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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_i). 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^{54}-dependent promoter activated by the response regulator NtrC. However, under P_i limitation, the expression was controlled from a σ^{70} 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.

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INTRODUCTION

Inorganic polyphosphates (polyPs) are linear polymers consisting of tens to hundreds of orthophosphate (P_i) residues linked by 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_i 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_i) 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_i 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.*,

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.

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 flagellum-dependent swimming and swarming 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 σ^{54}- and σ^{70}-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 (HP_i-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 Na^+/K^+ phosphate buffer, pH 7.0) was added to final concentrations of 0.1, 0.2, 0.5 and 5.0 mM, 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 Δ*ppx* and Δ*phoB* strains (Choi & Schweizer, 2005), respectively. To achieve complementation of the Δ*ppx* strain, the *ppx* gene was amplified by PCR using the primers $P1_{(500pb)}$ and *ppx*-Dwn (Table S1). The obtained amplicon was cloned into pUC18-mini-Tn7T-Gm using the restriction enzymes *Spe*I and *Sac*I. This plasmid, pUC18-*ppx*C, 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 (Nievas *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 *atra*G::*lacZ* fusion and *traR* (Cha *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 Essar *et al.* (1990) and measured at OD_{520}. To measure pyoverdine production, the absorbance of the culture supernatants was determined at OD_{403} (Yeom & Park, 2012).

Rhamnolipids production. For detection of biosurfactant rhamnolipids, *P. aeruginosa* cells were grown as described by 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 QIAquick 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 QuikChange Mutagenesis Kit (Stratagene). The primer 12.a 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:

Table 1. Bacteria strains and plasmids used in this study

Strain/plasmid	Genotype/description	Reference/source
Strains		
Escherichia coli		
XL10-Gold	Tet^r Δ(*mcrA*) *183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F′ *proAB lacIqZ*Δ*M15* Tn*10*(Tet^r) Amy Cam^r]	Stratagene
BL21-CodonPlus(DE3)-RIPL	F$^-$ *ompT hsdS*($r_B^-m_B^-$) *dcm*$^+$ Tet^r *gal λ*(DE3) *endA* Hte [*argU proL* Cam^r] [*argU ileY leuW* Strep/Spec^r]	Stratagene
Pseudomonas aeruginosa PAO1		
PAO1-WT	Prototrophic WT strain	
P(1–8)::*lacZ*	PAO1 with a chromosomal integration of mini-Tn*7*T carrying the fusions P(1–8)::*lacZ*	This study
Δ*ntrC*	PAO1 Δ*ntrC*	Massimelli *et al.* (2011)
Δ*rpoN*	PAO1 Δ*rpoN*	Heurlier *et al.* (2003)
Δ*ntrC* P1::*lacZ*	PAO1 Δ*ntrC* with a chromosomal integration of mini-Tn*7*T carrying the fusion pP1::*lacZ*	This study
Δ*rpoN* P1::*lacZ*	PAO1 Δ*rpoN* with a chromosomal integration of mini-Tn*7*T carrying the fusion pP1::*lacZ*	This study
Δ*ppx*	PAO1 Δ*ppx*	This study
Δ*ppx*C	PAO1 Δ*ppx* complemented with the *ppx* gene	This study
Δ*phoB*-P1/P8::*lacZ*	PAO1 Δ*phoB* with a chromosomal integration of mini-Tn*7T* carrying the fusions P1 or P8::*lacZ*	This study
*Agrobacterium tumefaciens*NTL4 (pZLR4)	Carries the plasmid pZLR4, which contains the *atra*G::*lacZ* fusion and *traR*	Cha *et al.* (1998)
Plasmids		
pUC18-mini-Tn*7*T-Gm-*lacZ*	Gm^r on mini-Tn*7*T; *lacZ* transcriptional fusion vector	Choi & Schweizer (2006)
pUC18-mini-Tn*7*T-Gm	Gm^r on mini-Tn*7*T	Choi & Schweizer (2006)
pTNS2	Ap^r; helper vector encoding the site-specific Tn*7* transposition pathway	Choi & Schweizer (2006)
pFLP2	Ap^r; Flp recombinase-encoding vector	Choi & Schweizer (2005)
pDONR221	Km^r; Gateway entry or donor vector	Invitrogen
pEX18ApGW	Ap^r; gene replacement vector, compatible with Gateway system	Choi & Schweizer (2005)
pPS856	Gm^r; vector carrying gentamycin resistance gene	Choi & Schweizer (2005)
pP1–8::*lacZ*	Gm^r, Ap^r; pUC18-mini-Tn*7*T-Gm-*lacZ* with a *Spe*I/*Xho*I fragment	This study
pET-15b	Ap^r, T7 promoter, multiple cloning sites, His-tag-coding sequence	Novagen
pET-15b::*ntrC*	1400 bp *Eco*RI/*Nde*I fragment containing the *ntrC* gene cloned into pET-15b	This study
pUC18-*ppx*C	2018 bp *Spe*I/*Sac*I fragment containing the *ppx* gene plus 500 bp upstream	This study

