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ORIGINAL PAPER

Effect of organic and inorganic nitrogenous compounds on RDX degradation and cytochrome P-450 expression in *Rhodococcus* strain YH1

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Abstract We hypothesized that biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX)—a widely used explosive contaminating soil and groundwater—by *Rhodococcus* strain YH1 is controlled by the presence of external nitrogen sources. This strain is capable of degrading RDX while using it as sole nitrogen source under aerobic conditions. Both inorganic and organic nitrogen sources were found to have a profound impact on RDX-biodegradation activity. This effect was tested in growing and resting cells of strain YH1. Nitrate and nitrite delayed the onset of RDX degradation by strain YH1, while ammonium inhibited it almost completely. In addition, 2,4,6-trinitrotoluene (TNT) inhibited RDX degradation and growth of strain YH1. On the other hand, tetrahydrophthalamide did not influence biodegradation or growth. Growth on RDX induced the expression of a cytochrome P-450 enzyme that is suggested to be involved in the first step in the aerobic pathway of RDX degradation, as identified by SDS-PAGE analysis. Ammonium and nitrite strongly repressed cytochrome P-450 expression. Our findings suggest that effective RDX bioremediation by strain YH1 requires the design of a treatment scheme that includes initial removal of ammonium, nitrite, nitrate and TNT before RDX degradation can take place.

Keywords Cytochrome P-450 expression · RDX degradation · *Rhodococcus* · TNT degradation · TNT toxicity

Abbreviations

HMX	octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine
RDX	hexahydro-1,3,5-trinitro-1,3,5-triazine
THPI	1,2,3,6-tetrahydrophthalimide
TNT	2,4,6-trinitrotoluene

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Introduction

The biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), 2,4,6-trinitrotoluene (TNT) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) has received a great deal of attention because they are three of the most widely used nitro-organic explosives which have been detected in soil and groundwater (Fig. 1). The extensive manufacture, use and disposal of RDX have resulted in severe

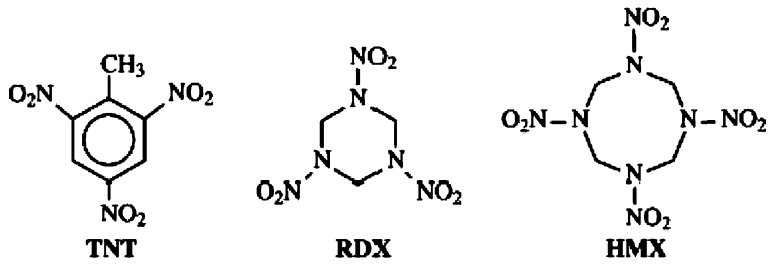

Fig. 1 Molecular structures of TNT, RDX and HMX

environmental contamination in many locations (Haas et al. 1990). RDX is known to affect the central nervous system in mammals (Kaplan et al. 1965) and a standard of 10 μg l^{-1} for ingestion from drinking water has been proposed (Etnier 1989). The U.S. Environmental Protection Agency (EPA) has classified RDX as a class C possible human carcinogen (Faust 1994).

The high nitrogen content of RDX, in both its heterocyclic ring and nitro groups (Fig. 1), suggests that it is a potentially good nitrogen source for microorganisms, as well as a good electron acceptor for them under anaerobic conditions (Ronen et al. 1998; Brenner et al. 2000). Indeed, anaerobic biodegradation of RDX and a possible degradation pathway have been demonstrated (McCormick et al. 1981; Hawari et al. 2000; Halasz et al. 2002). Biodegradation of RDX also occurs under aerobic conditions, and several bacterial strains have been isolated that degrade RDX and use it as a nitrogen source in pure cultures (Binks et al. 1995; Coleman et al. 1998). These studies showed that the initial degradation step is the formation of nitrite, through enzymatic activity, which is consumed by the bacteria. The participation of cytochrome P-450 in the aerobic degradation of RDX by *Rhodococcus* sp. was first suggested by Tekoah et al. (1999). In this study about 30% mineralization of RDX carbon by *Rhodococcus* YH1 was demonstrated using ^{14}C labeled compound. Further analysis of degradation products indicated that they are identical to those described by Fournier et al. (2002). Bhushan et al. (2003) were able to identify a transformation product from the degradation of RDX by another *Rhodococcus* strain (DN22) and showed that it is similar to the transformation product of RDX catalyzed by cytochrome P-450 2B4 from rabbit livers. Seth-Smith et al. (2002) reported the isolation of a *Rhodococcus rhodochrous* strain that degrades RDX when provided as the sole nitrogen source and suggested the involvement of a constitutively expressed cytochrome P-450 in this degradation. They isolated a gene cluster that contains three open reading frames coding for a putative reductase, a cytochrome P-450 protein with a flavodoxin domain at the N terminus and an acetyl coenzyme A synthase. However, the cytochrome P-450-like gene product was not detected and the regulation of its expression was not studied. Moreover, understanding the fate RDX in the environment requires a thorough knowledge of the parameters influencing its biodegradation in soil and groundwater. Nutrients like carbon and nitrogen can influence the biodegradation of pollutants in the environment and in this case, it is important to investigate the effect of alternative nitrogen sources on the degradation of RDX.

