

PnrA, a new nitroreductase-family enzyme in the TNT-degrading strain *Pseudomonas putida* JLR11

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Summary

Nitroreductases are a group of proteins that catalyse pyridine nucleotide-dependent reduction of nitroaromatic compounds, showing significant human health and environmental implications. In this study we have identified the nitroreductase-family enzymes PnrA and PnrB from the TNT-degrading strain *Pseudomonas putida*. The enzyme encoded by the *pnrA* gene was expressed in *Escherichia coli*, purified to homogeneity and shown to be a flavoprotein that used 2 mol of NADPH to reduce 1 mol of 2,4,6-trinitrotoluene (TNT) to 4-hydroxylamine-2,6-dinitrotoluene, using a ping-pong bi-bi mechanism. The PnrA enzyme also recognized as substrates a number of other nitroaromatic compounds, i.e. 2,4-dinitrotoluene, 3-nitrotoluene, 3- and 4-nitrobenzoate, 3,5-dinitrobenzamide and 3,5-dinitroaniline expanding the substrates profile from previously described nitroreductases. However, TNT resulted to be the most efficient substrate examined according to the V_{\max}/K_m parameter. Expression analysis of *pnrA*- and *pnrB*-mRNA isolated from cells growing on different nitrogen sources suggested that expression of both genes was constitutive and that its level of expression was relatively constant regardless of the growth substrate. This is in agreement with enzyme-specific activity determined with cells growing with different N-sources.

Introduction

Nitroreductases are a group of enzymes that catalyse the reduction of nitro groups on aromatic compounds. These proteins have recently raised enormous interest because of their central role in mediating nitroaromatic toxicity (Homma-Takeda *et al.*, 2002; Padda *et al.*, 2003; Sarlauskas

et al., 2004), their potential use in bioremediation (Hannink *et al.*, 2001), biocatalysis (Kadiyala *et al.*, 2003) and their utility in activating prodrugs in directed anticancer therapies (Denny, 2002; Knox *et al.*, 2003). Recently, it has been shown that these enzymes have also chromate reduction activity (Kwak *et al.*, 2003). Although the physiological role of these enzymes is unknown, two types of nitroreductases have been described according to their response to oxygen (Asnis, 1957; McCalla *et al.*, 1978; Bryant *et al.*, 1981). Oxygen-sensitive nitroreductases mediate single-electron transfers, producing nitro-anion free radicals that, in the presence of oxygen, can be rapidly reoxidized in a futile redox cycle through which superoxide is generated (Peterson *et al.*, 1979; Angermaier and Simon, 1983). Oxygen-insensitive nitroreductases are widely distributed in nature and are flavoproteins that have been suggested to mediate the sequential transfer of two electrons from NADH or NADPH to the nitro moiety of nitrosubstituted compounds (Bryant *et al.*, 1981). Intermediates produced biologically through this pathway include nitroso and hydroxylamine derivatives, which are responsible for the toxicity of nitroaromatic compounds when reacting with biological molecules (Haack *et al.*, 2001; Sarlauskas *et al.*, 2004).

Two oxygen-insensitive nitroreductases, NfsA and NfsB, have been characterized in *Escherichia coli* (Bryant *et al.*, 1981; Zenno *et al.*, 1996a,b; Whiteway *et al.*, 1998). NfsA is the major oxygen-insensitive nitroreductase, and uses NADPH as an electron source, whereas NfsB is a reductase that can use either NADH or NADPH as a source of reducing equivalents (Bryant and DeLuca, 1991; Zenno *et al.*, 1996a,b). Oxygen-insensitive nitroreductases have been described in other enterobacteriaceae such as *Salmonella enterica* serovar Typhimurium (Watanabe *et al.*, 1990; Nokhbeh *et al.*, 2002), *Enterobacter cloacae* (Bryant and DeLuca, 1991; Haynes *et al.*, 2002) and *Helicobacter pylori* (Goodwin *et al.*, 1998), and in other microorganisms such as *Vibrio harveyi* (Lei *et al.*, 1994), *Vibrio fischerii* (Zenno *et al.*, 1994; Riefler and Smets, 2002), *Rhodobacter capsulatus* (Blasco and Castillo, 1993), *Thermus thermophilus* (Park *et al.*, 1992), *Pseudomonas pseudoalcaligenes* (Somerville *et al.*, 1995), *Pseudomonas putida* (Park and Kim, 2000; Hughes and Williams, 2001) and *Selenomonas ruminantium* (Anderson *et al.*, 2002).