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*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 $P1_{(500)}$::*lacZ* to $P8_{(149)}$::*lacZ*, respectively. Colony PCR using the primers P_{Tn7R} and $P_{glmS\text{-}down}$ was used to confirm the chromosomal Tn*7* insertions. The gentamycin 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 over-expressed 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/w) 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/) was used to identify σ^{54}-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) was used to identify σ^{70}-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 Δ*ppx* and the complemented strain Δ*ppx*C.

Survival in P_i-deficient medium. The PAO1-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: 8.8×10^7 c.f.u. ml^{-1} versus T_{24}: 3.5×10^8 c.f.u. ml^{-1}). In this strain, polyP may be used eventually as a source of P_i. The Δ*ppx* 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: 3.8×10^7 c.f.u. ml^{-1} versus T_{24}: 2.3×10^5 c.f.u. ml^{-1}). The behaviour of Δ*ppx*C 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 Δ*ppx* (OD_{570} 0.27 ± 0.06) relative to the WT strain (OD_{570} 1.93 ± 0.22). Biofilm production was almost recovered in Δ*ppx*C (Fig. 1a). The total amount of rhamnolipids determined in the culture supernatant of Δ*ppx* (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 Δ*ppx*C the value was ~83 % (4.53 ± 0.35 mg ml^{-1}) with respect 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). Δ*ppx* 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 Δ*ppx*C values (Fig. 1b).

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

Extracellular blue/green pigments. The Δ*ppx* strain presented only 10 % of pyocyanin and 18 % of pyoverdine of the WT or Δ*ppx*C 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^+ [(↑P_i)/S/NH_4^+], 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 (Sonnleitner *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 (↑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

