Exopolyphosphatase of *Pseudomonas aeruginosa* is essential for the production of virulence factors, and its expression is controlled by NtrC and PhoB acting at two interspaced promoters

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The exopolyphosphatase (Ppx) of *Pseudomonas aeruginosa* is encoded by the PA5241 gene (*ppx*). Ppx catalyses the hydrolysis of inorganic polyphosphates to orthophosphate (P<sub>i</sub>). In the present work, we identified and characterized the promoter region of *ppx* and its regulation under environmental stress conditions. The role of Ppx in the production of several virulence factors was demonstrated through studies performed on a *ppx* null mutant. We found that *ppx* is under the control of two interspaced promoters, dually regulated by nitrogen and phosphate limitation. Under nitrogen-limiting conditions, its expression was controlled from a σ<sup>54</sup>-dependent promoter activated by the response regulator NtrC. However, under P<sub>i</sub> limitation, the expression was controlled from a σ<sup>70</sup> promoter, activated by PhoB. Results obtained from the *ppx* null mutant demonstrated that Ppx is involved in the production of virulence factors associated with both acute infection (e.g. motility-promoting factors, blue/green pigment production, C6–C12 quorum-sensing homoserine lactones) and chronic infection (e.g. rhamnolipids, biofilm formation).

Molecular and physiological approaches used in this study indicated that *P. aeruginosa* maintains consistently proper levels of Ppx regardless of environmental conditions. The precise control of *ppx* expression appeared to be essential for the survival of *P. aeruginosa* and the occurrence of either acute or chronic infection in the host.

**INTRODUCTION**

Inorganic polyphosphates (polyPs) are linear polymers consisting of tens to hundreds of orthophosphate (P<sub>i</sub>) residues linked to energy-rich phosphoanhydride bonds. There are numerous reports indicating that polyP is essential for the growth of micro-organisms, their responses to stresses and other environmental factors, and the virulence of pathogens (reviewed by Rao *et al.*, 2009). PolyP is synthesized by polyphosphate kinases (Ppks) that catalyse the reversible transfer of the terminal phosphate (γ) of ATP to the polyP chain (Kornberg *et al.*, 1999). The polymer can be hydrolysed by the exopolyphosphatase (Ppx) that cleaves processively P<sub>i</sub> residues from the termini of the polyP chain (Akiyama *et al.*, 1993).

*Pseudomonas aeruginosa* is a highly versatile motile organism that survives in a wide variety of environments, and causes diseases in insects, plants and animals, including humans. PolyPs and Ppks are clearly related to the virulence of *P. aeruginosa* and other pathogens as both are essential for swimming, swarming and twitching motilities, biofilm development, and quorum sensing (Rashid & Kornberg, 2000; Rashid *et al.*, 2000a, b). PolyP and Ppks, in particular, are also involved in the adaptation of micro-organisms to changes in their surroundings, such as phosphate (P<sub>i</sub>) deficiency or nitrogen starvation. In various bacteria, including numerous pathogens, the *ppk* gene is part of the Pho regulon and is upregulated in response to a low external P<sub>i</sub> concentration (Kato *et al.*, 1993; Geissdörfer *et al.*, 1998; Ault-Riché *et al.*, 1998; Rao *et al.*, 1998; Kornberg *et al.*, 1999; Lee *et al.*, 2000).

**Abbreviations:** AHL, N-acyl homoserine lactone; Cho, choline; DR, direct repeat sequences; IHF, integration host factor; polyP, inorganic polyphosphate; Ppk, polyphosphate kinase; Ppx, exopolyphosphatase; RACE, rapid amplification of cDNA ends; S, succinate; TSS, transcription start site.

Two supplementary figures and one supplementary table are available with the online version of this paper.

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2006; Silby et al., 2009). It was also reported that in Escherichia coli, under nitrogen-limiting conditions, ppk expression was activated by the NtrC two-component response regulator (Ault-Riché et al., 1998).

Despite the large amount of literature available on Ppk, little is known about the role of Ppx in the physiology of harmless or pathogenic bacteria. It has been reported that Ppx is essential for the pathogenesis of Mycobacterium tuberculosis (Thayil et al., 2011), Bacillus cereus (Shi et al., 2004) and Neisseria meningitidis (Zhang et al., 2010). It was suggested that Ppx may be involved in type III secretion system of P. aeruginosa (Dacheux et al., 2002).

Choline (Cho) is an essential nutrient in eukaryotes and it is a compound readily available to bacteria during infections. Our previous studies on the enzymes related to Cho metabolism in P. aeruginosa indicated that this quaternary ammonium compound may be considered a factor that promotes pathogenesis in this opportunistic bacterium (Lisa et al., 1994, 2007; Beassoni et al., 2008; Massimelli et al., 2011; Sánchez et al., 2012). Recently, we also demonstrated that Cho metabolism is controlled by the intracellular balance between carbon and nitrogen, and consequently regulated by the global regulators NtrC and CbrB (Massimelli et al., 2011). Preliminary studies carried out in our laboratory suggested that Cho may play a role in the intracellular accumulation of polyP. All these findings led us to study Ppx at the molecular level and to determine if it is involved in P. aeruginosa pathogenesis, as well as the relationship of ppx expression with Cho, a nitrogen-limited source and P_i deprivation conditions.