The most widely used nitroaromatic compound is 2,4,6-trinitrotoluene (TNT) (Hartter, 1985). The recalcitrance of

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this explosive in the environment is mainly attributed to the symmetric localization of the nitro groups of the aromatic ring, an arrangement that limits attack by classic dioxygenase enzymes involved in the microbial metabolism of aromatic compounds. In contrast with the lack of reports on oxygenolytic metabolism of TNT by living organisms, there are numerous studies of the reductive attack of TNT, leading to the formation of Meisenheimer complex via hydride-transfer or direct reduction of the nitro groups (Duque *et al.*, 1993; Haïdour and Ramos, 1996; Esteve-Núñez and Ramos, 1998; Esteve-Núñez *et al.*, 2001).

We have previously shown that *P. putida* JLR11 uses TNT as an N-source under anaerobic conditions, and that under these conditions TNT serves also as a terminal electron acceptor for conserving energy in the absence of oxygen (Esteve-Núñez *et al.*, 2000). *Pseudomonas putida* JLR11 is also able to reduce TNT to 4-hydroxylamino-derivatives in the cytoplasm regardless of growth under aerobic or anaerobic conditions (Esteve-Núñez and Ramos, 1998). This reaction seems to be a process in which electrons are diverted to the reduction of TNT without energy conservation. In this report we have identified the cytoplasmic PnrA protein of the TNT-degrading strain *P. putida* JLR11 as a nitroreductase able to transfer four electrons to TNT and other nitroaromatic compounds to produce the corresponding hydroxylamino derivatives.

Results

Identification of a soluble nitroreductase activity in cell-free extracts of *P. putida* JLR11 under different growth conditions

Pseudomonas putida JLR11 was grown on minimal medium with either ammonium or TNT as nitrogen source or a mixture of both under aerobiosis and in absence of oxygen. Cell-free extracts from these cultures showed a constitutive nitroreductase activity regardless of the growth conditions. Given that membrane-associated TNT reduction was only present in cells grown under anaerobiosis with TNT as electron acceptor (Esteve-Núñez, 2000), we considered that the nitroreductase activity produced under aerobic conditions could be mainly attributed to the cytoplasmic reduction of TNT. To test this hypothesis we fractionated cell-free extracts from ammonium-grown cells and from cells grown in presence of TNT cultured under aerobic or anoxic conditions. We found that cells showed a similar TNT-reductase activity associated with the cytoplasmic fraction regardless of the culture conditions (Fig. 1).

Cloning of the *P. putida* JLR11 TNT-nitroreductase genes

Random DNA sequences of *P. putida* JLR11 revealed >98% identity when compared with the corresponding

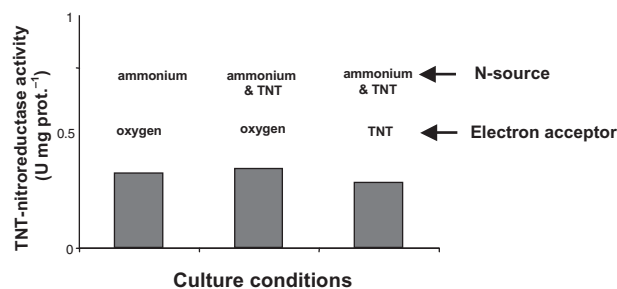


Fig. 1. Cytoplasmic TNT-nitroreductase activity and *pnrA* gene expression in the strain JLR11 grown with different nitrogen sources.

