 μg of protein.

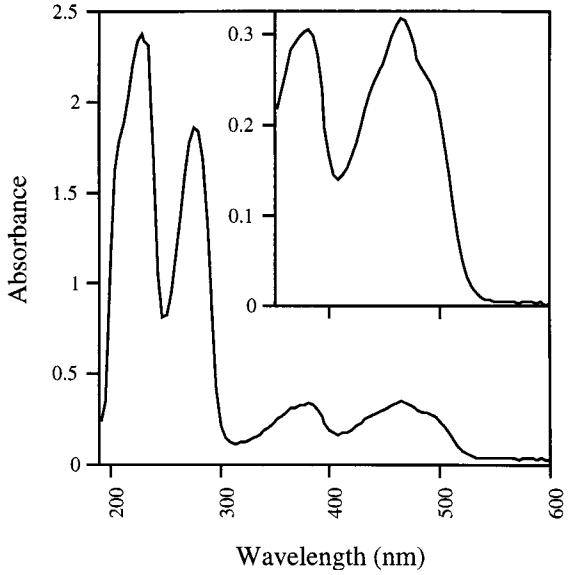

FIG. 5. Absorbance spectrum of purified PETN reductase. The absorbance spectrum of purified PETN reductase (1.55 mg/ml) was measured in 6 mM phosphate buffer, pH 7.0, containing 10 mM NaCl, against a blank consisting of the same buffer.

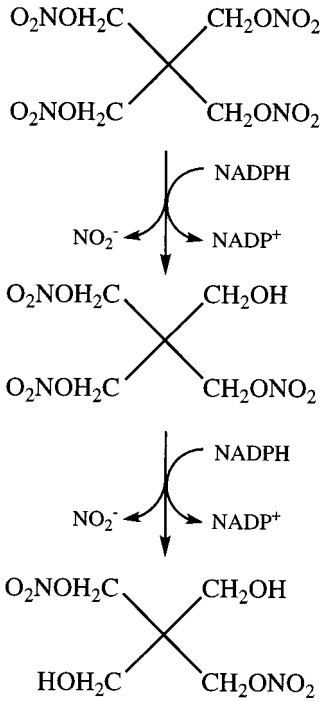

FIG. 6. Reactions catalyzed by PETN reductase.

aqueous solutions (approximately 6.6 μM [16]), the low carbon content of PETN, and the known recalcitrance of pentaerythritol, it was felt that PETN was less likely to be used as a carbon source than as a nitrogen source. Since nitrate esters are rare in nature, soil which had been contaminated with explosives over a period of years was used as an inoculum. Of eight soil samples tested, one showed bacterial growth with PETN as sole nitrogen source. From this culture was isolated *E. cloacae* PB2, which was found to be capable of aerobic growth in pure culture with PETN as sole nitrogen source.

E. cloacae PB2 apparently utilizes two atoms of nitrogen per molecule of PETN, producing pentaerythritol dinitrate, which is subsequently oxidized to the dialdehyde. An enzyme activity, designated PETN reductase, was found to reductively liberate nitrite from PETN with the production of pentaerythritol trinitrate and pentaerythritol dinitrate.

PETN reductase was purified by ion-exchange and affinity chromatography. The purified enzyme was found to be a monomeric flavoprotein with an M_r of approximately 40,000, binding FMN noncovalently.

Known microbial transformations of nitrate esters have been reviewed by White and Snape (23). Denitration with the production of alcohols is typical and has been reported for PETN (8) as well as GTN, EGDN, and other nitrate esters (7, 23). Denitration of nitrate ester vasodilators also occurs in mammalian tissues, in which it is typically reductive, catalyzed by glutathione *S*-transferase (21), heme-containing enzymes such as cytochrome P-450 (3), a dithiothreitol-dependent enzyme (13), or several other systems. Denitration of GTN by the white-rot fungus *Phanaerochaete chrysosporium* is also reductive (19). Thus, reductive rather than hydrolytic denitration of nitrate esters appears to be the rule (23), although a recent report described the denitration of GTN by strains of *Bacillus cereus* and *Enterobacter agglomerans* in reactions that apparently did not require dissociable or depletable cofactors (11).

To the best of our knowledge, this is the first report of the purification of a pyridine nucleotide cofactor-dependent nitrate ester reductase. Similar enzymes may be responsible for the denitration of nitrate esters in other systems. In this regard, it is interesting that the organism in question, *E. cloacae*, is not found only in soil but is also known to be an inhabitant of the mammalian gut, since intestinal microflora have been shown to denitrate PETN and GTN. Further study of this enzyme may also cast light upon the question of the origin of enzymes apparently specialized for the metabolism of xenobiotic compounds.