Here, we have provided evidence that Ppx of P. aeruginosa is required for flagellar-dependent swimming and swarm motility, and for the production of certain virulence factors such as biofilm, rhamnolipids, pyocyanin and pyoverdine, and the quorum-sensing C6–C12 N-acyl homoserine lactones (AHLs). We also demonstrated that ppx expression is mediated by both σ24- and σ70-dependent promoters, activated by NtrC under nitrogen limitation and by PhoB under conditions of low P_i availability, respectively. Our results highlighted the contribution of Ppx in the maintenance of intracellular levels of polyP in P. aeruginosa.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. P. aeruginosa strain PAO1 and its derivatives were grown in Luria broth (LB) medium (Sambrook & Russell, 2001) or high P_i basal salt medium (HPi-BSM) (Lisa et al., 1994). The low-P_i medium described previously (Lucchesi et al., 1989) was modified by adding no exogenous P_i and termed BSM(−P_i). P_i (as NaPO_4) was added to final concentrations of 0.1, 0.2, 0.5 and 5.0 mM, respectively. When necessary, Carbon and nitrogen sources were added to a final concentration of 20 mM. E. coli strain XL10-Gold (Stratagene) was used for plasmid maintenance and E. coli strain BL21-CodonPlus (Stratagene) was used to overexpress NtrC(S161F). All E. coli strains were grown in LB medium containing 150 µg ampicillin ml⁻¹. Liquid cultures were incubated at 37 °C with shaking. The primers UpF-ppx-Gw, UpR-ppx-Gm, DwnF-ppx-Gm, DwnR-ppx-Gw and UpF-phoB-Gw, UpR-phoB-Gm, DwnF-phoB-Gm, DwnR-phoB-Gw (Table S1, available in Microbiology Online) were used to construct Appx and ΔphoB strains (Choi & Schweizer, 2005), respectively. To achieve complementation of the Appx strain, the ppx gene was amplified by PCR using the primers P1(500bp) and ppx-Dwn (Table S1). The obtained amplicon was cloned into pUC18-mini-Tn7-Gm using the restriction enzymes SphI and SacI. This plasmid, pUC18-pxpC, was inserted into the bacterial chromosome as described by Choi & Schweizer (2006) and Choi et al. (2006).

**Biofilm assay.** Biofilm formation capacity was determined macroscopically (Nieves et al., 2012). Briefly, glass tubes were inoculated with 800 µl LB medium (OD_600 0.5) and incubated with shaking for 24 h at 37 °C. Cells were removed, and the tubes were washed three times with saline solution, stained with crystal violet 0.1 % (w/v) for 15 min and rinsed to remove excess dye. Biofilm formation was quantified by solubilization of crystal violet with 1 ml ethanol 95 % (v/v) for 20 min and posterior measurement of absorbance at OD_570.

**Motility assay.** LB medium plates containing agar 0.3 or 0.5 % (w/v) were used for swimming and swarming assays, respectively. The plates were point-inoculated with an LB overnight culture with a sterile toothpick and incubated at 37 °C for 24 h. Motility was assessed by measuring the diameter of the zones formed by bacterial cells migrating away from the inoculation point.

**Quorum-sensing assay.** Agrobacterium tumefaciens strain NTL4 (pZLR4) was used to detect AHLs with long acyl chains (C6–C12). This strain carries the plasmid pZLR4, which contains the atrC::lacZ fusion and traR (Chu et al., 1998). A positive result was defined as the presence of a blue halo around a colony indicative of hydrolysis of X-Gal.

**Pyocyanin and pyoverdine production assays.** Cells were grown in LB broth for 24 h at 37 °C with maximum aeration. The levels of pyocyanin and pyoverdine were determined in the supernatants. Pyocyanin was extracted from the supernatant by the method of Silva et al. (2010) with 3 % (v/v) glycerol and 0.6 % (w/v) NaNO_3, as carbon and nitrogen sources, respectively. Rhamnolipids were measured in the cell-free culture medium by the phenol/sulphuric acid method (Dubois et al., 1956) and quantified in terms of rhamnose concentration (mg ml⁻¹).

**β-Galactosidase activity.** The activity of this enzyme was measured as described by Miller (1972).

**DNA methodology.** Genomic and plasmid DNA isolation were performed by using commercial kits from Promega and Qiagen, respectively. Restriction enzymes and T4 ligase (Promega) were applied according to the manufacturer's instructions. DNA fragments were purified from agarose gels with a QAquick Kit (Qiagen). To ensure that no errors were introduced by the PCR or subcloning procedures, all PCR products were sequenced by Macrogen. For site-directed mutagenesis, promoter regions were mutated using the QuickChange Mutagenesis Kit (Stratagene). The primer 12a was employed for −154 A/C substitution; to determine the transcription start sites (TSSs) of the ppx gene, a modified 5' rapid amplification of cDNA ends (RACE) methodology was used as described by Mendoza-Vargas et al. (2009), using two specific primers for the ppx gene.

