ENVIRONMENTAL BIOTECHNOLOGY

Denitration of 2,4,6-trinitrotoluene by *Pseudomonas aeruginosa* ESA-5 in the presence of ferrihydrite

Laurent Eyers · Ben Stenuit · Spiros N. Agathos

Abstract Denitration of 2,4,6-trinitrotoluene (TNT) was evaluated in oxygen-depleted enrichment cultures. These cultures were established starting with an uncontaminated or a TNT-contaminated soil inoculum and contained TNT as sole nitrogen source. Incubations were carried out in the presence or absence of ferrihydrite. A significant release of nitrite was observed in the liquid culture containing TNT, ferrihydrite, and inoculum from a TNT-contaminated soil. Under these conditions, *Pseudomonas aeruginosa* was the predominant bacterium in the enrichment, leading to the isolation of *P. aeruginosa* ESA-5 as a pure strain. The isolate had TNT denitration capabilities as confirmed by nitrite release in oxygen-depleted cultures containing TNT and ferrihydrite. In addition to reduced derivatives of TNT, several unidentified metabolites were detected. Concomitant to a decrease of TNT concentration, a release of nitrite was observed. The concentration of nitrite peaked and then it slowly decreased. In the absence of TNT, the drop in the concentration of nitrite in oxygen-depleted cultures was lower when ferrihydrite was provided, suggesting that ferrihydrite inhibited the utilization of nitrite by *P. aeruginosa* ESA-5.

Keywords Biodegradation · Denitration · Trinitrotoluene · Pseudomonas · Ferrihydrite · Nitrite

L. Eyers · B. Stenuit · S. N. Agathos (✉)
Unit of Bioengineering, Catholic University of Louvain,
Place Croix du Sud 2/19,
1348 Louvain-la-Neuve, Belgium
e-mail: spiros.agathos@uclouvain.be

Introduction

2,4,6-Trinitrotoluene (TNT) is an environmental pollutant frequently found at sites associated with military activities. TNT is toxic to many organisms, from bacteria to humans, and has mutagenic activities (George et al. 2001). Therefore, intensive research has been conducted to identify microorganisms degrading this compound. Due to its xenobiotic character and structural features, TNT is very recalcitrant to microbial attack. The capacity to reduce the nitro groups of TNT is a ubiquitous reaction among many microorganisms. However, the reduced TNT metabolites (aminodinitrotoluene (ADNT) and diaminonitrotoluene (DANT) isomers) are not degraded further and are as toxic as TNT itself. A promising catabolic pathway consists in denitration (defined as the release of nitrite from TNT or other TNT metabolites) because related aromatic molecules with less than three nitro groups can be completely mineralized by bacteria (e.g., Nishino et al. 2000).

Few reports describe the release of nitrite from TNT. Stenuit et al. (2005) provided a detailed review of these reports. Briefly, nucleophilic addition of a hydride ion to the TNT molecule with the formation of hydride–Meisenheimer complex has been observed under aerobic conditions (Esteve-Nunez et al. 2001). Nitrite release from the hydride–Meisenheimer complex and formation of dinitrotoluene compounds were reported using a *Pseudomonas* sp. (Duque et al. 1993; Haïdour and Ramos 1996), although this could not be confirmed by Vorbeck et al. (1998). Pak et al. (2000) showed, through in vitro experiments with a purified oxidoreductase from a *Pseudomonas fluorescens* strain, that protonated dihydride–Meisenheimer complexes and aryl cations formed from hydroxylaminodinitrotoluene compounds reacted with each other to form a dimeric molecule with the release of nitrite. This was confirmed by Stenuit et

al. (2006) with whole cells and cell-free extracts of *Escherichia coli*. Another aerobic denitration pathway from a dihydroxylamino-nitrotoluene metabolite of TNT was proposed by Fiorella and Spain (1997) using a *Pseudomonas pseudoalcaligenes* strain, whereas Kalafut et al. (1998) reported the production of nitrite and 2-amino-4-nitrotoluene from TNT using Gram-positive and Gram-negative bacteria. Aerobic denitration of TNT and formation of dinitrotoluene compounds were reported by Kim et al. (2002) and by Martin et al. (1997) with a *Klebsiella* and a *Pseudomonas savastanoi* strain, respectively. Recently, oxidative denitration of TNT by a bacterial consortium with 3-methyl-4,6-dinitrocatechol as a metabolite was reported (Tront and Hughes 2005). All of these studies have shown that denitration of TNT occurred under aerobic conditions.

Microbial biodegradative reactions of TNT in the presence of terminal electron acceptors other than oxygen have also been described. With terminal electron acceptors such as sulfate or carbon dioxide, TNT is rapidly reduced to 2,4,6-triaminotoluene (Esteve-Nunez et al. 2001). This is due to the low redox potential associated with these acceptors. Under such redox conditions, the reduction of TNT prevents its denitration. Under anoxic conditions, Esteve-Nunez and Ramos (1998) reported the denitration of TNT using a *Pseudomonas putida* strain. In this work, a temporary release of nitrite was detected and particular denitrated compounds including 2-nitro-4-hydroxy-benzoic acid and 4-hydroxy-benzoic acid were identified.