In this study, we investigated the effect of different organic and inorganic nitrogen sources on RDX biodegradation by strain YH1, identified a cytochrome P-450 gene product, and determined the effect of different nitrogen sources on the accumulation of this protein. Our results clearly demonstrate the inhibitory effect of the different nitrogenous compounds on RDX biodegradation by *Rhodococcus* YH1.

Materials and methods

Chemicals

RDX, TNT and HMX were obtained from Israeli military industries (95% pure). 2-Methyl-1,2-di-3-pyridyl-1-propanone (metyrapone) and 1,2,3,6-tetrahydrophthalimide (THPI) were purchased from Sigma-Aldrich (Rehovot, Israel).

Bacteria and growth conditions

Rhodococcus strain YH1 (Brenner et al. 2000) was grown on nitrogen-free basal mineral medium

containing (per l): 1 g K_2HPO_4, 0.5 g KH_2PO_4; 200 mg $MgSO_4 \cdot 7H_2O$, 10 mg $CaCl_2 \cdot 2H_2O$, 5 mg $FeSO_4 \cdot 7H_2O$, 2.5 mg $MnSO_4 \cdot 4H_2O$, 500 μg $Na_2MoO_4 \cdot 2H_2O$, 500 μg $CuSO_4 \cdot 5H_2O$ (pH 7.0), supplemented with various nitrogen sources (20 mg l^{-1} RDX, 3.82 g l^{-1} NH_4Cl, 7.22 g l^{-1} KNO_3 or 0.608 g l^{-1} KNO_2) and cyclohexanone (0.95 g l^{-1}) as the carbon source. RDX was first dissolved in cyclohexanone and then the other constituents were added. All cultures were shaken at 200 rpm, 30°C, and bacterial growth was measured as turbidity at 600 nm (HP 8452 spectrophotometer, Agilent, Palo Alto, CA).

Preparation of crude extracts and protein analysis

Cells grown on RDX and cyclohexanone were harvested by centrifugation (14,000 *g*, 30 min, 4°C) at the late exponential phase of growth (OD_{600} ca. 0.8 after 5 days), followed by washing (×3) in mineral medium (as above), unless otherwise specified. Frozen cells (−80°C) were fractured in the presence of 0.5 mM (final concentration) phenylmethylsulfonyl fluoride (PMSF, 40 mM in isopropanol stock solution) by passage (×3) through a pre-chilled modified French Press at 20,000 psi. Unbroken cells and debris were removed by centrifugation at 24,000 *g* for 5 min at 4°C. Soluble proteins were separated from the membrane fraction by centrifugation at 200,000 *g*, 5 h, 10°C (Centrikon T-1080, Kontron, Milan, Italy). Proteins for SDS-PAGE were further precipitated from the soluble fraction by 20% (final concentration) trichloroacetic acid and collected by centrifugation (24,000 *g*, 1 h, 10°C), washed (×2) with ethanol and resuspended overnight at 4°C in 5 M urea buffer (5 M urea, 50 mM Tris–HCl pH 8.8, 1.5 mM EDTA, 0.2% SDS). Undissolved proteins were removed by centrifugation in a microcentrifuge (24,000 *g*, 20 min, 4°C). The supernatant was assayed for protein concentration using bovine serum albumin as the standard (Lowry et al. 1951). Protein samples (100 μg) were separated by SDS-PAGE using a 10% acrylamide resolving gel and a 5% stacking gel with a ratio of acrylamide to bis-acrylamide of 37.5:1 run at 150 V for 5 h. Proteins were either stained using Gelcode Blue stain reagent (Pierce, Rockford, IL) or electroblotted (1 h, 400 mA) onto a polyvinylidene difluoride (PVDF) membrane. Protein bands of interest were excised and subjected to N-terminal and tryptic fragment amino-acid sequencing.