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Table 1. Bacteria strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Genotype/description</th>
<th>Reference/source</th>
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<tr>
<td>Strains Escherichia coli</td>
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<tr>
<td>XL10-Gold</td>
<td>Tet&lt;sup&gt;+&lt;/sup&gt; Δ(mcrA) 183 Δ(mcrCB-hadSMR-mrr)173 endA1 supE44 thi-1 recA1</td>
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<td></td>
<td>gyrA96 relA1 lac Hte [F&lt;sup&gt;+&lt;/sup&gt; proAB lacIqZΔM15 Tn10 Tet&lt;sup&gt;+&lt;/sup&gt;] Amy Cam&lt;sup&gt;+&lt;/sup&gt;]</td>
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<td>BL21-CodonPlus(DE3)-RIPL</td>
<td>F&lt;sup&gt;+&lt;/sup&gt; ompT hadS&lt;sup&gt;+&lt;/sup&gt;Δ(mcrB) lacI&lt;sup&gt;+&lt;/sup&gt; lacZ&lt;sup&gt;+&lt;/sup&gt; 7 Tet&lt;sup&gt;+&lt;/sup&gt; gal λ(DE3) endA Hte [argU proU Cam&lt;sup&gt;+&lt;/sup&gt;] [argU ileY leuW Strep/Spec&lt;sup&gt;+&lt;/sup&gt;]</td>
<td>Stratagene</td>
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<td>Pseudomonas aeruginosa PAO1</td>
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<td>PAO1-WT</td>
<td>Protoprototic WT strain</td>
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<td>P(1–8) :: lacZ</td>
<td>PAO1 with a chromosomal integration of mini-Tn&lt;sup&gt;+&lt;/sup&gt;7 carrying the fusions P(1–8) :: lacZ</td>
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<td>PAO1 ΔntrC</td>
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<td>ΔrpoN</td>
<td>PAO1 ΔrpoN</td>
<td>Heurlier et al. (2003)</td>
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<td>Plasmids</td>
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<td>pUC18-mini-Tn&lt;sup&gt;+&lt;/sup&gt;7-Gm-lacZ</td>
<td>Gm&lt;sup&gt;+&lt;/sup&gt; mini-Tn&lt;sup&gt;+&lt;/sup&gt;7; lacZ transcriptional fusion vector</td>
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<td>Gm&lt;sup&gt;+&lt;/sup&gt; mini-Tn&lt;sup&gt;+&lt;/sup&gt;7</td>
<td>Choi &amp; Schweizer (2006)</td>
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<td>pTNS2</td>
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<td>pFLP2</td>
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<td>pEX18AmpGW</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;; gene replacement vector, compatible with Gateway system</td>
<td>Choi &amp; Schweizer (2005)</td>
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<td>pPS856</td>
<td>Gm&lt;sup&gt;+&lt;/sup&gt;; vector carrying gentamicin resistance gene</td>
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<td>pP1-8 :: lacZ</td>
<td>Gm&lt;sup&gt;+&lt;/sup&gt;, Ap&lt;sup&gt;+&lt;/sup&gt;; pUC18-mini-Tn&lt;sup&gt;+&lt;/sup&gt;7-Gm-lacZ with a Spel/Xhol fragment</td>
<td>This study</td>
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<td>pET-15b</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;, T7 promoter, multiple cloning sites, His-tag-coding sequence</td>
<td>Novagen</td>
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<td>pUC18-ppxC</td>
<td>2018 bp Spel/Sacl fragment containing the ppx gene plus</td>
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<td>500 bp upstream</td>
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ppx-tssA and ppx-tssB (Table S1). For TSS1, a polyA tail was added at the 5′-RNA end; for TSS2, both polyA and polyC were added by terminal transferase (New England BioLabs). Additionally, a double-stranded oligonucleotide was ligated at the 5′-RNA end instead of the polynucleotide tail to confirm the mapping by a different strategy.

Construction of plasmids harbouring the putative promoter region of the ppx gene. P1–P8 DNA fragments were PCR amplified from genomic DNA templates with the following forward primers: Up-2DR and Dwn-prom for P1; Up-1DR and Dwn-prom for P2; Up-prom and Dwn-prom for P3; Up-EBP and Dwn-prom for P4; Up-1DR and Dwn-12 for P5; Up-1DR and Dwn-1 for P6; Up-2DR and Dwn-1 for P7; and Up-pho and Dwn-prom for P8 (Table S1). The PCR products, P1(500 bp), P2(368 bp), P3(355 bp), P4(307 bp), P5(191 bp), P6(219 bp), P7(351 bp), and P8(149 bp), were digested and then individually ligated into pUC18-mini-Tn<sup>+</sup>7-Gm-lacZ to obtain P(1–8) :: lacZ. These plasmids and pTNS2 were cotransformed into P. aeruginosa by electroporation (Choi et al., 2006), and the resulting strains containing the integrated DNA fragments were termed P1<sub>500</sub> :: lacZ to P8<sub>149</sub> :: lacZ, respectively. Colony PCR using the primers P<sub>Tn7R</sub> and P<sub>pho-B-down</sub> was used to confirm the chromosomal Tn<sup>+</sup>7 insertions. The gentamicin marker was excised as described (Choi & Schweizer, 2005).

Overexpression and purification of P. aeruginosa mutated NtrC. The NtrC(S161F) protein from P. aeruginosa PAO1 was overexpressed and purified after generating the S161F mutation using the pET-15b :: ntrC plasmid as template and the ntrC-m1 and ntrC-m2 primers (Table S1). Following overexpression of the protein in E. coli BL21-CodonPlus, His-tagged NtrC(S161F) was purified according to the manufacturer’s protocol (Qiagen). The purity was estimated visually through SDS-PAGE.

DNA-binding studies. The P2(368) and P4(307) DNA fragments obtained by PCR were used in protein-DNA binding assays. The DNA fragments were incubated independently with 4 μg His6-NtrC(S161F) in 50 mM Tris/HCl (pH 8.0), 750 mM KCl, 2.5 mM EDTA, 0.5% (v/v) Triton X-100, 1 mM DTT and 4% (v/v) glycerol for 20 min at 28 °C as described by Leech et al. (2008).
Electrophoretic mobility shift assays were conducted using a 5 % non-denaturing polyacrylamide gel in Tris/HCl (pH 8.0) as upper phase and sodium acetate buffer (pH5.0) as lower phase. Tris/borate-EDTA (pH 8.0) was used as running buffer at 200 V for 2 h at 4 °C.