Given that iron is one of the most abundant elements in the Earth's crust and ferric iron oxides are widespread in anoxic habitats, TNT denitration pathways in the presence of ferric iron Fe(III) in oxygen-depleted medium represents valuable environmental implications. However, an anoxic TNT denitration in the presence of iron species has never been reported. Therefore, the objective of this study was to investigate the denitration of TNT in oxygen-depleted enrichment cultures containing TNT as sole nitrogen source and ferrihydrite (the latter mineral embraces a variety of structurally ill-defined, poorly crystallized ferric iron species, ubiquitous at and near the Earth's surface, which are also commonly referred to as amorphous ferric oxyhydroxide (Straub et al. 2005)). Another objective was to identify bacteria enriched under these conditions and characterize their physiology towards the denitration of TNT.

Materials and methods

Chemicals

TNT was obtained from Nobel Explosives (Châtelet, Belgium). ADNT isomers (2-amino-4,6-dinitrotoluene (2-A-4,6-DNT) and 4-amino-2,6-dinitrotoluene (4-A-2,6-

DNT)) were synthesized as previously described (Van Aken and Agathos 2002). Formylamido-dinitrotoluene (fADNT) compounds were obtained by reacting formic acid with ADNT compounds (Hawari et al. 1999). 2, 4-Diamino-6-nitrotoluene (2,4-DA-6-NT) and azoxy compounds (2,2′-azoxy-4,4′,6,6′-tetranitrotoluene and 4,4′-azoxy-2,2′,6,6′-tetranitrotoluene) were graciously donated by Dr. D. Bruns-Nagel (University of Marburg, Germany). TNT hydride–Meisenheimer complexes were synthesized by reacting TNT with sodium borohydride (Pak et al. 2000). Other chemicals were purchased from Sigma (St Louis, MO).

Soil inocula

Samples were collected from TNT-contaminated soil (29.1 ± 2.8 g TNT per kilogram of dry soil) and uncontaminated soil (TNT was not detected by high-performance liquid chromatography (HPLC) with a detection limit of 1 mg TNT per kilogram of soil) at a site in Bourges, France that had been used for TNT destruction over the past 20 years (Eyers et al. 2004b). The uncontaminated soil, which was sampled a few meters from the contaminated soil, was not polluted due to the presence of a protecting wall at the destruction field. Both sets of samples were collected from the top 5-cm layer of soil and homogenized. Other soil characteristics (pH, soil texture, and carbon content) are described elsewhere (George et al. 2008).

Enrichment cultures

Enrichment cultures were carried out in 250-ml serum bottles using 2 g of soil per 150 ml of M8 minimal medium (Haïdour and Ramos 1996) spiked with TNT as sole nitrogen source at a concentration of 4,400 μM (i.e., ten times its limit of solubility in water). Oxygen was removed in all liquid cultures. To remove oxygen, standard anaerobic techniques were used. In brief, argon was passed through heated reduced copper filings to remove traces of oxygen and boiling M8 medium was flushed with this oxygen-free argon for 2 h. The carbon source was a mixture of glucose, acetate, succinate, and pyruvate (6 mM each). Ferrihydrite was freshly prepared as described by Lovley and Phillips (1988) and, when used, it was provided at a concentration of 400 mM. The prepared ferrihydrite was indeed amorphous and its chemical composition was $Fe(OH)_3$ (Régis M., personal communication). Biotic control cultures were carried out without TNT to determine potential release of nitrogen species in the form of nitrate, nitrite, or ammonium. Additional biotic controls consisted in cultures with 4-A-2,6-DNT replacing TNT. Abiotic controls were established by adding $HgCl_2$ (200 ppm) in the presence or absence of carbon source. Other abiotic controls contained

ferrihydrite and TNT alone. The pH of each liquid culture was adjusted to 6.8. Cultures were sealed with a rubber stopper and an aluminum crimp. They were incubated in the dark at 35°C on an orbital shaker set at 170 rpm. Experiments were carried out in triplicate.

DNA extraction, PCR amplification, and denaturing gradient gel electrophoresis

For denaturing gradient gel electrophoresis (DGGE), 2 ml of the enrichment cultures were centrifuged and the pellet was used for DNA extraction. DNA was extracted with the UltraClean Soil DNA isolation kit from Mo Bio (Carlsbad, CA, USA) following the manufacturer's instructions. Polymerase chain reaction (PCR) amplification of a portion of the 16S ribosomal RNA (rRNA) gene was carried out with primers P63f containing a 40-bp GC-clamp attached to its 5′-end and P518r (Eyers et al. 2004a) in a thermal cycler (Perkin-Elmer, Norwalk, CT, USA). The PCR mix (100 μl) contained 50 μl of Red'y'Star mix (Eurogentec, Seraing, Belgium), 0.25 μM of each primer, and 100 ng of template DNA. An initial denaturation step of 10 min at 94°C was followed by 30 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C. A final extension at 72°C for 10 min was done. DGGE was performed with a D-Code System (Bio-Rad, Hercules, CA USA). PCR products (~600 ng) were loaded on a 6% polyacrylamide gel with a denaturing gradient of 35% to 55%. The electrophoresis was carried out at 150 V for 7 h in 0.5 X Tris–acetate–ethylenediaminetetraacetic acid buffer maintained at 60°C. Gels were stained with ethidium bromide and photographed on a transillumination table.