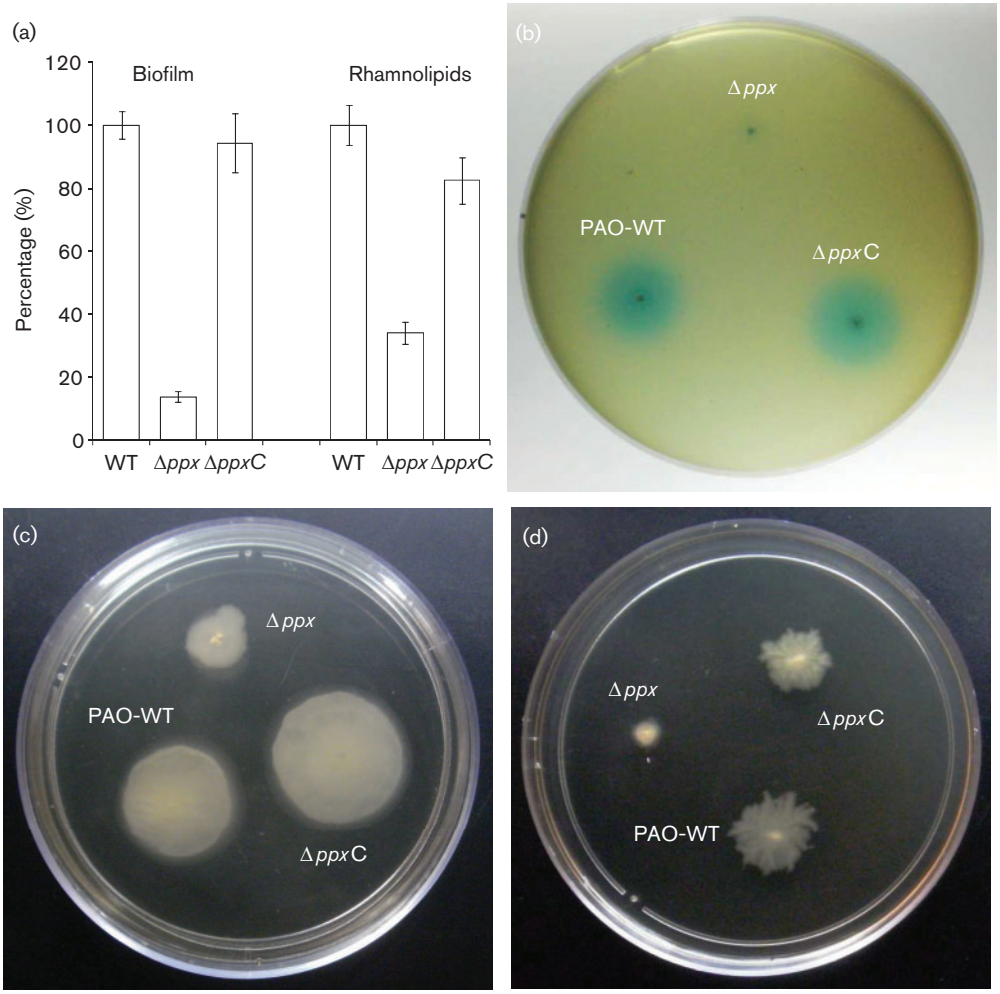


Fig. 1. Biofilm development, amount of rhamnolipids, quorum sensing and motility behaviour of *P. aeruginosa* PAO1-WT, Δ*ppx* and Δ*ppx*C strains. (a) Percentage of biofilm production in both strains after 24 h of incubation and staining with crystal violet measured at OD_{570} (100 %=1.93±0.1). The amount of rhamnolipids was determined in culture supernatants in terms of rhamnose (mg ml^{-1}) (100 %=5.57±0.30). Data represent mean±SD; n=3. (b) Representative quorum-sensing bioassay results of PAO1-WT, Δ*ppx* and Δ*ppx*C. (c) Representative swimming phenotypes of PAO1-WT, Δ*ppx* and Δ*ppx*C on a swim plate (LB medium plus 0.3 % agar) after 48 h of incubation. (d) Representative swarming phenotypes of PAO1-WT, Δ*ppx* and Δ*ppx*C on a swarm plate (LB medium plus 0.5 % agar) after 72 h of incubation.

phase (~7 h). Registered values were threefold higher than those observed for bacteria grown with NH_4^+ (310± 50 MU) (Fig. 3b). Based in these results, we conclude that the activation of *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 (*pchP*) expression (Massimelli *et al.*, 2011).

Effect of different P_i concentrations on *ppx* expression. Concentrations of $P_i \leqslant 0.2$ mM in the growth media were defined as P_i limitation conditions. Therefore, we performed experiments using $P1_{(500)}$::*lacZ* cells grown in BSM(–P_i)/S/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 β-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 β-galactosidase activity at this time reached a similar level (~600±180 MU) under all culture conditions tested (Fig. 3c, d).