**Bioinformatic analysis.** The Promscan.pl Perl script (Studholme & Dixon, 2003) ([http://molbiol-tools.ca/promscan/](http://molbiol-tools.ca/promscan/)) was used to identify σ24-dependent promoters with a scoring matrix derived from a compilation (Barrios et al., 1999). **PRODORIC** was used to determine the integration host factor (IHF) consensus (Münch et al., 2003). The *BPROM* tool from the SoftBerry server ([http://linux1.softberry.com/](http://linux1.softberry.com/)) was used to identify σ70-dependent promoters. NtrC- and PhoB-binding consensuses were determined by using the sequences described by Hervás et al. (2008) and Shinagawa et al. (1987), respectively.

**RESULTS**

**Effects of a null mutation of the ppx gene on virulence factors**

To evaluate the phenotypic effects of Ppx on some of the virulence factors we used a mutant strain Δppx and the complemented strain ΔppxC.

**Survival in P_i-deficient medium.** The PA01-WT and mutant strains grew similarly in LB and HP_i-BSM. The WT strain grew very poorly after 8 or 24 h incubation in a culture medium without the external addition of P_i, BSM(−P_i) (cell density at initial time, T_0: 8.8 x 10^7 c.f.u. ml^{-1} versus T_24: 3.5 x 10^8 c.f.u. ml^{-1}). In this strain, polyP may be used eventually as a source of P_i. The Δppx mutant strain did no grow in this P_i-deficient medium and the survival after 8 or 24 h declined by ~25–30 % of the initial value (T_0: 3.8 x 10^7 c.f.u. ml^{-1} versus T_24: 2.3 x 10^5 c.f.u. ml^{-1}). The behaviour of ΔppxC was similar to the WT.

**Biofilm development and rhamnolipids production.** Biofilm production was analysed for the two strains grown in LB medium for 24 h. Crystal violet staining revealed a significant decrease (~86%) in biofilm production in Δppx (OD_{570} 0.27 ± 0.06) relative to the WT strain (OD_{570} 1.93 ± 0.22). Biofilm production was almost recovered in ΔppxC (Fig. 1a). The total amount of rhamnolipids determined in the culture supernatant of Δppx (1.88 ± 0.2 mg ml^{-1}) was ~34% of the value observed for the WT strain (5.50 ± 0.18 mg ml^{-1}), whereas in ΔppxC the value was ~83% (4.53 ± 0.35 mg ml^{-1}) with respect to the WT strain (Fig. 1a).

**Autoinducer biosynthesis.** The WT strain was able to synthesize AHL-like molecules with long (C6–C12) acyl chains as detected using the biosensor strain *A. tumefaciens* NTL4 (Fig. 1b). Δppx was defective in the synthesis of long acyl chain AHL molecules as revealed by the large decrease (>95%) in the blue halo surrounding the colony when compared with WT and ΔppxC values (Fig. 1b).

**Swimming and swarming motility.** We evaluated the flagellum-dependent swimming and swarming motility of WT, Δppx and ΔppxC strains on LB semisolid agar medium. The swimming motility of Δppx was ~55–75 % lower than that of the WT (Fig. 1c). The Δppx strain also presented a decreased swarming motility: ~65–80% with respect to the WT (Fig. 1d). The ΔppxC strain showed similar motility behaviour as the WT (Fig. 1c, d).

**Extracellular blue/green pigments.** The Δppx strain presented only 10% of pyocyanin and 18% of pyoverdine of the WT or ΔppxC registered pigment values (data not shown).

**Expression of the ppx gene under various nutritional stress conditions**

The above observations demonstrated that Ppx, similar to Ppk and polyP, is involved in the pathogenesis of *P. aeruginosa*. We then studied various nutritional conditions that the bacterium could find in the host cell, such as carbon, nitrogen and P_i limitation. To study ppx expression under different nutritional stress conditions, a 500 bp DNA fragment, termed P1_{(500)}, was fused to *lacZ* and integrated into the chromosome of the *P. aeruginosa* PAO1-WT strain. This fragment carried the intergenic region (183 nt) of the divergent PA5241 (ppx) and PA5240 (trxA) genes plus 47 and 198 nt downstream of the ATG initiation codons of ppx and PA5240 (TAC), respectively (Fig. 2). The resulting strain, termed P1_{(500)}::lacZ, was grown in the appropriate culture medium and the β-galactosidase activities were compared with those of the cells grown in HP_i-BSM with succinate (S) and NH_4^+ ([NH_4^+]S/NH_4H^+), i.e. the preferred carbon and nitrogen sources (Fig. 3).

**Effect of carbon and nitrogen sources on *P. aeruginosa* PAO1 ppx expression.** Since the transcription of CbrB-dependent genes is low in the presence of the preferential carbon source (S), intermediate in the presence of glucose and high with the less favourable substrate mannitol (Somleitner et al., 2009), we investigated if ppx expression is under the control of the carbon-source-sensitive two-component system CbrAB (Li & Lu, 2007). Thus, cells were grown with S, glucose or mannitol. β-Galactosidase activities were ~310 ± 50 MU in all the tested conditions, suggesting that ppx expression is independent of CbrB. To evaluate the effect of nitrogen stress condition we replaced the preferential nitrogen source, NH_4^+, by the non-preferential nitrogen sources Cho, His or nitrate (Fig. 3a, b) and also by arginine, betaine or dimethylglycine (data not shown). Under all of these conditions, the β-galactosidase activities were greatly increased. Briefly, after exhaustion of the intracellular nitrogen by growing the cells in (\uparrow P_i)/S medium without addition of an external nitrogen source, the culture was divided and the non-preferential nitrogen compounds were added. Finally, ppx expression was compared with that from culture with NH_4^+. In all cases, the β-galactosidase activity increased in parallel with growth and reached very similar levels (~900 ± 80 MU) at the end of the exponential growth.