sequence in *P. putida* KT2440 (A. Caballero and J.L. Ramos, unpublished results). Two sequences of known nitroreductases from *E. coli*, *nfsA* and *nfsB* (Zenno *et al.*, 1996a,b), were used to find similar genes in the sequenced genome of *P. putida* KT2440 (Nelson *et al.*, 2002) using BLASTP programs. Two open reading frames (ORFs) from the *Pseudomonas* genome matched the ones in *E. coli*, and were called *pnrA* and *pnrB* (*P*seudomonas *N*itro*R*eductase A and B) according to the amino acid identity of the translated products with *NfsA* (30%) and *NfsB* (52%) respectively. The sequence of the *pnrA* and *pnrB* genes of *P. putida* KT2440 was used to design primers to amplify, by polymerase chain reaction (PCR), the *pnrA* and *pnrB* genes of strain JLR11 as described in the *Experimental procedures*. *Escherichia coli* DH5 α bearing pNAJ and pNBj was grown on Luria-Bertani (LB) medium, and cell-free extracts were prepared to measure the nitroreductase activity associated with the cloned genes using the following substrates: 2,4,6-trinitrotoluene, 2,4-dinitrotoluene, 4-nitrotoluene and 4-nitrobenzoate (Fig. 2). PnrB did show negligible activity over the nitroaromatic assayed. Cell-free extract from *E. coli* DH5 α bearing the pUC19 plasmid was used as control for NADPH-dependent TNT-nitroreductase activity, showing a specific activity lower than 10 nmoles NADPH oxidized per milligram of protein per minute. In contrast, we found high, NADPH-dependent TNT-nitroreductase activity associated with the *pnrA* gene product (the specific activity was 520 nmoles NADPH oxidized per milligram of protein per minute) but not with the *pnrB* gene (less than 20 nmoles NADPH oxidized per milligram of protein per minute). Therefore we decided to concentrate on characterizing the enzyme made by the *pnrA* gene product.

The *pnrA* and *pnrB* genes are expressed constitutively in *P. putida* JLR11

The enzymatic analysis carried out with cytoplasmic fraction revealed that TNT reduction was constitutive. If the *pnrA* gene product was responsible for this activity, one would also expect its expression to be constitutive.

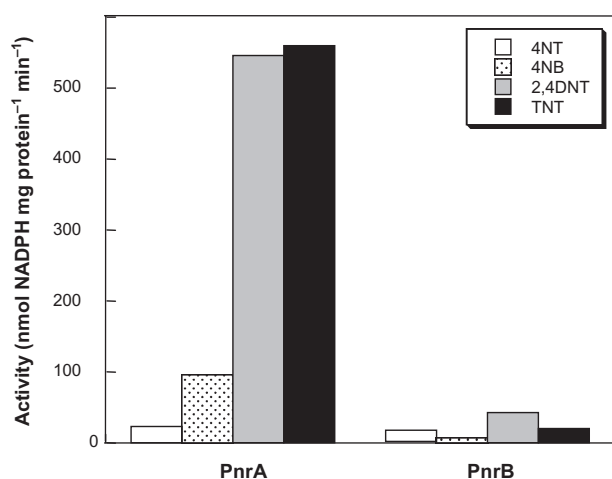


Fig. 2. Nitroaromatic substrates profile for PnrA and PnrB. *Escherichia coli* DH5 α bearing *pnrA* and *pnrB* were grown on LB medium, and nitroreductase activity associated with the cloned genes was measured for the following substrates: 2,4,6-trinitrotoluene (TNT), 2,4-dinitrotoluene (2,4DNT), 4-nitrotoluene (4NT) and 4-nitrobenzoate (4NB).

Pseudomonas putida JLR11 cells were grown on minimal medium with different nitrogen sources (ammonium, TNT and ammonium plus TNT), and total RNA extracted to determine the relative amount of the *pnrA* and *pnrB* mRNA. The results obtained are shown in Table 1. The level of expression of both genes was similar regardless of the N-source used for growth.

Purification and kinetic properties of PnrA

The *pnrA* gene encodes a 275-amino-acid polypeptide with an apparent mass of 28 kDa. The PnrA protein was purified to homogeneity from extracts of *E. coli* DH5 α (pNAJ), as described in *Experimental procedures*. The purification procedure yielded 36% of the initial enzymatic activity and regularly produced about 0.05 mg PnrA per litre of culture of homogeneous protein, which was purified about 366-fold (Table 2).

The molecular weight of PnrA was estimated by SDS-PAGE to be 28 kDa. The mass of the native protein was

estimated by gel filtration to be 57 kDa. Therefore the PnrA enzyme is active as a homodimer.

Solutions of the PnrA nitroreductase were yellow, and the spectrum obtained for the protein was consistent with that of a flavoprotein. The flavin cofactor was subsequently characterized by high-performance liquid chromatography (HPLC) and identified as flavine mononucleotide (FMN) (Fig. 3).