Bands of the DGGE to be sequenced were excised from the gel with a scalpel, placed in 100 μl of sterile water, and incubated overnight at 4°C. A 5-μl volume of the DNA diffused in water served as a template for PCR amplification. The PCR products were purified with a Qiaquick PCR purification kit (Qiagen, Valencia, CA USA) before being sent for sequencing with primer P518r. Sequences were aligned to 16S rRNA sequences obtained from the National Center for Biotechnology Information GenBank database by using the Basic Local Alignment Search Tool 2.2.10 search program (Altschul et al. 1997). 16S rRNA gene sequences of DGGE bands 1 to 4 (Fig. 2) are available in the GenBank database under accession numbers DQ641676 to DQ641679, respectively.

Isolation of *Pseudomonas aeruginosa*, taxonomic identification, and pure cultures

After 55 days of incubation, a sample of the enrichment culture with the TNT-contaminated soil inoculum, TNT, and ferrihydrite was used to inoculate *Pseudomonas* isolation agar plates (Difco Laboratories Inc., Detroit, MI,

USA). Several colonies were picked up and analyzed by DGGE. One of these selected colonies was chosen for additional characterization in pure culture and was named *P. aeruginosa* strain ESA-5.

For sequencing of the 16S rRNA gene of the isolated strain, its DNA was extracted with the UltraClean Soil DNA isolation kit from Mo Bio. PCR amplification was done with primers Bact11F (Kane et al. 1993) and S-D-Bact-1512-a-A-16 (Fry et al. 1997) and amplicons were purified under conditions described above. Amplicons were sent for sequencing with primers Bact11F, Univ907r (Amman et al. 1992), and S-D-Bact-1512-a-A-16. The 16S rRNA gene sequence of *P. aeruginosa* ESA-5 is available in the GenBank database under accession number DQ641680.

P. aeruginosa ESA-5 was pregrown aerobically in 150 ml of M8 minimal medium containing glucose, acetate, succinate and pyruvate (6 mM each), and ammonium (5 mM). When the OD_{600} was close to 0.6, cells were harvested by centrifugation ($6,000 \times g$, 15 min, and 4°C) and the pellet was washed three times with M8 minimal medium. The washed pellet was used to inoculate cultures to an OD_{600} of 0.225. Pure culture experiments were performed with TNT (at 380 or 4,400 μM) as sole nitrogen source and ferrihydrite (400 mM) under oxygen-depleted conditions. To investigate the utilization of nitrite by *P. aeruginosa*, it was provided as sole nitrogen source (1,000 μM spiked) under various conditions in oxygen-depleted or aerobic cultures in the presence or absence of ferrihydrite. The carbon source was a mixture of glucose, acetate, succinate, and pyruvate (6 mM each), except when mentioned. The pH of each culture was adjusted to 6.8 and cultures were incubated in the dark at 35°C on an orbital shaker set at 170 rpm. Experiments were carried out in triplicate.

Analytical methods

TNT and its metabolites were extracted from liquid culture samples by mixing 1 ml of the sample with 1 ml of acetonitrile. After centrifugation ($18,000 \times g$, 20 min), the supernatant was analyzed using a HPLC system (multi-solvent delivery system, controller, and autosampler (Waters, Milford, MA, USA)). The column was a 4-μm C18 Nova-Pak, 3.9×300 mm (Waters). The mobile phase consisted of 30% acetonitrile and 70% phosphate buffer (10 mM, pH 3.2) and the flow rate was maintained at 1 ml/min. After 2 min, a linear gradient was applied to reach a composition of 90% acetonitrile and 10% phosphate buffer in 18 min. Then, a linear gradient was set for 5 min to reach the initial mobile phase composition and this composition was maintained for 15 min. Compounds were monitored by a photodiode array detector (Waters). TNT

metabolites were identified and quantified by comparing their ultraviolet (UV) spectra and peak absorbance with authentic standards.

The concentrations of nitrite, nitrate, and ammonium were quantified in the supernatants obtained after centrifugation of the samples ($18,000 \times g$, 20 min). Nitrite concentration was colorimetrically determined using a nitrite Spectroquant® kit from Merck (Darmstadt, Germany). To avoid the interference of colored samples, a supernatant without the addition of reactants for the determination of nitrite was used as reference. Nitrite was also quantified by HPLC with an IC-PAK Anion HC column (Waters). The mobile phase was a borate–gluconate eluent (pH 8.5) with the following constituents (in milliliter per liter of bidistilled water): 40 ml of *n*-butanol, 240 ml of acetonitrile, and 40 ml of borate–gluconate concentrate. One liter of the borate–gluconate concentrate contained 16 g of sodium gluconate, 18 g of boric acid, 25 g of sodium tetraborate decahydrate, and 250 ml of glycerol. Nitrite was detected with a UV detector (Pharmacia, Uppsala, Sweden) set at 210 nm. Nitrate and ammonium concentrations were spectrophotometrically determined with a nitrate and ammonium Spectroquant® kit (Merck), respectively. TNT interfered with ammonium determination and was removed by passing the samples through a Sep-Pak C18 cartridge (Waters).