The cytochrome P-450 gene fragment was amplified by applying two degenerate PCR primers prepared based on the amino-acid sequence of the N terminus and an internal tryptic fragment of the polypeptide protein y1: a forward primer (5′-GGNACNGARACNGGNAAYGC-3′) and a reverse primer (5′-TCRTCRAANACYTCNGGR-3′). The PCR (100 μl) was composed of 1X buffer (Sigma Rehovot, Israel), 5 units of *Taq* polymerase (Red *Taq* polymerase, Sigma Rehovot, Israel), 0.4 μM of each primer, 200 μM each of four deoxynucleotides and 2 μg genomic DNA. PCR included 30 cycles of 1.5 min at 94°C, 1 min at 42°C, 2 min at 72°C and a final 5-min extension at 72°C. The amplified fragment was cloned into the pCR II-TOPO vector (Invitrogen, Carlsbad, CA) for sequencing.

Effect of different nitrogen sources on RDX biodegradation

Bacteria were grown in mineral medium with different organic nitrogen sources (7.56 mg l^{-1} TNT-N, 7.56 mg l^{-1} HMX-N or 7.56 mg l^{-1} THPI-N) and inorganic nitrogen sources (NH_4^+, NO_3^- or NO_2^-) in the presence of RDX. RDX and TNT analyses were performed by HPLC (Ronen et al. 1998).

Statistics

The presented results are representative of two to three similar experiments. Each experiment was run in triplicate.

Results

Effect of inorganic nitrogen sources on RDX degradation

Isolation of the RDX-degrading strain YH1 has been previously described (Brenner et al. 2000). This strain can utilize RDX as a sole nitrogen source while using cyclohexanone as the carbon source. YH1 is an obligate aerobic bacterium and cannot degrade RDX in the absence of oxygen. 16S rDNA sequences of strain YH1 (1455 bp, Genbank accession no. AF103733) show high similarity to several *Rhodo-*

coccus strains, the highest (100%) to *Rhodococcus ruber* (strain DSM43338T, accession no. X80625).

RDX degradation was determined in the presence of nitrate, nitrite and ammonium (Fig. 2). Two parameters were used to asses the effect of the abovementioned nitrogen sources: the lag time precede the on set degradation and the rate of RDX depletion relative to growth. RDX degradation was fastest when it served as the sole nitrogen source; nitrate and nitrite delayed its degradation and in the presence of ammonium, RDX biodegradation was almost completely inhibited. Nitrite was more efficient in delaying RDX degradation. A concentration of 100 mg l^{-1} NO_2^--N was as effective as 1,000 mg l^{-1} NO_3^--N. It was not possible to use higher concentrations of nitrite due to its toxicity to the growth of strain YH1. The initial increase in RDX concentration was due to dissolution of RDX traces into the medium.