The molar ratio of NADPH to reduced TNT was 1.9, consistent with the 4-hydroxylamieno-2,6-dinitrotoluene detected as product of TNT reduction and occurred in a pH range between 6.5 and 8.5, with optimal pH at 7.2 and optimal temperature at 25°C. The K_m for NADPH was $20 \pm 2 \mu\text{M}$.

We determined the V_{max} and K_m values of PnrA for a serie of mono- and dinitrotoluenes, 2,4,6-trinitrotoluene, 3- and 4-nitrobenzoate, 3,5-dinitroaniline and 3,5-dinitrobenzamide (Table 3). The highest V_{max}/K_m value was obtained with TNT, which indicates that of all the nitroaromatic compounds we assayed, this was the best substrate for the enzyme. The V_{max}/K_m values for 2,4-DNT and 3,5-dinitrobenzamide were similar to those found for TNT, suggesting that these two compounds are also good substrates for the enzyme. In contrast with the results with 2,4-DNT, those obtained with 2,6-DNT revealed that it was a poor substrate. Mononitrotoluenes and nitrobenzoates were also poor substrates for the enzyme. The enzyme's preference for the reduction of nitro groups at the *para* position is in agreement with the more negative charge of

Table 1. Expression of *pnrA* and *pnrB* genes via RT-PCR.

Source of nitrogen	Level of expression	
	<i>pnrA</i>	<i>pnrB</i>
Ammonium	100%	100%
Ammonium + TNT	96%	79%
TNT	126%	64%

Values are the mean of three replicates and show the level of expression relative to the gene expression in M9 media with ammonium as sole nitrogen source.

Table 2. Purification yield of PnrA *E. coli* DH5 α cells with pNAJ.

Purification step	Vol (ml)	Total activity (U)	Total protein (mg)	Specific activity (U mg prot. ⁻¹)	Yield (%)	Purification factor
Crude extract	50	436	700	0.62	100	1.0
Cytopl. fraction	45	483	594	0.81	110	1.25
Ammonium sulfate 30–90% (dialysed)	70	400	420	0.95	92	1.53
Hiprep XL chromatography	50	296	65	4.56	68	7.36
Phenyl sepharose	15	230	11.4	20.25	53	32.66
Red sepharose	15	102	0.4	226.66	36	365.58

One unit of nitroreductase activity corresponded to the oxidation of 1 μmol NADPH per minute.