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phase (~7 h). Registered values were threefold higher than those observed for bacteria grown with \( \text{NH}_4^+ \) (310 ± 50 MU) (Fig. 3b). Based in these results, we conclude that the activation of \( \text{ppx} \) expression, observed when cells were grown in Cho, was due to the effect of nitrogen limitation rather than to the utilization of Cho as a carbon or nitrogen source, as occurred with phosphorylcholine phosphatase gene (\( \text{pchP} \)) expression (Massimelli et al., 2011).

**Effect of different \( P_i \) concentrations on \( \text{ppx} \) expression.** Concentrations of \( P_i \leq 0.2 \) mM in the growth media were defined as \( P_i \) limitation conditions. Therefore, we performed experiments using \( \text{P}1(500):\text{lacZ} \) cells grown in BSM(−\( P_i \))/S/\( \text{NH}_4^+ \) medium with or without the addition of \( P_i \) at concentrations ranging from 0.1 to 5.0 mM (Fig. 3c). As expected, bacterial growth was proportional to the amount of \( P_i \) added and no growth occurred without the addition of \( P_i \). The maximum level of \( \beta \)-galactosidase activity was observed after 2–3 h of incubation without adding \( P_i \) and declined as the concentration of \( P_i \) was increased (Fig. 3d). In the presence of 0.5 or 5.0 mM \( P_i \), the cells reached the stationary phase after ~8 h of growth and \( \beta \)-galactosidase activity at this time reached a similar level (~600 ± 180 MU) under all culture conditions tested (Fig. 3c, d).

**Transcriptional organization of the \( \text{P. aeruginosa} \) PAO1 \( \text{ppx} \) gene**

Identification of functional motifs in the \( \text{ppx} \) regulatory region. To obtain insights into the molecular mechanisms
Fig. 2. DNA sequence of the 384 nt upstream and 47 nt downstream of the ATG start codon of the ppx gene of *P. aeruginosa* PAO1. The conserved -24/-12 and -35/-10 elements of the $\sigma^{54}$- and $\sigma^{70}$-dependent promoters are indicated by grey boxes. NtrC-binding site and the start codons of PA5240 (CAT) and ppx gene (ATG) are indicated in bold. DR2/DR1 and the putative IHF-binding site are indicated by open and black boxes, respectively. The potential NtrC- and PhoB-binding sites are underlined. The consensus sequence of the Pho box, according to Monds et al. (2006), is indicated by double underline. The TSSs determined by 5'-RACE analyses, TSS1 and TSS2, are indicated by arrows. The numbers to the left of the sequences indicate nucleotide positions in the *P. aeruginosa* genome and numbers to the right indicate positions relative to the ATG start codon of ppx.

Exopolyphosphatase of *P. aeruginosa*

5900 503 ggtatcctgtttctcctcgatgTTCAGCTTCGACGCACGCCTCTATCGTATGATCCTGCTGTTGAA -322

NtrC

5900 565 gcacctgtccacacccggggccgtagactcatTTTGCA -260

5900 627 accagccacgaggtcgccggtccgcttcacgagctctgctgtgacgatgtaaggctgtaa -198

IHF

5900 689 AATAATTCCTTGTAAATTCTCTCGTATGCTGACGCAGTTG -136

TSS2

5900 751 cactcatatcccccctctctggcgcgtcgggtcaggtatctctatgtgctgt -74

5900 813 ccgctgtatccggtagtgcgctgcgttgctgtgctgcgtgctgcctacgtgctgcctat cggtgcc -12

35

5900 875 cggattgcttcggtttggcagccattggcggtcagggggttcctgccggtcaggggtcctgccggtcaggggt -50

ppx

TSS1

5900503

5900565

5900627

5900689

5900751

5900813

5900875

-35

Ext. -10

Pho box

TSS Mapping. To determine experimentally whether the two promoters identified in the regulatory region of ppx