Detection of cytochrome P-450

The inhibitory effect of the inorganic nitrogen compounds on RDX degradation by strain YH1 led us to examine the effect of these compounds on the expression of cytochrome P-450, which has been suggested to be involved in the first step of the aerobic RDX-degradation pathway (Tekoah et al. 1999; Fournier et al. 2002; Seth-Smith et al. 2002). Analysis of the soluble-protein fraction by SDS-PAGE revealed the accumulation of a protein polypeptide (y1) in cells of strain YH1 grown on RDX but not in those grown on ammonium (Fig. 3) or those which were nitrogen-starved (data not shown). Amino-acid sequences were generated from the N terminus of the y1 polypeptide (TDVTVLFGTETGNAE) and from an internal tryptic fragment (FMIGAANRDPEVFDD). Degenerate PCR primers were synthesized based on the amino-acid fragment sequences of y1 and used in a PCR to amplify a 1409-bp DNA fragment (Genbank accession no. AF406983). Upon translation, the 120 amino acids at the N terminus showed 100% identity to the flavodoxin domain of the *xplA* gene from *R. rhodochrous* and 303 amino acids at the C terminus showed 100% identity to the cytochrome P-450 domain of *xplA* from *R. rhodochrous* (Seth-Smith et al. 2002); 100% identity was also found in the DNA sequences. Although we do not have the entire gene, it is safe to conclude that the y1 polypeptide is encoded by a gene that is identical to *xplA*.

Interestingly, the accumulation of the y1 polypeptide was significantly reduced in the presence of

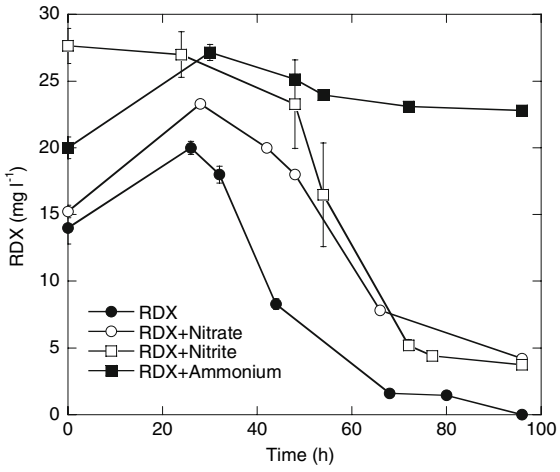

Fig. 2 Effect of nitrate (1000 mg l^{-1} NO_3^--N), nitrite (100 mg l^{-1} NO_2^--N) and ammonium (1000 mg l^{-1} NH_4^+-N) on RDX degradation

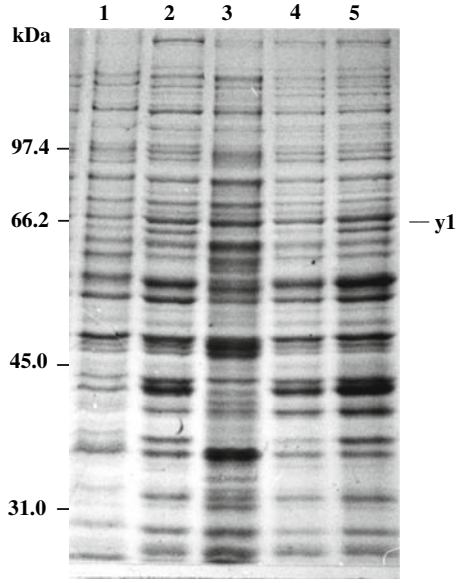

Fig. 3 Effect of RDX, ammonium and nitrite on the accumulation of the polypeptide protein y1 inYH1 cells grown on these nitrogen sources. Soluble proteins (100 µg) were subjected to SDS-PAGE. Lane 1, 20 mg l^{-1} RDX + 10 mg l^{-1} NO_2^--N; lane 2, 20 mg l^{-1} RDX + 1 mg l^{-1} NO_2^--N; lane 3, 20 mg l^{-1} RDX + 1000 mg l^{-1} NH_4^+-N; lane 4, 1000 mg l^{-1} NH_4^+ -N and lane 5, 20 mg l^{-1} RDX

ammonium, nitrite (Fig. 3) and nitrate (data not shown). To support the possible involvement of cytochrome P-450 enzyme in the degradation of RDX by strain YH1, we examined the effect of metyrapone, a specific inhibitor of cytochrome P-450 (Guengerich & MacDonald 1990), on the growth of strain YH1 and RDX degradation. Metyrapone inhibited the growth of strain YH1 on glucose and RDX as the nitrogen source, while it only slightly affected the growth of cells grown on glucose and ammonium as the nitrogen source (Fig. 4a). In addition, metyrapone almost completely inhibited RDX degradation (Fig. 4b).