The concentration of Fe^{2+} was colorimetrically determined with an iron Spectroquant® kit (Merck) on samples containing ferrihydrite. Samples were diluted to a total iron final concentration of approximately 20 mM in 2 M HCl and the iron precipitate was dissolved for 45 min. Then, 50 μl were diluted in 950 μl dH₂O, and 67.5 μl of solution 'Fe-2' (1,10-phenanthroline solution provided with the iron Spectroquant® kit) was added. Fe^{2+} concentration was determined by measuring the absorbance at 510 nm after a 10-min reaction.

Results

Enrichment cultures

The release of nitrite was investigated in enrichment cultures degassed with argon and with TNT (4,400 μM) as sole nitrogen source. When ferrihydrite was omitted, a relatively low concentration of nitrite was detected with the uncontaminated soil (initial nitrite concentration was 1.0 μM and reached 50.5 μM after 56 days of incubation). With the TNT-contaminated soil, the concentration of nitrite was not significant (0.8 to 2.0 μM over the same timescale). When ferrihydrite was present, a concentration of nitrite of 236.0 μM after 56 days was measured (Fig. 1) with the uncontaminated soil inoculum. Nitrite concentra-
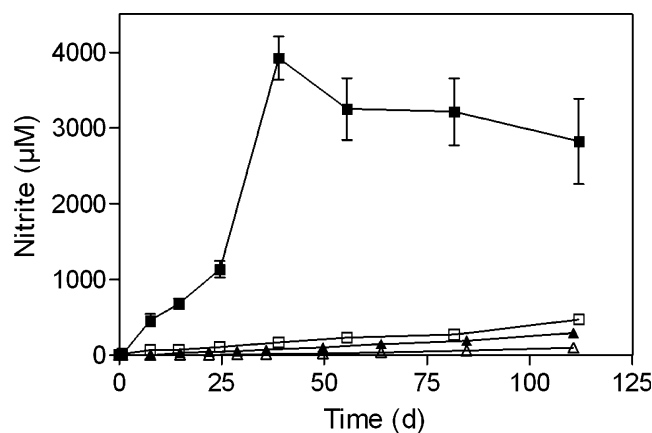

Fig. 1 Nitrite release in the presence of ferrihydrite and TNT (4,400 μM). TNT-contaminated soil inoculum (*closed squares*), uncontaminated soil inoculum (*open squares*). Abiotic controls (200 ppm HgCl₂ and omission of carbon source) with inoculum from TNT-contaminated soil (*closed triangles*) and uncontaminated soil (*open triangles*). *Error bars* represent the standard deviation of triplicates

tion reached 475.0 μM after 112 days. Even more remarkable was the evolution of nitrite concentration with the TNT-contaminated soil inoculum. Nitrite concentration peaked to 3,923.8 μM after 39 days and decreased thereafter (2,825.7 μM after 112 days, Fig. 1). The accuracy of this measurement was confirmed by anionic chromatography (results not shown). When TNT was omitted or replaced by 4-A-2,6-DNT, the concentration of nitrite measured (between 0.8 and 2.9 μM during the entire time course of the experiment) was not significant with either inoculum, indicating that TNT was at the origin of the release of nitrite. Neither nitrate nor ammonium was detected in these samples. Abiotic controls consisting of the uncontaminated or TNT-contaminated inoculum, TNT, ferrihydrite, and HgCl₂ in the absence of carbon source were also incubated. The concentrations of nitrite detected in these controls were much lower than those attained biotically (Fig. 1). Similar results were obtained when the carbon source was added to abiotic controls or when ferrihydrite and TNT were the only two chemicals in the medium (results not shown). Taken together, these results indicated that (1) the marked release of nitrite observed in the presence of ferrihydrite resulted from a biotic denitration of TNT and (2) the release of nitrite was more significant with the TNT-contaminated soil inoculum.

DGGE analysis

DGGE fingerprints of the enrichment cultures with TNT as sole nitrogen source and supplemented with ferrihydrite are depicted in Fig. 2. Two faint bands were initially observed with the uncontaminated soil inoculum. Subsequently, three brighter bands appeared after 34 days. At day 55, the
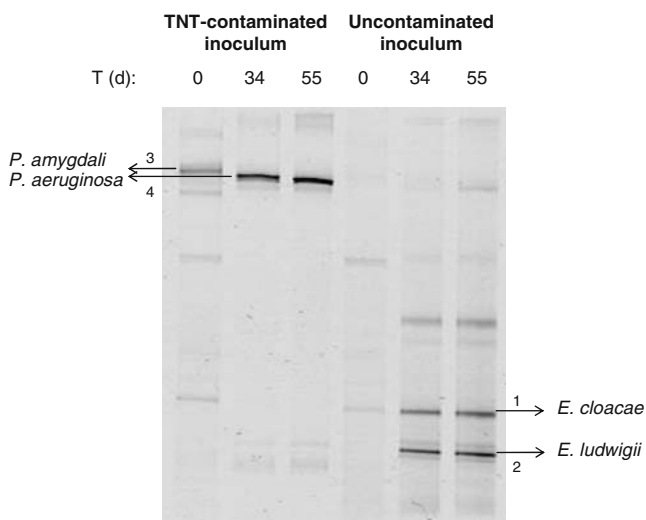
Fig. 2 DGGE fingerprints of 16S rRNA gene fragments amplified from DNA extracted from enrichment cultures. Samples were collected from enrichment cultures with TNT, ferrihydrite, and TNT-contaminated or uncontaminated soil inoculum. *Arrows* indicate bands that were extracted and sequenced