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REFERENCES

1. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
2. **Cook, A. M., and R. Hutter.** 1981. s-Triazines as nitrogen sources for bacteria. J. Agric. Food Chem. **29:**1135–1143.
3. **Delaforge, M., D. Servent, P. Wirsta, C. Ducrocq, D. Mansuy, and M. Lenfant.** 1993. Particular ability of cytochrome P-450 CYP3A to reduce glyceryl trinitrate in rat liver microsomes: subsequent formation of nitric oxide. Chem.-Biol. Interact. **86:**103–117.
4. **Fazekas, A. G., and K. Kokai.** 1971. Extraction, purification and separation of tissue flavins for spectrophotometric determination. Methods Enzymol. **18B:**385–398.
5. **Hall, D. R., P. S. Beevor, D. G. Campion, D. J. Chamberlain, A. Cork, R. D. White, A. Almestar, and T. J. Henneberry.** 1992. Nitrate esters: novel sex pheromone components of the cotton leafperforator, *Bucculatrix thurberiella* Busck. (Lepidoptera: Lyonetiidae). Tetrahedron Lett. **33:**4811–4814.
6. **Kaplan, D. L.** 1992. Biological degradation of explosives and chemical agents. Curr. Opin. Biotechnol. **3:**253–260.
7. **Kaplan, D. L.** 1989. Biotransformation pathways of hazardous energetic organo-nitro compounds, p. 155–181. *In* D. Kamely, A. Chakrabarty, and G. S. Omenn (ed.), Biotechnology and biodegradation. Gulf Publishing, Houston, Tex.
8. **King, S. Y. P., and H. L. Fung.** 1984. Rapid microbial degradation of organic nitrates in rat excreta. Drug Metab. Dispos. **12:**353–357.
9. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
10. **Luria, S. E.** 1960. The bacterial protoplasm: composition and organisation,

p. 1–31. *In* I. C. Gunsalus and R. Y. Stanier (ed.), The bacteria, vol. 1. Academic Press, New York.
11. **Meng, M., W.-Q. Sun, L. A. Geelhaar, G. Kumar, A. R. Patel, G. F. Payne, M. K. Speedie, and J. R. Stacy.** 1995. Denitration of glycerol trinitrate by resting cells and cell extracts of *Bacillus thuringiensis*/*cereus* and *Enterobacter agglomerans*. Appl. Environ. Microbiol. **61:**2548–2553.
12. **Obata, H., D. Akizuki, J. Tanishita, and T. Tokuyama.** 1983. Isolation and characterisation of pentaerythritol utilising bacterium. J. Ferment. Technol. **61:**121–126.
13. **Ogawa, N., T. Hirose, M. Tsukamoto, K. Fukushima, T. Suwa, and T. Satoh.** 1995. Purification and characterization of glutathione-independent denitration enzyme of organic nitrate esters in rabbit hepatic cytosol. Biol. Pharm. Bull. **18:**1352–1355.
14. **Pfennig, N., and K. D. Lippert.** 1966. Vitamin B_{12} requirement of phototrophic sulphur bacteria. Arch. Microbiol. **55:**245–256.
15. **Roberts, J. M.** 1990. The atmospheric chemistry of organic nitrates. Atmos. Environ. **24A:**243–287.
16. **Rosenblatt, D. H., E. P. Burrows, W. R. Mitchell, and D. L. Parmer.** 1991. Organic explosives and related compounds, p. 195–234. *In* O. Hutzinger (ed.), The handbook of environmental chemistry 3 (G). Springer-Verlag KG, Berlin.
17. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
18. **Scheideler, L., and H. Ninnemann.** 1986. Nitrate reductase activity test: phenazine methosulfate-ferricyanide stop reagent replaces postassay treatment. Anal. Biochem. **154:**29–33.
19. **Servent, D., C. Ducrocq, Y. Henry, A. Guissani, and M. Lenfant.** 1991. Nitroglycerin metabolism by *Phanaerochaete chrysosporium*: evidence for nitric oxide and nitrite formation. Biochim. Biophys. Acta **1074:**320–325.
20. **Steuckart, C., E. Berger-Preiss, and K. Levsen.** 1994. Determination of explosives and their biodegradation products in contaminated soil and water from former ammunition plants by multiple development high performance thin layer chromatography. Anal. Chem. **66:**2570–2577.
21. **Taylor, I. W., C. Ioannides, and D. V. Parke.** 1989. Organic nitrate reductase: reassessment of its subcellular localization and tissue distribution and its relationship to the glutathione transferases. Int. J. Biochem. **21:**67–71.
22. **Urbanski, T.** 1985. Chemistry and technology of explosives, vol. 2. Pergamon Press, New York.
23. **White, G. F., and J. R. Snape.** 1993. Microbial cleavage of nitrate esters: defusing the environment. J. Gen. Microbiol. **139:**1947–1957.