Effect of organic nitrogen sources on YH1 growth and RDX degradation

Strain YH1 was cultured on aromatic organic nitrogen compounds as sole nitrogen source: it failed to grow on TNT, HMX, pyridine, atrazine and cyanuric acid; significant growth was only obtained on THPI, a metabolite of the fungicide Captan (Hour et al. 1998), with a lag of 50 h relative to its growth with RDX (data not shown). To examine the effect of THPI, HMX and TNT on RDX degradation, strain YH1 was grown in RDX medium supplemented with THPI, HMX or TNT. The growth of strain YH1 and degradation of RDX were not affected by THPI or HMX, but they were completely inhibited by TNT (Fig. 5). In addition, strain YH1 failed to grow on medium containing NH_4^+ and TNT. However, when strain YH1 was first grown on ammonium for 40 h (OD_{600} ca. 0.4) and then TNT was added, the culture continued to grow and TNT was degraded (data not shown). Following this observation, strain YH1 was first grown on ammonium for 32 h (OD_{600} ca. 0.2) and then TNT and RDX were added simultaneously. The culture continued to grow (data not shown) and TNT was degraded while RDX concentration remained unchanged (Fig. 6a). The lack of RDX degradation may stem from the presence of ammonium, TNT or TNT-degradation products. To verify this, stationary cells of strain YH1 grown in ammonium (100 mg l^{-1} NH_4^+-N) were washed three times in nitrogen-free mineral medium and then incubated with RDX and TNT. In this case (Fig. 6b), the onset of RDX degradation started only after the TNT had been completely degraded. HPLC chromatograms of these experiments revealed the appearance of two new peaks with identical retention time, of 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene.

Discussion

Rhodococcus strain YH1 is capable of RDX degradation when it is present as the sole nitrogen source (Brenner et al. 2000). RDX degradation was delayed in the presence of nitrate and nitrite and almost completely inhibited in the presence of ammonium (Fig. 2). However, when amended with nitrogen-containing organic compounds (Fig. 5) as nitrogen sources, degradation was only affected by TNT. This observation supports the possibility that ammonium,

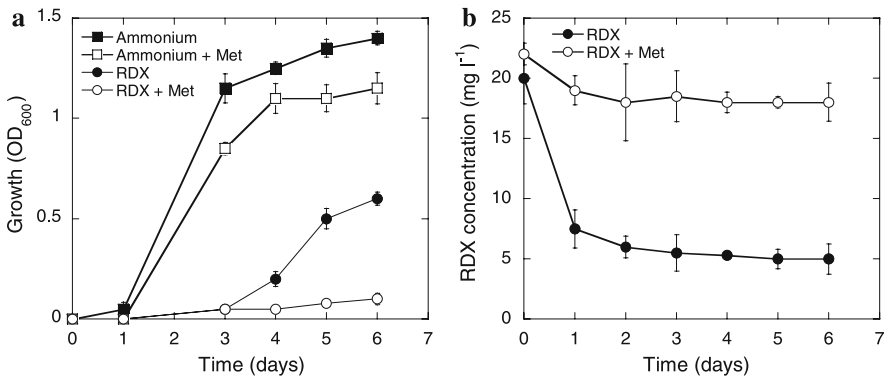

Fig. 4 Effect of the cytochrome P-450 inhibitor metyrapone (Met; 100 μM) on growth of strain YH1 (**a**) and RDX degradation (**b**). Growth was measured in the presence of RDX (20 mg l^{-1}) or ammonium (NH_4Cl, 23.8 mg l^{-1}) as the nitrogen source and glucose (1 g l^{-1}) as the carbon source. The effect on RDX degradation was tested in resting cells (no carbon source)