DGGE fingerprint was similar to the one after 34 days of incubation. Two of these three bands were sequenced (bands 1 and 2, Fig. 2) and were found to correspond to *Enterobacter cloacae* (100% identity on a 442-bp fragment) and to *Enterobacter ludwigii* (100% identity on 442 bp). With the TNT-contaminated soil inoculum, five bands were initially observed. Among them, a brighter band (band 3) corresponded to *Pseudomonas amygdali* (99.8% identity on 409 bp). At day 34, this band disappeared. Instead, another bright band (band 4) was detected and corresponded to *P. aeruginosa* (99.8% identity on 428 bp). This dominant band was still present after 55 days of incubation.

Isolation and characterization of *P. aeruginosa* ESA-5

At day 55, a sample of the enrichment culture with the TNT-contaminated soil inoculum, TNT, and ferrihydrite was taken and used to inoculate *Pseudomonas* isolation agar plates. This selective medium was used given the predominance of *Pseudomonas* species that had been revealed by the DGGE analysis (Fig. 2). All the colonies on the plates appeared similar. Several of them were picked up and analyzed by DGGE. For each colony, a single band was detected and migrated at the same position as the *P. aeruginosa* band of the enrichment culture with the TNT-contaminated soil inoculum. Subsequently, one of these analyzed colonies was chosen for additional characterization. When the strain was grown in peptone–glycerol medium (Degiorgi et al. 1996), a blue pigment was extracted. Under acidic conditions, this pigment turned red. This behavior is typical of pyocyanin (Cox 1986), a

pigment associated with *P. aeruginosa*. Sequencing of the 16S rRNA gene of this isolate confirmed that it was a *P. aeruginosa* strain (99.9% identity on 1,422 bp). This strain produced pyoverdine group I (Cornelis P. and Matthijs S., personal communication), a feature of *P. aeruginosa* PAO1 (Meyer et al. 1997). Our isolate was named *P. aeruginosa* strain ESA-5.

Denitration of TNT by *P. aeruginosa* ESA-5

P. aeruginosa ESA-5 was used to inoculate liquid cultures containing TNT (4,400 μM) as sole nitrogen source. In the presence of ferrihydrite, a significant release of nitrite was detected. After 9 days, 281.5 μM of nitrite was measured. Neither nitrate nor ammonium was detected. When ferrihydrite or TNT was not provided, nitrite concentration values were negligible (i.e., between 0.7 and 1.8 μM on a 9-day timescale). Abiotically (i.e., inoculated cultures, absence or presence of carbon source, presence of TNT, ferrihydrite, and $HgCl_2$), nitrite concentration values were also not significant (i.e., between 0.8 and 2.8 μM). These results indicated, once again, that the release of nitrite from TNT had a biotic origin and that this release was only detected in the presence of ferrihydrite.

To test the influence of the carbon source in the observed release of nitrite from TNT by *P. aeruginosa* ESA-5 in the presence of ferrihydrite, each carbon source (glucose, succinate, acetate, or pyruvate) was added individually (final concentration of 25 mM) instead of a mixture of four carbon sources. With acetate, succinate, and pyruvate, no release of nitrite was detected. With glucose, a significant release of nitrite was observed (200.8 μM after 7 days).

Different TNT metabolites were detected. At the starting time of the incubation, TNT was the only metabolite identified (Fig. 3a). After 15 days of incubation, ADNT (2-A-4,6-DNT and 4-A-2,6-DNT), DANT (2,4-DA-6-NT and 2,6-DA-4-ANT), and azoxy (2,2′-azoxy-4,4′,6,6′-tetranitrotoluene and 4,4′-azoxy-2,2′,6,6′-tetranitrotoluene) compounds were detected (Fig. 3b). fADNT compounds were also identified, as well as three additional unidentified metabolites (Fig. 3b). No production of TNT hydride–Meisenheimer complexes was detected.