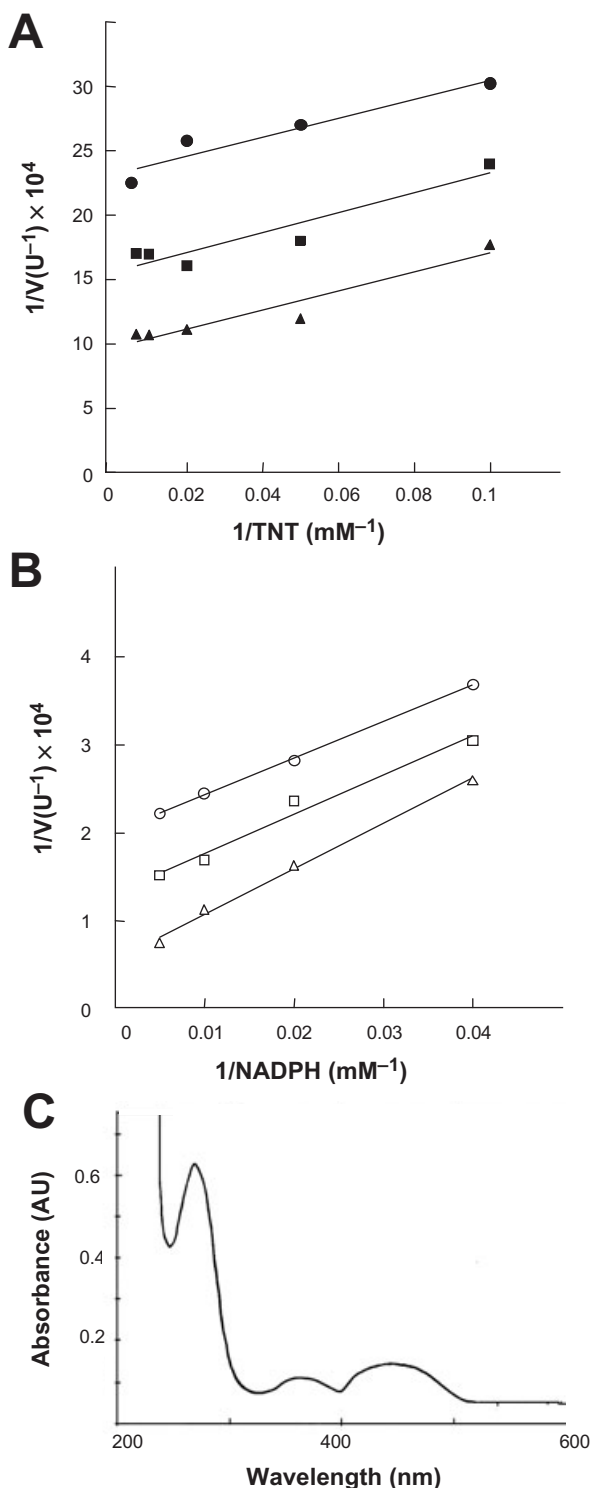


Fig. 3. Double-reciprocal plots of initial velocity versus concentrations of TNT (A) or NADPH (B). NADPH-TNT nitroreductase activity was assayed with constant concentrations of NADPH (25 μM , open triangles; 50 μM , open squares; 100 μM , open circles) or TNT (5 μM , filled circles; 10 μM , filled squares, 50 μM , filled triangles). C. Spectrum of the homogenous PnrA protein. The PnrA spectrum has peaks at 445, 370, and 270 nm, whereas peaks of free FMN are at 448, 375, and 224 nm.

Table 3. Kinetic constants for purified PnrA nitroreductase.

Substrate	K_m (μM)	V_{max} (U mg^{-1})	V_{max}/K_m
2,4,6-trinitrotoluene	5	6400	1280
2,4-dinitrotoluene	4	3500	875
3,5-dinitrotoluene	10	3800	380
2,6-dinitrotoluene	500	3150	6
2-nitrotoluene	No activity	No activity	Not applicable
2-nitrobenzoate	No activity	No activity	Not applicable
3-nitrotoluene	30	700	20
3-nitrobenzoate	30	2800	90
4-nitrotoluene	No activity	No activity	Not applicable
4-nitrobenzoate	20	3400	170
3,5-dinitrobenzamide	7	6500	930
3,5-dinitroaniline	9	5400	600

Values are the average of four independent assays. Standard deviations were below 10% of the given values.

the nitro group, as reported by Barrows and colleagues (1996).

Double-reciprocal plots of initial TNT reduction velocity versus NADPH concentrations showed parallel lines (Fig. 3), which suggests that the catalysis mode of PnrA TNT-nitroreductase is a ping-pong bi-bi mechanism, as described for other nitroreductases (Bryant and DeLuca, 1991; Zenno *et al.*, 1996a,b).

Discussion

Comparative analysis of PnrA with other nitroreductases

The PnrA nitroreductase of *P. putida* strain JLR11 is a flavoprotein that reduces TNT to 4-hydroxylamino-2,6-dinitrotoluene. This enzyme exhibits a number of similarities and differences with other nitroaromatic reductases, which explains its substrate specificity with nitroarenes. PnrA is a homodimer made of two 28 kDa subunits. Nitroreductase enzymes in the range of 55–60 kDa have been reported for other microorganisms (Bryant *et al.*, 1981; Bryant and DeLuca, 1991; Zenno *et al.*, 1996a,b). The phylogenetic analysis suggested PnrA to be close to the NfsA/Frp nitroreductase subgroup (Fig. 4A). To better understand the substrate range of PnrA we aligned the amino acid sequences of the *P. putida* strain JLR11 PnrA protein with the most similar nitroreductases such as NfsA from *E. coli*, Frp from *V. harveyi* and SnrA from *S. enterica* (Fig. 4B). The degree of identity with these proteins was around 20% and the degree of similarity was around 36%. The alignment revealed a number of well-conserved residues in all four proteins along the sequence, in consonance with their common activity. The crystal structure of NfsA and Frp defined them as homodimeric globular proteins (Tanna *et al.*, 1996; Kobori *et al.*, 2001; Haynes *et al.*, 2002).