responsible for nitrogen and P$_i$ control of ppx expression, we performed *in silico* analyses of the regulatory ppx region (Fig. 2). Interestingly, two consensus promoter sequences were identified. (i) A putative -24/-12 motif located at -160 bp upstream of the ppx ATG start codon with a score of 0.81, similar to the $\sigma^{54}$ factor described by Barrios et al. (1999). This putative promoter lacks the conserved G and C at positions -24 and -12, respectively. There are some examples of functional promoters lacking these positions in *P. aeruginosa* and other bacteria (Wang & Gralla, 1998). (ii) A putative $\sigma^{70}$-dependent promoter located at -44 TTGGCAGN$_{15}$TGGCAGGAT$_{15}$ upstream of ppx (Fig. 2) presenting the tripartite delineation of this class of promoters ($\sigma^{54}$ TTA -50/12 -16 bp/ -15 TGT -12 -11 ETAAT -7) described by Del Peso-Santos et al. (2012). In addition, we detected: (i) a conserved IHF-binding site at -200/-192; (ii) a 6 bp palindromic sequence resembling a NtrC-binding site at -365 GGCAGCGN$_{15}$GTCGCCCA -289; (iii) two direct repeat (DR) sequences (TTCAAGCTTGC) from -347 to -338 (DR1) and from -362 to -353 (DR2) upstream of ppx and with unknown function; and (iv) a putative Pho-binding site at -52 (CTGCAACN$_{15}$GCTGACC) -35, where underlined nucleotides match the consensus Pho-binding site from the start site (Fig. 2). In several micro-organisms, the Pho-binding sites display a 7 bp sequence interspaced by 4 bp/7 bp (CTGCCAGCTTGC/ (Makino et al., 1996; Monds et al., 2006). The -35 element of the putative $\sigma^{70}$ promoter (TTGGCG, indicated by a grey box in Fig. 2) was overlapped with this Pho box, as proposed by Makino et al. (1996). The -24/-12 region belongs to a unique class of promoters that requires an activator protein for its expression. These activators, called enhancer-binding proteins, activate transcription by binding distant sites (enhancers), located normally >100 bp upstream of the $\sigma^{54}$ promoter (Morett & Segovia, 1993). Activation takes place by direct interaction of the enhancer-binding protein with the $\sigma^{54}$-RNA polymerase holoenzyme bound at the -24/-12 promoter. This interaction between protein complexes located at distant sites on the DNA is facilitated by bending of the intervening DNA stimulated by the binding of the IHF at sites located between the promoter and the enhancer (Delic-Attree et al., 1996). The presence of a $\sigma^{54}$ promoter and both IHF- and NtrC-binding sites in the regulatory region of ppx is compatible with a nitrogen control exerted directly by the latter protein. However, the identification of a putative Pho-binding site overlapping a -35 region of a $\sigma^{70}$-dependent promoter is consistent with the mechanism of regulation exerted by this transcription factor (Makino et al., 1996; Blanco et al., 2011). Thus, the *in silico* analyses strongly suggested that nitrogen and P$_i$ control of ppx expression is exerted by the global regulators NtrC and PhoB acting upon two different promoters. 

TSS Mapping. To determine experimentally whether the two promoters identified in the regulatory region of ppx
are functional, we carried out TSS mapping experiments using a modified 5′ RACE assay. Two initiation events located 8 and 140 nt upstream of the ATG start codon were identified. TSS1 and TSS2 were located 6 and 13 nt downstream of the putative s70 and s54 promoters described above (Fig. 2). Both TSSs were detected using at least two different strategies (Fig. S1). Thus, TSS mapping provided further support for the functionality of the two different putative promoters detected upstream of the ppx gene.

**Determination of the minimal DNA sequence required for ppx expression and the importance of each regulatory region**

To study the relevance of each putative regulatory motif identified upstream of ppx (Fig. 2), we constructed several strains with DNA fragments of lengths shorter than P1(500) and termed P2::lacZ to P8::lacZ (Fig. 4) integrated into the chromosome. β-Galactosidase activities were determined in three different culture media: (i) HP1-BSM/S/NH4+ [↑P1]/S/NH4+, a culture condition with all the nutritional requirements in which both PhoB and NtrC are inactive; (ii) (↑P1)/S/Cho, a culture condition with an excess of Pi and a limiting nitrogen source in which PhoB is inactive, but NtrC is active; and, (iii) BSM(–Pi)/S/NH4+, a Pi-limited condition with NH4+ in which PhoB is active, but NtrC is inactive.

In (↑P1)/S/NH4+ medium, β-galactosidase activity of the strains P1(500)::lacZ, P3(355)::lacZ and P8(149)::lacZ was similar: 313 ± 32, 269 ± 46 and 229 ± 47 MU, respectively (Fig. 4), indicating that the region encompassing the s70 promoter is sufficient to almost fulfil expression.

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Fig. 3. Effect of nitrogen and Pi starvation on ppx gene expression. (a) Growth of P1::lacZ cultured in (↑Pi)/S. At the time indicated by the arrow, the culture was divided into four subcultures, and 20 mM NH4+, Cho, His or nitrate (Nit) was added to each subculture. Samples were collected at various times to measure OD660 and β-galactosidase activity (expressed as MU). (b) Time course of β-galactosidase activity during the growth of cells described in (a). (c) Growth of P1::lacZ cultured in (–Pi)//S/NH4+. At the time indicated by the arrow, the culture was divided into five subcultures, made up to Pi concentrations of 0.0, 0.1, 0.2, 0.5 and 5.0 mM. Samples were collected at various times to measure β-galactosidase activity. (d) Time course of β-galactosidase activity during the growth of cells described in (c). Data represent mean ± sd; n = 3.
Consequently, we observed that in the strains P6(219)::lacZ and P7(351)::lacZ, β-galactosidase activities were reduced >75% (69 ± 13 and 72 ± 22 MU, respectively). The latter two strains have the putative −35/-10 σ54 promoter deleted. Thus, in high Pi and NH4+, ppx expression is dependent mainly on this promoter. In (↑ Pi)/S/Cho, the β-galactosidase activity of strains P1(500)::lacZ, P2::lacZ(368), P3(355)::lacZ, P4(307)::lacZ and P5(191)::lacZ decreased progressively (982 ± 80, 640 ± 49, 631 ± 52, 320 ± 38 and 63 ± 13 MU, respectively). P2::lacZ(368) (with only DR1) and P3(355)::lacZ (without DRs) displayed only 65% of the activity of P1(500)::lacZ (Fig. 3). Therefore, the absence of a single DR (as in P2) or both DRs (as in P3) produced the same mild effect on ppx expression. The activity of P4(307)::lacZ (320 ± 38 MU) was almost 67% less that of P1(500)::lacZ, indicating that the putative NtrC-binding site is required for full ppx expression under this nitrogen-limiting growth condition. It was confirmed by using the mutant strain ΔntrC with the P1(500)::lacZ fusion as its reported activity (342 ± 25 MU) was similar to that obtained with P4(307)::lacZ (320 ± 38 MU). Thus, both the lack of the NtrC-binding site or the removal of ntrC have a similar effect on ppx expression. Direct evidence of the interaction of NtrC with its putative binding site was obtained by electrophoretic mobility shift assays. When 4 µg purified His-NtrC was preincubated with the P2(368) fragment containing the palindromic NtrC-binding region, a retarded complex was observed. As anticipated, this complex was not detected with the P4(307) DNA fragment that does not carry the putative NtrC-binding site (Fig. S2). These results demonstrated not only the role of the NtrC protein, but also its DNA-binding site, in the expression of ppx under nitrogen-limiting growth conditions.