Transcriptional organization of the *P. aeruginosa* PAO1 *ppx* gene

Identification of functional motifs in the *ppx* regulatory region. To obtain insights into the molecular mechanisms

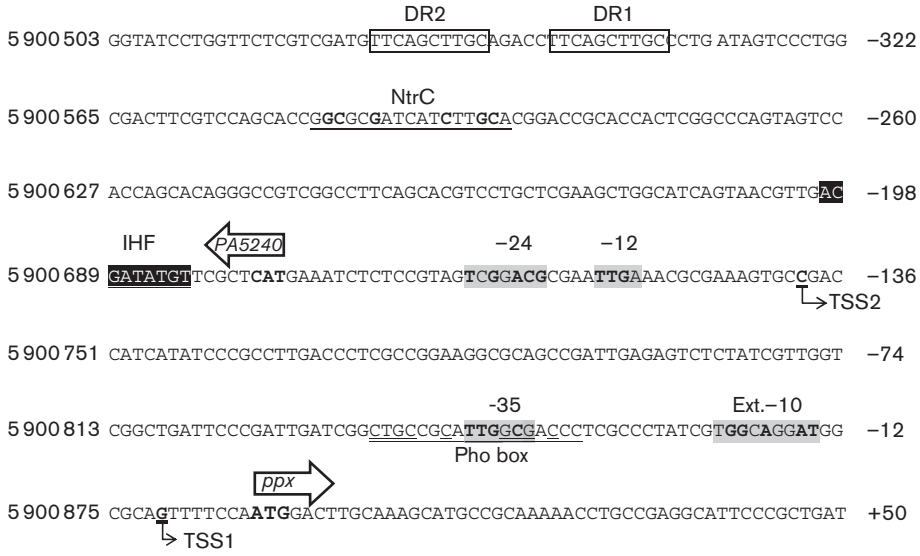


Fig. 2. DNA sequence of the 384 nt upstream and 47 nt downstream of the ATG start codon of the *ppx* gene of *P. aeruginosa* PAO1. The conserved −24/−12 and −35/−10 elements of the σ^{54}- and σ^{70}-dependent promoters are indicated by grey boxes. NtrC-binding site and the start codons of PA5240 (CAT) 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*.

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 $^{-168}$**T**C**GG**A**CG**N$_4$**TTG**A^{-154} upstream of the *ppx* ATG start codon with a score of 0.81, similar to the σ^{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 σ^{70}-dependent promoter located at $^{-44}$**TTG**G**C**GN$_{15}$**TGG**-C**A**GG**AT**$^{-15}$ upstream of *ppx* (Fig. 2) presenting the tripartite delineation of this class of promoters ($^{-35}$TTGACA^{-30}/12–16 bp/$^{-15}$TGGT^{-12}/$^{-11}$ATAAT^{-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 $^{-305}$G**GC**GC**G**N$_5$**C**TT**GC**A^{-289}; (iii) two direct repeat (DR) sequences (TTCAGCTTGC) 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 (CTGCCGCN$_4$GCGACCC) −35, where underlined nucleotides match the consensus Pho-binding site from the ATG start site (Fig. 2). In several micro-organisms, the Pho-binding sites display a 7 bp sequence interspaced by 4 bp/7 bp (CTGCAACN$_4$GCGTCAT/C) (Makino *et al.*, 1996; Monds *et al.*, 2006). The −35 element of the putative σ^{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 σ^{54} promoter (Morett & Segovia, 1993). Activation takes place by direct interaction of the enhancer-binding protein with the σ^{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 σ^{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 σ^{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*