In order to determine the kinetics of TNT transformation and production of identified TNT metabolites, the concentration of initially spiked TNT was reduced to 380 μM (i.e., below its limit of solubility). After 5 days of incubation, the concentration of TNT was below detection limit (Fig. 4). A temporary accumulation of ADNT compounds was observed, which reached a maximum concentration of 162.6 μM after 5 days. The concentration of DANT metabolites was 24.8 and 191.6 μM after 5 and 15 days, respectively. The concentration of azoxy metabolites was low during the time course of the experiment (below
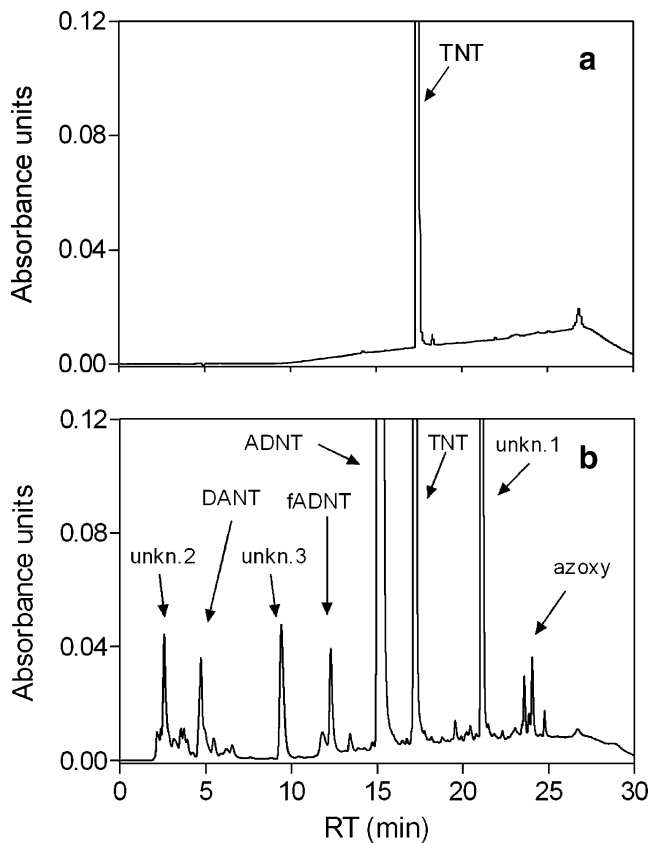
Fig. 3 HPLC chromatogram of *P. aeruginosa* ESA-5 cultures in the presence of ferrihydrite and TNT (4,400 μM). Samples from cultures after 0 day (**a**) and 15 days (**b**) of incubation. Glucose was the sole carbon source. UV detection was set at 230 nm

16 μM). The concentration of fADNT was not determined but was likely low given their respective HPLC areas which remained small compared to ADNT and DANT compounds (e.g., Fig. 3b).

In the presence of ferrihydrite, the release of nitrite was lower with 380 μM of TNT (Fig. 5) compared to 4,400 μM. Concomitant to TNT utilization (Fig. 4), nitrite concentration increased and peaked at 53.5 μM after 3 days. Then, the concentration of nitrite slowly decreased reaching 4.2 μM by day 15, thus indicating nitrite utilization. Again, nitrite concentration was not significant when ferrihydrite was omitted (0.1 to 1.7 μM, Fig. 5). In its presence, the concentration of Fe^{2+} was low until day 3 (0.4 to 0.6 mM, Fig. 5). After that point, a significant increase in the concentration of Fe^{2+} was observed, concomitant with a decrease in the concentration of nitrite. The concentration of Fe^{2+} was maximal (3.8 mM) after 15 days.

Utilization of nitrite by *P. aeruginosa* ESA-5

In order to understand the role of ferrihydrite, *P. aeruginosa* ESA-5 was inoculated in oxygen-depleted liquid medium containing ferrihydrite and nitrite as sole nitrogen source instead of TNT. The concentration of nitrite decreased from an initial concentration of 1,012.5 to 27.5 μM in 15 days (Fig. 6a). The initial concentration of Fe^{2+} was 0.2 mM and peaked to 3.3 mM at day 15. Abiotically, nitrite concentration remained stable and no Fe^{2+} production was observed (results not shown). Interestingly, in oxygen-depleted cultures containing nitrite in the absence of ferrihydrite, nitrite utilization was much faster than in its presence (Fig. 6b). Under these conditions, a residual level of 2.4 μM was measured after just 1 day of incubation. Aerobically, in the presence of ferrihydrite, nitrite utilization was also faster as 3.7 μM of nitrite was detected after 8 h. Fe^{2+} concentration was below detection limit (results not shown). Aerobically, in the absence of ferrihydrite, nitrite concentration was below detection limit after 8 h.
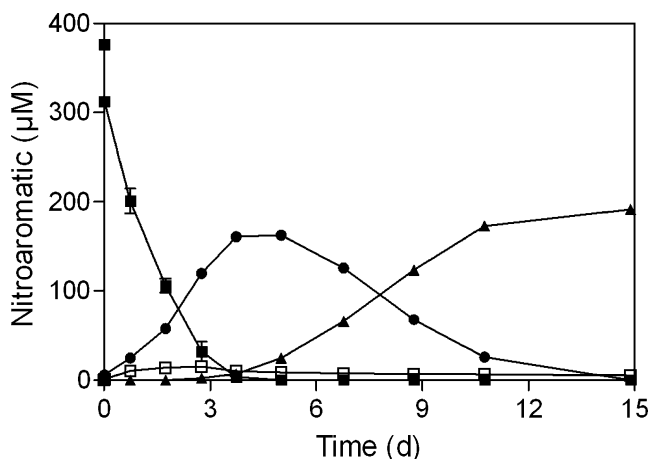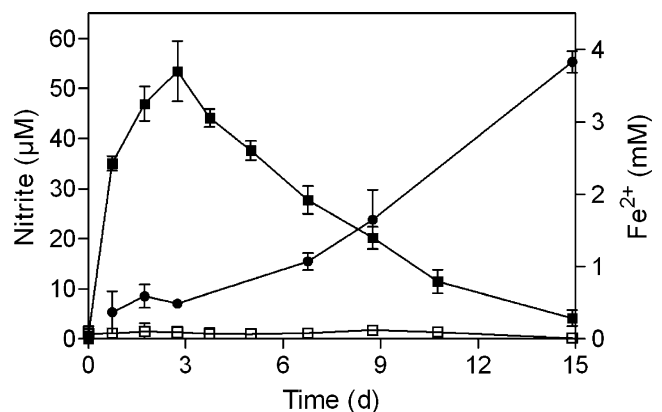