Nitroreductase has three conserved domains for interaction with the electron donor, the cofactor, and the nitroaromatic compounds that function as substrates for

arginine, lysine and histidine, are involved in the recognition of the 2'-phosphate group of NADPH through a hydrogen bond (Wilson *et al.*, 1992; Thorn *et al.*, 1995), so we conclude that His203 in PnrA could play the same role that Arg203 does in the rest of the nitroreductases.

Comparison of active-site residues involved in the binding of nitroaromatic compounds in each of the chains revealed that those located in one side of the NsfA-active centre (Arg15, Ser39, Ser40, Gln67, Arg133, Lys167 and Arg225) were well conserved among the aligned proteins. In contrast, residues in the other side of the active centre (Tyr199, Tyr200, Thr219, Lys222 and Ser224) were not conserved in the aligned sequences. It is therefore tempting to propose that the difference in the profile of those protein substrates could be caused by the different residues on respective sides of the active site. This could explain why 3- and 4-nitrobenzoate are substrates for PnrA but not for NfsA (Zenno *et al.*, 1996a).

The physiological role of the nitroreductases described above as similar to PnrA is unknown, but not necessarily associated with the reduction of these compounds *in vivo*, although their ubiquity in many different microorganisms suggests that they might share a common role in, for example, physiological redox sensing or detoxification. Recently the promoter region of the *nfsA* gene was identified and characterized in terms of its response to the superoxide-generating compound paraquat, and classified as a class I SoxS-dependent promoter (Liochev *et al.*, 1999; Paterson *et al.*, 2002). Our analysis of the genome of *P. putida* revealed that the strain does not contain a homologue to the *soxS* gene, and whether or not the constitutive expression of *pnrA* can be modified upon oxidative stress is unknown.

Our *in vitro* results showed that PnrA uses four electrons to reduce TNT and other nitroaromatic compounds, and that the resulting products are the corresponding hydroxylamino derivatives. Cell-free extract assays with 2,4-dinitrotoluene and 4-nitrobenzoate also resulted in the production of the corresponding hydroxylamino derivatives, which supports that PnrA could play an important role in the initial cytosolic reduction of nitroaromatic compounds.

The partial reduction of nitroaromatic compounds to hydroxylamino derivatives has been identified as a key reaction in productive catabolic routes of some mononitroaromatic as 4-nitrobenzoate or nitrobenzene (Groenewegen and de Bont, 1992; Nishino and Spain, 1993) and have been also suggested to participate in the nitrogen metabolism of TNT (Fiorella and Spain, 1997; Hughes *et al.*, 1998; Vorbeck *et al.*, 1998). More recently, we have used mutants in the nitrogen metabolism of TNT to show that JLR11 exerts different modes of attack on the TNT ring, resulting in the release of nitrogen as nitrite or ammonium (Caballero *et al.*, 2005). These results can be

explained by a partial reduction of the nitro group, a function that could be performed by PnrA. However, microbial nitroreduction is better known as a mechanism that converts the original nitro compounds in reactive molecules able to cause chemical mutagenesis and carcinogenesis (Homma-Takeda *et al.*, 2002; Padda *et al.*, 2003; Sarlauskas *et al.*, 2004). The mutagenic properties of some of these hydroxylaminoaromatic compounds are currently applied in anticancer treatments, with the use of bacterial nitroreductases as effective tools for the *in situ* production of the active drugs. The most widely studied prodrug used for gene-directed enzymatic prodrugs therapies (GDEPT) is CB1954, a dinitrobenzamide derivative that is a substrate activated by *E. coli* nitroreductases to generate a difunctional agent, which forms DNA-DNA interstrand cross-links and inhibits the tumours transfected with the bacterial gene (Grove *et al.*, 2003; Green *et al.*, 2004). According with the V_{\max}/K_m values for the different nitroaromatic compounds we have tested, dinitrobenzamide resulted to be the best substrate for PnrA after TNT, which suggests a potential use of this enzyme in this kind of gene-directed therapies.

A more recent application of these enzymes is the phytodetoxification of TNT by transgenic tobacco plants expressing a *En. cloacae* nitroreductase. The plants show a striking increase in ability to tolerate, take up and accumulate TNT-derivatives (Hannink *et al.*, 2001). However, the TNT-polluted soils are also contaminated with other related nitroaromatics generated in the process of manufacture, use or disposal of the explosive (Harterter, 1985). Thus, the wide profile of nitroaromatic substrates shown by PnrA makes this enzyme a suitable candidate for making transgenic plants to phytoremediate environments polluted with mixtures of nitroaromatic compounds.