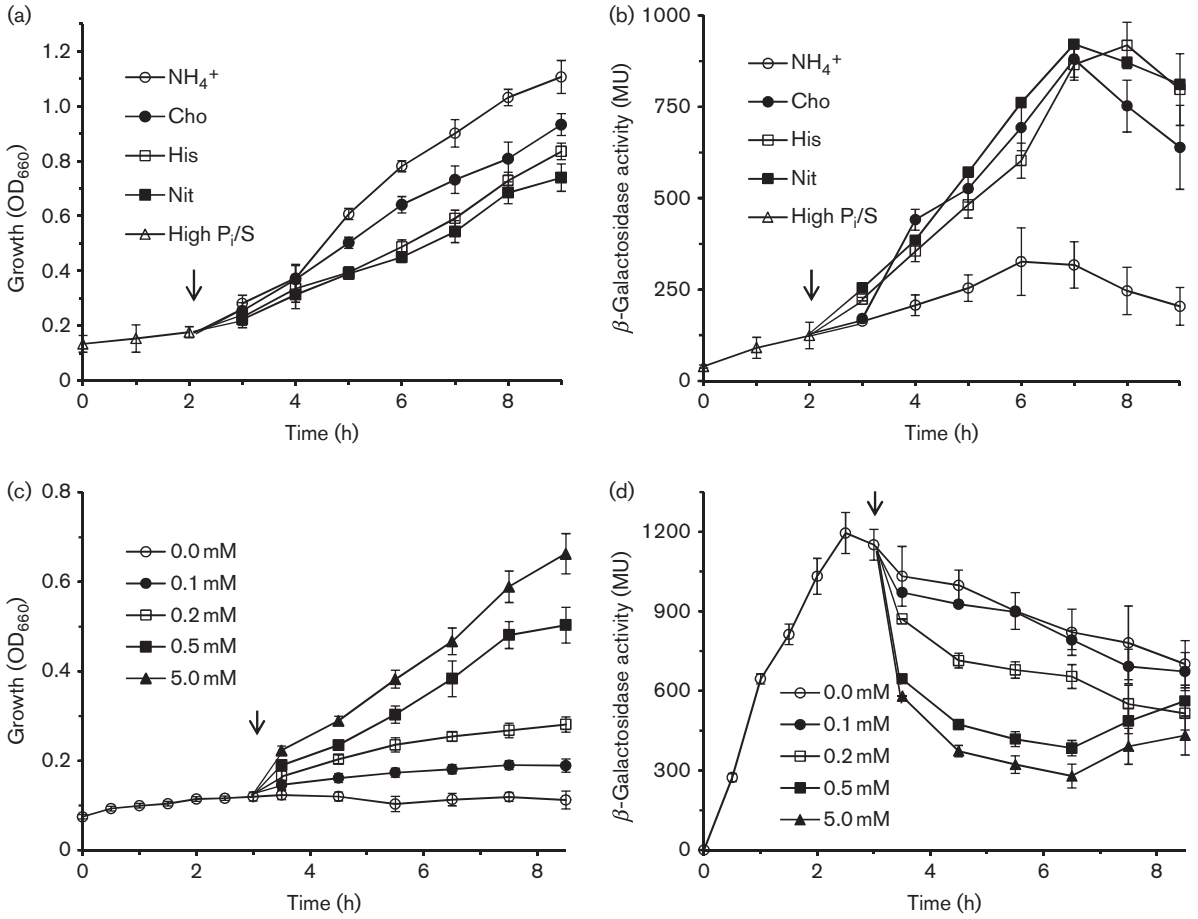


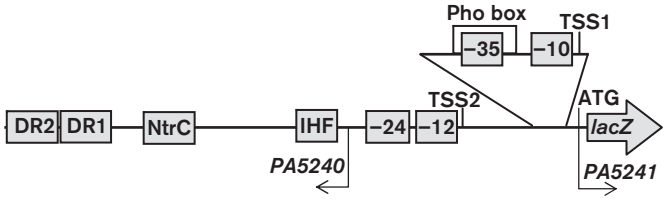
Fig. 3. Effect of nitrogen and P_i starvation on *ppx* gene expression. (a) Growth of P1::*lacZ* cultured in (↑ P_i)/S. At the time indicated by the arrow, the culture was divided into four subcultures, and 20 mM NH_4^+, Cho, His or nitrate (Nit) was added to each subculture. Samples were collected at various times to measure OD_{660} 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 (–P_i)/S/NH_4^+. At the time indicated by the arrow, the culture was divided into five subcultures, made up to P_i 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.

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 σ^{70} and σ^{54} 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) HP_i-BSM/S/NH_4^+ [(↑ P_i)/S/NH_4^+], a culture condition with all the nutritional requirements in which both PhoB and NtrC are inactive; (ii) (↑ P_i)/S/Cho, a culture condition with an excess of P_i and a limiting nitrogen source in which PhoB is inactive, but NtrC is active; and, (iii) BSM(–P_i)/S/NH_4^+, a P_i-limited condition with NH_4^+ in which PhoB is active, but NtrC is inactive.