Fig. 4 Evolution of the concentration of nitroaromatic compounds of *P. aeruginosa* ESA-5 cultures in the presence of ferrihydrite and TNT (380 μM). TNT disappearance (*closed squares*) and production of DANT (*closed triangles*), ADNT (*closed circles*), and azoxy (*open squares*) metabolites. *Error bars* represent the standard deviation of triplicates

Fig. 5 Nitrite release and Fe^{2+} production by *P. aeruginosa* ESA-5 in the presence of TNT (380 μM). Nitrite release (*closed squares*) and Fe^{2+} production (*closed circles*) with ferrihydrite. Nitrite concentration (*open squares*) without ferrihydrite. *Error bars* represent the standard deviation of triplicates
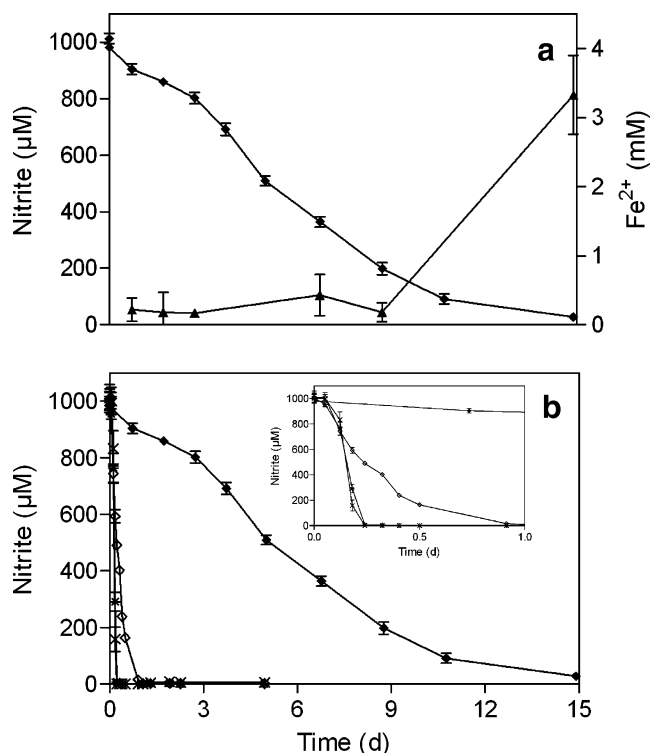
Fig. 6 Nitrite utilization by *P. aeruginosa* ESA-5 in the absence of TNT. **a** Nitrite utilization (*closed lozenges*) and Fe^{2+} production (*closed triangles*) in oxygen-depleted medium with ferrihydrite. **b** Nitrite utilization in oxygen-depleted medium with ferrihydrite (*closed lozenges*), oxygen-depleted medium without ferrihydrite (*open diamonds*), aerobic medium with ferrihydrite (*asterisk* crosses), aerobic medium without ferrihydrite (*x crosses*). The *inset* shows in more detail the kinetics during the first day of incubation. *Error bars* represent the standard deviation of triplicates

Discussion

A significant release of nitrite from TNT was detected under specific biotic conditions. We measured a nitrite concentration of 3,923.8 μM with a TNT-contaminated soil inoculum (Fig. 1) under oxygen-depleted medium containing ferrihydrite and TNT as sole nitrogen source. This concentration was one order of magnitude higher than with an uncontaminated soil inoculum. In an experiment with ferrogenic aquifer sediments packed into columns fed with a TNT solution and without acetate, Hofstetter et al. (1999) observed a transformation of TNT into ADNT and DANT compounds. Mass balances indicated complete transformation of TNT into these reduced compounds (67% of 2-A-4,6-DNT, 21% of 4-A-2,6-DNT, and 11% of DANT). Hofstetter et al. (1999) observed no denitration of TNT. When acetate was added to stimulate indigenous consortia of iron-reducing bacteria, TNT was rapidly and completely transformed to 2,4,6-triaminotoluene (TAT). The authors suggested a mechanism by which Fe(III) is reduced to Fe(II) due to microbial activity with the concomitant regeneration of Fe(III) after reduction of

nitroaromatic compounds. Here, with ferrihydrite and non-polluted or contaminated soil, formation of TAT was not observed. Instead, ADNT and DANT metabolites accumulated in the medium without being further transformed. When TNT was absent or replaced by 4-A-2,6-DNT as sole nitrogen source, no nitrite was detected, confirming that the nitrite detected in the medium originated from TNT. When ferrihydrite was absent, nitrite concentration was not significant. Abiotically, we detected some nitrite whose concentration was not significant compared to what was observed biotically with the TNT-contaminated soil inoculum.