P5(191)::lacZ, which does not carry the −12 σ54 promoter motif, retained only 9% of reporter activity in comparison with P2(368)::lacZ. In support of the functionality of the σ54-dependent promoter, the level of β-galactosidase activity of P1(500)::lacZ in the mutant ΔrpoN strain was reduced by 81% (186 ± 15 and 982 ± 80 MU, respectively). Interestingly, ppx expression in both the strain devoid of the σ54 factor and in the fusion lacking the −12 σ54 promoter element was lower than detected in the absence of NtrC or its binding site, suggesting that a certain level of expression from this promoter occurs even in the absence of its cognate regulator NtrC. This result may be indicative of crosstalk with others of the many enhancer-binding proteins present in P. aeruginosa. Since this putative promoter does not have the conserved C at position −12, we generated an A→C substitution (TTGA→TTGC) at this position by site-directed mutagenesis to increase the similarity to the canonical −24/−12 promoters and integrated it into the chromosome. We anticipated that this

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**Fig. 4.** Schematic diagram of the ppx promoter region. The sizes of the P(1–8)::lacZ constructs are indicated by lines. The DNA fragments were integrated into the PAO1-WT chromosome, and β-galactosidase activities (in MU) were measured in cells grown under (i) (↑ Pi)/S/NH4+, (ii) (↑ Pi)/S/Cho or (iii) (–Pi)/S/NH4+. Cells grown under ↑ Pi were harvested after 7 h of growth. Cells grown under –Pi were collected after 2 h of incubation because no growth was detected. Data represent mean ± SD; n=3.
mutation would result in an enhanced promoter activity; it resulted in ~25% increase of ppx expression (814 ± 41 and 650 ± 12 MU, respectively), as expected. These results indicate that even when this promoter lacks two critical positions it is still active and drives expression of ppx under nitrogen-limiting conditions in a NtrC-dependent manner. In support of this, P1(500)::lacZ and P7(351)::lacZ cells showed similar activities (982 ± 80 and 913 ± 69 MU, respectively), indicating that under these conditions ppx expression does not depend on the σ70 promoter.

Taking into account all the above observations, we concluded that the minimal DNA sequence required for the σ54-dependent promoter was P7(351) (Fig. 4) and that NtrC activated this promoter under nitrogen limitation.

Analysis of P(1–8)::lacZ strains in (–Pi)/S/NH4+ medium helped us to confirm experimentally the putative pho box and the promoter directing expression from TSS1. As shown in Fig. 4, the maximum β-galactosidase activity (~1130 ± 80 MU) was observed in cells containing the DNA fragments P1(500), P2(368), P3(355), P4(307) and P8(149). In contrast, the reporter activities observed in cells lacking the predicted pho box [P5(191) to P7(351)] were ~98 ± 18 MU. All strains with DNA fragments that contained the ~131 downstream region displayed the highest reporter activity from a σ70-dependent promoter under these culture conditions. The activation of this promoter by PhoB was confirmed in cells of PAO1-WT and ΔphoB containing the insertion P8(149)::lacZ. Under Pi-limiting growth conditions (iii), β-galactosidase activities were 1175 ± 34 and 127 ± 71 MU, respectively (Fig. 4).

In conclusion, two ppx promoters were identified: one was shown to be transcribed by σ54 and activated by NtrC, and the other was under the control of σ70 and activated by PhoB.

