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 as 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 (Hannink 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; Angermair 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 fisheri (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 ruminatum (Anderson et al., 2002).

The most widely used nitroaromatic compound is 2,4,6-trinitrotoluene (TNT) (Hartter, 1985). The recalcitrance of
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 oxygenlytic 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; Haidour 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 which electrons are diverted to the reduction of TNT with-

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 (Pseudomonas NitroReductase 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 DH5α 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 not show negligible activity over the nitroaromatic assayed. Cell-free extract from E. coli DH5α 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.
PnrA, a nitroreductase-family enzyme

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 DH5α(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-hydroxylamino-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<sub>m</sub> for NADPH was 20 ± 2 µM.

We determined the V<sub>max</sub> and K<sub>m</sub> values of PnrA for a series of mono- and dinitrotoluenes, 2,4,6-trinitrotoluene, 3- and 4-nitrobenzoate, 3,5-dinitroaniline and 3,5-dinitrobenzamide (Table 3). The highest V<sub>max</sub>/K<sub>m</sub> 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<sub>max</sub>/K<sub>m</sub> 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

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

<table>
<thead>
<tr>
<th>Source of nitrogen</th>
<th>pnrA Level of expression</th>
<th>pnrB Level of expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Ammonium + TNT</td>
<td>96%</td>
<td>79%</td>
</tr>
<tr>
<td>TNT</td>
<td>126%</td>
<td>64%</td>
</tr>
</tbody>
</table>

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.

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**Table 2. Purification yield of PnrA E. coli DH5α cells with pNAJ.**

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

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

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

### Table 3. Kinetic constants for purified PnrA nitroreductase.

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

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

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.
the enzyme. The binding site for the FMN cofactor in NsfA involves residues Arg15, Ser39, Gln67, Tyr128, Gly130, Gly131 and Lys167. All these residues are well conserved in the corresponding alignment of PnrA with NsfA. This is in agreement with our identification of FMN as a cofactor for PnrA.

Our results show that NADPH and not NADH was the preferred electron donor for PnrA. The use of NAPDH as electron donor is a common feature in this subgroup and the presence of critical residues have been analysed. The key role of Arg203 in NADPH binding of NsfA was established in a mutant in which Arg was replaced by Ala. This resulted in a mutant that had a Km value for NADPH, which was 33-fold higher than that of the wild-type enzyme (Kobori et al., 2001). Arg203 is conserved in all nitroreductases aligned in Fig. 4 except for PnrA, which showed a histidine residue. However, it has been previously reported that positively charged residues, such as...
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, Gin67, 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 class I SoxS-dependent promoter (Liochev et al., 1996b). This 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 to the $V_{\text{max}}/K_m$ 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 photodetoxification 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 (Hartter, 1985). Thus, the wide profile of nitroaromatic substrates shown by PnrA makes this enzyme a suitable candidate for making transgenic plants to phyto remediate 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-Ñuñez and Ramos, 1998). This strain grows on minimal medium in the presence of 50 μg 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 μg 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 (5000 g, 10 min), washed with 30 ml of the latter buffer at a flow rate of 1 cm s⁻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 dialysed against 50 mM phosphate buffer (pH 7.0), equilibrated with 50 mM phosphate buffer (pH 7.0), and the desalted extract was applied 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 (ε = 6.3 × 10³ M⁻¹ cm⁻¹). Protein concentration was determined with the Bradford protein assay, using bovine serum albumin as the standard.

Nucleic 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'-ATTCTAGAGTTATGAGCGTATATCGGGAAGGAGCT (designed with a XbaI site, underlined) and 5'-AAGGATCCGACCCTGGAACCTTGAGTGTCG (designed with a BamHI site, underlined). The primers used to amplify the *pnrB* gene were 5'-GCTCTAGAGGATTCTGAGGCTGAGC (designed with a XbaI site, underlined) and 5'-AAGGATCCGAGAATTCTGCAAAATCCTGGC (designed with a BamHI site, underlined). Polymerase chain reaction products were separated in horizontal agarose gels, recovered from the gels, digested with XbaI and BamHI and cloned into pUC19 digested with XbaI and BamHI. 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 OD₆₅₀ₐₚ 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'-CTGAAACCCTGGCTTG-3'); *pnrB*: pnrB3 (5'-CCACGCAGCTCAAGCAGGACTG-3') and for rRNA 16S: rRNA16SRev (5'-GAAAATCCACACGCTCTACCC-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 iTM SYBR® Green Supermix from Bio-Rad, and 200 nM of every primer. For *pnrA*: PnrART52 (5'-AGCGATGTCGAGGACTTGCG-3') plus PnrART3 (see above); for *pnrB*: PnrB52 (5'-GTACTGCTTGGCTGGTGC-3') plus PnrB3 (see above); for rRNA 16S: rRNA16SFWd (5'-AAACCTGTACCGG GAT-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 Zennino and colleagues (1996a). An aliquot (10 ml) of an over-night 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) 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) 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) 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) 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) and dialysed against the same buffer for 24 h.
concentrated with a Centricron membrane, PnrA was purified to homogeneity by Superoxide 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.

References