Therefore, the study and characterization of enzymes that reduce nitro groups to hydroxylamino is relevant to complete our understanding of nitroaromatic metabolism in order to minimize the toxicity of this compound in the environment, as well as maximize its curative properties in chemotherapy treatments.

Experimental procedures

Organisms, culture media and growth conditions

Pseudomonas putida strain JLR11 was grown on M9 minimal medium with glucose (0.1–0.5% wt/vol) as a C-source (Esteve-Núñez and Ramos, 1998). This strain grows on minimal medium in the presence of 50 $\mu\text{g ml}^{-1}$ kanamycin (Km). When TNT or nitrate were used as the sole N-source, they were supplied at 100 mg l^{-1} or 10 mM, respectively, and ammonium was omitted from the M9 minimal medium. The strain was grown at 37°C in LB medium with 100 $\mu\text{g ml}^{-1}$ ampicillin (Ap) when required. Protein was measured as described by Bradford (1976).

Analytical methods

Products resulting from TNT reduction were analysed after removing the proteins with 10% (v/v) trichloroacetic acid. The protein-free supernatants were then analysed by HPLC on a Hewlett-Packard model 1050 chromatograph equipped with a diode-array detector and a 5 µm C18RP column (UltraCarb C30 Phenomenex; 15 cm by 4.6 mm) as described (Esteve-Núñez and Ramos, 1998).

Cell-free extracts and enzyme assays

Pseudomonas putida JLR11 was grown on M9 minimal medium with ammonium or TNT as the sole N-source. Cells were harvested by centrifugation (5000 g, 10 min), washed once in 50 mM phosphate buffer (pH 7.0), re-suspended in 5 ml of the same buffer and disrupted in a French press at 120 Mpa. Cell debris was removed by centrifugation (10 000 g, 10 min) and the clear supernatant was used as a source of cell-free extract. For fractionation the cell-free extract was centrifuged at 90 000 g for 60 min. PnrA activity was assayed at 25°C in 50 mM phosphate buffer (pH 7.0), supplied with 0.3 mM NADPH (or NADH), 0.1 mM of a given electron acceptor, and a suitable amount of enzyme. The rate of the reaction was monitored based on the oxidation of NADPH at 340 nm ($\epsilon = 6.3 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$). Protein concentration was determined with the Bradford protein assay, using bovine seroalbumin as the standard.

Nucleid acid methods

Plasmids were isolated with a Quiagen kit. Plasmid DNA was sequenced on both strands with universal, reverse or specifically designed primers on an automatic DNA sequencer (ABI-PRISM 310; Applied Biosystems).

The primer sequences used to amplify by PCR the *pnrA* gene from *P. putida* JLR11 were 5'-ATTCTAGAGTTATGAGCGTATATCTCGG-3' (designed with a *Xba*I site, underlined) and 5'-AAGGATCCGCCACTGGAACCTTGAGTGTC-3' (designed with a *Bam*HI site, underlined). The primers used to amplify the *pnrB* gene were 5'-GCTCTAGAACAGTTCGAGGTCCGGTGAGC-3' (designed with a *Xba*I site, underlined) and 5'-AAGGATCCTGTGGATTTCGAAATCGCTG-3' (designed with a *Bam*HI site, underlined). Polymerase chain reaction products were separated in horizontal agarose gels, recovered from the gels, digested with *Xba*I and *Bam*HI and cloned into pUC19 digested with *Xba*I and *Bam*HI. The resulting plasmids were called pNAJ and pNBj for plasmids bearing the *pnrA* and *pnrB* genes respectively. The phylogenetic tree was generated with the PAUP program using a neighbour-joining method.