Because this significant nitrite concentration in the medium with the TNT-contaminated soil inoculum was taken as an indicator of a biotic nitrosubstituent cleavage of TNT, attempts to isolate a strain responsible for this TNT denitration activity were undertaken. A *P. amygdali* was initially a predominant bacterium. However, *P. amygdali* did not remain in the enrichment culture during the several-week-long incubation time. Instead, our culture conditions selected for a *P. aeruginosa*. This latter strain was responsible for TNT denitration (Figs. 3, 4, and 5). Some authors previously reported a TNT denitration activity with *P. aeruginosa* under aerobic conditions. For instance, Oh et al. (2003) observed a TNT denitration with a *P. aeruginosa* strain MX inoculated in an aerobic medium containing TNT (260 μM) and yeast extract (1 g/l). A nitrite concentration of 18 μM was measured in the inoculated medium compared to 9 μM in uninoculated medium. Kalafut et al. (1998) detected a marginal and transient amount of nitrite (13 μM) with another *P. aeruginosa* strain inoculated into an aerobic medium with TNT (440 μM). In both of these studies, the concentration of nitrite measured was relatively low. The culture conditions used in our study were different from those of Oh et al. (2003) and Kalafut et al. (1998). However, our results clearly show that the isolated *P. aeruginosa* strain ESA-5 had significant TNT denitration activities, given that TNT was the sole nitrogen source and that the concentration of nitrite released was much higher than those reported by these authors.

In the experiment with a *P. aeruginosa* strain ESA-5 liquid culture containing 380 μM of TNT as sole nitrogen source and ferrihydrite, we calculated a nitrogen mass balance of 53.4% (molar equivalent of initial TNT) after 15 days (data from Fig. 4), distributed as DANT (50.4%) and azoxy (3%, taking into account that an azoxy dimer corresponds to two molar equivalents of TNT). Therefore, the fate of 46.6% of the initial TNT remained unknown and likely included denitrated compounds. Several unidentified metabolites were detected (Fig. 3) which probably corresponded to denitrated metabolites.

The role of ferrihydrite in the denitration of TNT is not clear. However, a plausible hypothesis can be proposed based on the work of Cooper et al. (2003) and Coby and

Picardal (2005). In the study of Cooper et al. (2003), the presence of goethite (a crystalline form of Fe(III)) resulted in a tenfold decrease in the rate of nitrite reduction by *Shewanella putrefaciens* 200. When most of the nitrite was reduced, a significant production of Fe^{2+} (above 1 mM) was observed. With enrichment cultures, the authors showed that inhibition of nitrite reduction was not limited to *S. putrefaciens*. When nitrite and insoluble Fe(III) were both present, Cooper et al. (2003) and Coby and Picardal (2005) demonstrated that Fe^{2+} was microbially produced from Fe(III) and that Fe^{2+} abiotically reacted with nitrite to form N_2O. During this reaction, Fe^{2+} was oxidized back to insoluble Fe(III) that formed a dense coating on the surface of the cell as demonstrated with *S. putrefaciens* and *Paracoccus denitrificans* (Coby and Picardal 2005). This coating inhibited reduction of nitrite by physically blocking transport into the cell (Coby and Picardal 2005).

P. aeruginosa is a facultative anaerobe capable of utilizing O_2, nitrate (Carlson and Ingraham 1983), and nitrite (Williams et al. 1978) as terminal electron acceptors for carbon metabolism. *P. aeruginosa* can also reduce Fe (III) (Lovley 1987). A production of Fe^{2+} was only observed when most of the nitrite was eliminated and its concentration was low, i.e., approximately 30 μM (Figs. 5 and 6a). Also, the utilization rate of nitrite was significantly lower in the presence than in the absence of ferrihydrite (Fig. 6b). To some extent, these results are similar to the results of Cooper et al. (2003). If an insoluble Fe(III) coating was formed on the cell surface of *P. aeruginosa* strain ESA-5 inhibiting nitrite utilization, then the role of ferrihydrite was probably not directly linked to the denitration of TNT. In other words, its role would be limited to inhibiting the utilization of nitrite by *P. aeruginosa* strain ESA-5, thus making the release of nitrite from TNT more easily detectable. Therefore, nitrite release was probably detected because its production rate resulting from TNT denitration was faster than its utilization rate. In the absence of ferrihydrite, TNT denitration was probably also taking place but nitrite utilization rate (Fig. 6b) was likely faster than its production rate.

Although the exact role of ferrihydrite in the denitration of TNT remains to be determined, this study shows that both a TNT-contaminated soil inoculum and an isolated *P. aeruginosa* strain can denitrate TNT and release a significant concentration of nitrite in oxygen-depleted medium in the presence of ferrihydrite, a feature that has not been reported previously. Given that Fe(III) is commonly found in anoxic habitats, the results of this study are of significance for TNT bioremediation in such environments.

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