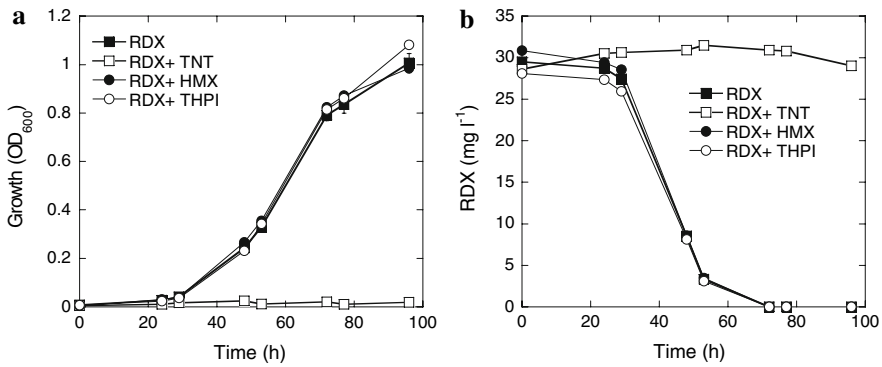

Fig. 5 Effect of TNT, HMX and THPI (7.56 mg N l^{-1}) on growth of strain YH1 and degradation of RDX

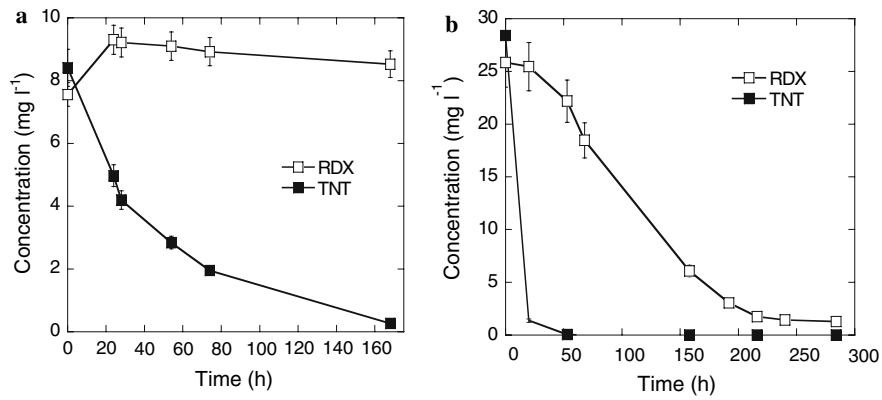

Fig. 6 Effect of TNT on RDX degradation. (**a**) RDX and TNT were added to cells growing on ammonium. (**b**) RDX and TNT were added to washed stationary cells that were grown on ammonium

nitrite and nitrate (and not any alternative organic nitrogen source) have a direct effect on the expression of the RDX-degradation mechanism. More importantly, our results suggest that in a mixture of explosives, TNT inhibits aerobic degradation of RDX. Nevertheless, *Rhodococcus* strain YH1 is able to overcome this effect by transforming TNT before degrading RDX. This observation has important consequences on the possibility of using strain YH1 in the bioremediation of RDX industrial wastes or contaminated sites where multiple inorganic or organic nitrogen species may be present.

RDX degradation activity was affected by inorganic nitrogen (ammonium; Fig. 1). This might be due to an inhibitory effect on the activity or accumulation of enzymes involved in RDX degradation. To elucidate the mechanism underlying this phenomenon, we looked at the protein profiles of YH1 cells grown with different nitrogen sources. Accumulation of a cytochrome P-450 protein was detected by SDS-PAGE in YH1 cells grown on RDX, but not in those grown on ammonium as the nitrogen source (Fig. 3), or in nitrogen-starved cells (not shown). The gene encoding the y1 protein had the same DNA sequence as *xplA*, characterized by Seth-Smith et al. (2002) and shown to confer RDX degradation upon transfer to a *Rhodococcus* strain that cannot use RDX as a nitrogen source. This fact strongly supports the involvement of y1 in strain YH1's RDX-degradation mechanism. Moreover, the inhibitory effect of metyrapone (Fig. 4) on RDX degradation and growth of strain YH1 on RDX as the nitrogen source supports the role of cytochrome P-450 in this strain's RDX-degradation process.

The expression of cytochrome P-450 protein was repressed by the presence of ammonium, nitrite (Fig. 3) and nitrate (not shown). This suggests that the RDX-biodegradation pathway is regulated by the cell's internal ammonium levels or the external levels of available nitrogen. The residual expression of cytochrome P-450 protein in the presence of nitrite and nitrate enabled significant degradation of RDX, in contrast to ammonium, with which RDX degradation was not observed. Ammonia may inhibit both cytochrome P-450 expression and its activity.