Real time PCR was performed to analyse the expression of *pnrA* and *pnrB*. For internal constitutive control rRNA 16S was also analysed. *Pseudomonas putida* JLR11 was grown in M9 minimal medium with glucose (0.5% w/v) as carbon source. Cells were collected in exponential phase by centrifugation, washed twice with phosphate buffer 50 mM pH 7.4 and transferred at an OD_{660 nm} of 0.5 to three different media: M9, M9 plus TNT and M8 with TNT as sole N-source. Glucose (0.5% w/v) was used as carbon source in all the media. After 1 h, 10 ml of each culture was extracted and total RNA was

isolated using TRI REAGENT® LS kit from Molecular Research Center. Construction of cDNA for real time PCR was performed by retrotranscription of the RNA using specific primers for *pnrA*: pnrART3 (5'-CTGAAACCGCCTCTGCCTCG-3'); *pnrB*: pnrB3 (5'-CCCACGCTCACGCAAACCC-3) and for rRNA 16S: rRNA16SRev (5'-GAAATTCACCACCTCTACC-3) and reverse transcriptase AMV from Roche Applied Biosciences and 1 µg of total RNA for every reaction. For real time PCR reaction, 1 µl of cDNA was mixed with iQ™ SYBR® Green Supermix from Bio-Rad, and 200 nM of every primer. For *pnrA*: PnrART52 (5'-AGCGATGTCCGAGGAGCTGGC-3) plus PnrART3 (see above); for *pnrB*: PnrB52 (5'-GTACTGCCTTGCTGGGTGCG-3) plus PnrB3 (see above); for rRNA 16S: rRNA16SFwd (5'-AAAGCCTGATCCAGCGAT-3) plus rRNA16SRev (see above). In the case of rRNA 16S a dilution 1/1000 of cDNA was utilized for real time amplification. The program for amplification was 7 min at 95°C, followed by 40 cycles of 15 s at 95°C, 30 s at 61°C and 20 s at 72°C. A melting curve was analysed in order to check that no unspecific fragments were amplified. Every gene was amplified in three different samples, and the real time reaction was repeated three times. For statistical treatment, a protocol described by Livak and Schmittgen (2001) was used.

Overexpression of the *pnrA* gene product in *E. coli* cells and purification of PnrA

The purification procedure is based on that described by Zenno and colleagues (1996a). An aliquot (10 ml) of an overnight LB culture of *E. coli* DH5α bearing pNAJ was added to 1 l of LB supplemented with 10 mg l⁻¹ Ap. The culture was incubated with shaking at 37°C for 12 h. Cells were harvested and cell-free extracts were prepared as described above. The crude extract was then centrifuged at 90 000 g for 60 min to obtain the cytosolic fraction, which was used to purify the enzyme. The crude extract was first fractionated with ammonium sulfate, and it was found that around 90% of the activity was precipitated between 30% and 90% saturation with (NH₄)₂SO₄. The precipitated proteins were re-suspended in 50 mM phosphate buffer (pH 7.0) and dialysed against the same buffer for 24 h. The desalted extract was applied through a superloop to a HiPrep Q XL anion-exchange column (Pharmacia) equilibrated with 50 mM phosphate buffer (pH 7.0), washed with 200 ml of the starting buffer (5 ml min⁻¹) and eluted with a 0 to 1 M linear gradient of KCl in 50 mM phosphate buffer (pH 7.0). Peak fractions with NADPH-TNT reductase activity, which eluted around 0.3 mM KCl, were pooled, and ammonium sulfate was added to reach a concentration of 1.7 M. The resulting mixture was loaded onto a Phenyl-Sepharose hydrophobic interaction column (Pharmacia), previously equilibrated with 50 mM phosphate buffer (pH 7.0) containing 1.7 M ammonium sulfate. The column was washed with 30 ml of the latter buffer at a flow rate of 0.5 ml min⁻¹, and eluted with a 1.7 to 0 M linear gradient of ammonium sulfate. Fractions with enzyme activity were pooled and dialysed against 50 mM phosphate buffer (pH 7.0). The sample was then applied onto a Red-Sepharose (Pharmacia) affinity chromatography column equilibrated with 50 mM phosphate buffer (pH 7.0), and the nitroreductase activity was eluted with a 0 to 3 M linear gradient of NaCl at 1 ml min⁻¹. After pooled peak fractions were

concentrated with a Centricon membrane, PnrA was purified to homogeneity by Superose 12 gel filtration, eluted with a 50 mM phosphate buffer (pH 7.0) supplemented with 150 mM NaCl at 0.2 ml min⁻¹, and then purified PnrA was pooled, disalted and stored at -20°C for further analysis.

Nucleotide sequence accession number

The sequences of the *pnrA* and *pnrB* genes were deposited at GenBank under accession numbers Q93AS3 and Q8KRI2 respectively.

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