DISCUSSION

There are few reports implicating Ppx in bacterial virulence. Dacheux et al. (2002) suggested that Ppx could be involved in a type III secretion system, which has been considered as a virulence determinant in P. aeruginosa. A reduction in swimming and swarming motility, biofilm formation, and sporulation efficiency was reported in a ppx null mutant of B. cereus (Shi et al., 2004). In N. meningitidis, a mutant lacking Ppx exhibited increased resistance to complement-mediated killing and it was reported that the biochemical activity of Ppx was necessary for interactions with the complement (Zhang et al., 2010). Finally, it was demonstrated that Ppx is required for long-term survival of M. tuberculosis in necrotic lung lesions (Thayil et al., 2011). Here, we demonstrated the relationship between Ppx and some factors implicated in the pathogenesis of P. aeruginosa by using a Δppx mutant strain. Impaired C12 AHL production in this strain suggests a failure in the expression of other virulence factors. The quorum system Las (responsible for the long acyl chain AHL C6–12 synthesis) not only controls the production of some virulence factors involved in acute infection, but also activates Rhl, the second quorum-sensing system of P. aeruginosa (Jimenez et al., 2012). Rhl controls the expression of genes responsible for the production of rhamnolipids, pyocyanin and pyoverdine (Jimenez et al., 2012). In the P. aeruginosa ppx null mutant, obtained here, there was lower production of these factors when compared with the PAO1-WT strain. The Δppx strain also had impaired swarming motility – a fact that may be related to the decreased production of rhamnolipids (Ciaiazza et al., 2005). Other effects of ppx gene inactivation that we registered in the present study were related to a decrease in biofilm formation and swimming motility – both mechanisms required for attachment to abiotic surfaces. All the results obtained with the Δppx mutant strain were reverted by the insertion of the ppx gene into the bacterial chromosome of the mutant strain. Thus, the results obtained here demonstrate that, similar to Ppk and polyP, Ppx is also involved in the production of factors associated with both acute infection (e.g. motility-promoting factors, blue/green pigments production, quorum-sensing AHL) and chronic infection (e.g. rhamnolipids, biofilm formation). The relationship between P. aeruginosa pathogenesis and the nutrient sources to sustain bacterial replication in infected tissues has been the subject of many studies. For example, Son et al. (2007) suggested that P. aeruginosa degrades amino acids (nitrogen depletion) and the principal lung surfactant lipid phosphatidylycholine (Pi depletion). Long et al. (2008) also observed a Pi depletion after surgery that was related with an increase in the virulence of P. aeruginosa. Zaborin et al. (2009) provided evidence that phosphate depletion induces virulence systems in P. aeruginosa associated with phosphate, quorum sensing and iron signalling. Here, we studied how the ppx gene is regulated transcriptionally in response to various nutritional conditions, including preferential carbon and nitrogen sources, carbon and nitrogen limitation, and with or without the addition of Pi. The nitrogen-limiting condition led us demonstrate that ppx expression is under the control of a σ54-dependent promoter and is activated by the response regulator NtrC. In silico analyses revealed a putative −24/+12 σ54 promoter element, and consensus sequences for the binding of both NtrC and IHF in the upstream region of ppx. We confirmed the functionality of these motifs through physiological and molecular studies. We found that ppx expression was reduced substantially in the ΔrpoN and ΔntrC strains containing the P1(500) DNA fragment, and the purified NtrC showed a direct binding with the P2(368) DNA fragment that contains the putative upstream activation sequence for the transcriptional factor, NtrC. ppx expression was also dependent on P1 concentration in the culture medium. During P1 starvation, the Pho regulon is activated and regulates genes involved in P1 homeostasis. There are many reports relating the ppk gene with the Pho regulon (Kato et al., 1993; Rao et al., 1998; Geissdörfer et al., 1998; Kornberg et al., 1999), but none so far.
Regarding such a relationship with the ppx gene. It is reasonable to assume that full ppx expression is necessary to degrade the internal polyP and to allow bacteria to obtain P_i for their growth. Deletion of phoB confirmed that PhoB is the activator of σ^54-RNA polymerase in the expression of ppx. Several of the general characteristics of promoters belonging to the Pho regulon (Shinagawa et al., 1987) are present in the ppx promoter, e.g. the putative Pho box sequence detected (−35/−52 from the ATG) shared 64% identity with the Pseudomonas fluorescens phoX promoter sequence (Monds et al., 2006), and 43% identity with the E. coli consensus sequence (Makino et al., 1996). Also, the mutant strain ΔphoB with the DNA fragments P1 or P8::lacZ showed lower promoter activity when compared with the WT strain grown under similar conditions. The role of the two inverted repeats (DR1 and DR2) identified here is still unknown, although their removal resulted in a 33–35% decrease of ppx expression. Thus, ppx could be possibly regulated by a third protein, as yet unidentified. Further molecular studies will be necessary to detect and identify this protein, and will clarify the contribution of DRs to ppx gene regulation.

Zago et al. (1999) studied the ppx promoter expression of P. aeruginosa under oxidative and osmotic stress conditions. They suggested that ppk–ppx genes are not coregulated and that Ppx activity would be only regulated by ppGpp, as with the E. coli enzyme. However, our results show that ppx expression is regulated at the transcriptional level under nutritional stress conditions, such as nitrogen and P_i starvation. Based on data presented here, and on recent observations made by Rao et al. (2009), Achbergerová & Nahalka (2011) and Österberg et al. (2011), we produced a hypothetical model to explain the transcriptional regulation of ppx gene expression under P_i- or nitrogen-limiting conditions (Fig. 5). In bacteria under nutritional stress, levels of ppGpp increase, resulting in the recruitment of free RNA polymerase in favour of formation of a holoenzyme with alternative sigmas, such as σ^54 (Jishage et al., 2002; Österberg et al. 2011). Under nitrogen starvation, the two-component NtrB/NtrC system is activated and ppGpp enables NtrC to activate the expression of ppx promoter through the σ^54-RNA polymerase (Fig. 5a). Under P_i-limiting conditions, the two-component PhoR/PhoB system is activated and, in turn, it activates the ppx gene encoding Ppx (Fig. 5b). Accumulated polyP may be hydrolysed by the processive action of Ppx, yielding P_i, plus a shorter polymer. In this regard, it is important to consider that Ppk2 of P. aeruginosa is >100-fold induced at the stationary phase, at which it preferentially catalyses the synthesis of GTP from short-chain polyP and GDP (Ishige et al., 2002).

Therefore, the maintenance of intracellular polyP levels may play a key role in bacterial survival. We found evidence of the inter-relationships between nutrient availability,
polyP levels and the enzymes regulating its metabolism, particularly Ppx. Deregulated polyP-mediated signalling results in a deficient response to nutritional stress and might also impair the production of *P. aeruginosa* virulence factors.

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