Seth-Smith et al. (2002) reported that the transcript of *xplA* is constitutively expressed in *R. rhodochrou*s 11Y. Accumulation of y1 protein was significantly higher when strain YH1 was grown on RDX as the nitrogen source compared to ammonium (Fig. 3). This suggests post-transcriptional regulation of the gene encoding the cytochrome P-450 involved in RDX degradation. However, although the ability to degrade RDX is ubiquitous in *Rhodococcus* spp., differences in the regulation of the RDX-degradation pathway among individual strains cannot be ruled out. For example, the genes encoding the cytochrome P-450 in our strain, YH1, and strain Y11 are genomic, whereas RDX degradation by strain DN22 involves a plasmid-encoded cytochrome P-450 (Coleman et al. 2002).

The effect of organic nitrogen sources presented a more complicated picture. As mentioned earlier, strain YH1 grew significantly on THPI (not shown), with no effect on RDX degradation (Fig. 5). On the other hand, although strain YH1 failed to grow on TNT and HMX as sole nitrogen source, it was capable of degrading TNT but not HMX, which is structurally more similar to RDX (Fig. 1). The inability of YH1 to degrade HMX is consistent with the known persistence of this compound in an aerobic environment (Hawari et al. 2001).

When TNT was added to a YH1 culture grown with ammonium, the culture continued to grow and TNT was degraded (not shown), with the formation of reduced intermediates. However, when strain YH1 was first grown on RDX until depletion, and then TNT and RDX were added, bacterial growth stopped and TNT was degraded (not shown). This suggests that even if TNT is degraded, it is not used as a nitrogen source, and the presence of TNT without ammonium is toxic to the growth of strain YH1 (ammonium cannot be replaced by RDX). Figure 6 shows that low concentrations of TNT, but not its degradation products, inhibit RDX degradation. Indeed, several studies have indicated that TNT is more toxic than its metabolites (Lachance et al. 1999; George et al. 2001; Oh et al. 2003). It was not possible to examine the effect of TNT on the expression of cytochrome P-450, since strain YH1 cannot grow in the presence of TNT without ammonium, which itself represses cytochrome P-450 expression (Fig. 3). The sensitivity of RDX degrading strains for TNT was tested on Luria Bertani agar by Seth-Smith (2002). The most sensitive strain, *R. rhodochrou*s 11Y, could not tolerate more than 0.1 mM ($\sim$23 mg l^{-1}) TNT. This result suggests a general toxic effect of TNT on the RDX degrading bacteria that appear to be consistent with our findings (Fig. 5).

The appearance of reduced metabolites of TNT indicated that YH1 most likely produces an oxygen-insensitive nitroreductase. This enzyme allows YH1 to overcome the toxic effect of TNT. This observation is unique and to the best of our knowledge, has never been reported for aerobic RDX-degrading strains. Although type 1 oxygen-insensitive nitroreductase from *Morganella morganii* strain B2 is known for its ability to reduce RDX (Kitts et al. 2000), this is not the case with the YH1 culture because no RDX nitroso derivatives were detected.

Nitroreductase in TNT-degrading bacteria such as *Pseudomonas putida* JLR11 is constitutively expressed, regardless of the electron acceptor (oxygen or TNT) and nitrogen source (ammonium or TNT) present in the culture medium (Caballero et al. 2005). Thus, the TNT-reduction activity in ammonium-grown cells of YH1 was expected (Fig. 6).

Conclusions

The results of this study show that ammonium, which represses the expression of a cytochrome P-450 enzyme suggested to catalyze the first step in the aerobic RDX-degradation pathway, completely inhibits its RDX degradation by *Rhodococcus* strain YH1. Moreover, TNT, which is toxic to the growth of strain YH1, is degraded by cells that were first grown on ammonium or RDX, and completely inhibits RDX degradation. Upon addition of TNT, the culture continues to grow in the presence of ammonium but this cannot be replaced by RDX. The ability of strain YH1 to degrade RDX resumes once the TNT is completely degraded, indicating the reversibility of TNT's toxic effect. Together, these results suggest that effective RDX degradation by *Rhodococcus* strain YH1 in wastewater must be preceded by the removal of ammonium and TNT.

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