In (↑ P_i)/S/NH_4^+ 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 σ^{70} promoter is sufficient to almost fulfil expression.



Strain:		β-Galactosidase activity (MU±SD)		
		(↑Pi) S/NH_4^+	(↑Pi) S/Cho	(−Pi) S/NH_4^+
PAO1-WT				
P1(500)::*lacZ*	−482 — +18	313 ± 32	982 ± 80	1195 ± 72
P2(368)::lacZ	−350 — +18	ND	640 ± 49	1098 ± 99
P3(355)::*lacZ*	−337 — +18	269 ± 46	631 ± 52	1181 ± 95
P4(307)::*lacZ*	−289 — +18	ND	320 ± 38	1035 ± 98
P5(191)::*lacZ*	−159 — +18	ND	63 ± 13	103 ± 15
P6(219)::*lacZ*	−141 — +18	69 ± 13	619 ± 32	95 ± 13
P7(351)::*lacZ*	−131 — +18	72 ± 22	913 ± 69	91 ± 25
P8(149)::*lacZ*	−131 — +18	229 ± 47	52 ± 12	1175 ± 34
PAO1-Δ*phoB*				
P1(500)::*lacZ*	−482 — +18	281 ± 61	913 ± 65	279 ± 15
P8(149)::*lacZ*	−131 — +18	167 ± 59	103 ± 48	127 ± 71

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) (↑ P_i)/S/NH_4^+), (ii) (↑ P_i)/S/Cho or (iii) (−P_i)/S/NH_4^+. Cells grown under ↑ P_i were harvested after 7 h of growth. Cells grown under −P_i were collected after 2 h of incubation because no growth was detected. 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 σ^{70} promoter deleted. Thus, in high P_i and NH_4^+, *ppx* expression is dependent mainly on this promoter. In (↑ P_i)/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 Δ*ntrC* 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 σ^{54} promoter motif, retained only 9 % of reporter activity in comparison with P2$_{(368)}$::*lacZ*. In support of the functionality of the σ^{54}-dependent promoter, the level of β-galactosidase activity of P1$_{(500)}$::*lacZ* in the mutant Δ*rpoN* strain was reduced by 81 % (186 ± 15 and 982 ± 80 MU, respectively). Interestingly, *ppx* expression in both the strain devoid of the σ^{54} factor and in the fusion lacking the −12 σ^{54} 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 (TTG**A**⟶TTG**C**) 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

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 σ^{70} promoter.

Taking into account all the above observations, we concluded that the minimal DNA sequence required for the σ^{54}-dependent promoter was $P7_{(351)}$ (Fig. 4) and that NtrC activated this promoter under nitrogen limitation.

Analysis of P(1–8)::*lacZ* strains in $(-P_i)$/S/NH_4^+ 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 σ^{70}-dependent promoter under these culture conditions. The activation of this promoter by PhoB was confirmed in cells of PAO1-WT and Δ*phoB* containing the insertion $P8_{(149)}$::*lacZ*. Under P_i-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 σ^{54} and activated by NtrC, and the other was under the control of σ^{70} 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 Δ*ppx* 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 Δ*ppx* strain also had impaired swarming motility – a fact that may be related to the decreased production of rhamnolipids (Caiazza *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 Δ*ppx* 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 phosphatidylcholine (P_i depletion). Long *et al.* (2008) also observed a P_i 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 P_i. The nitrogen-limiting condition led us demonstrate that *ppx* expression is under the control of a σ^{54}-dependent promoter and is activated by the response regulator NtrC. *In silico* analyses revealed a putative −24/–12 σ^{54} 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 Δ*rpoN* and Δ*ntrC* 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 P_i concentration in the culture medium. During P_i starvation, the Pho regulon is activated and regulates genes involved in P_i 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^{70}-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 Δ*phoB* 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,

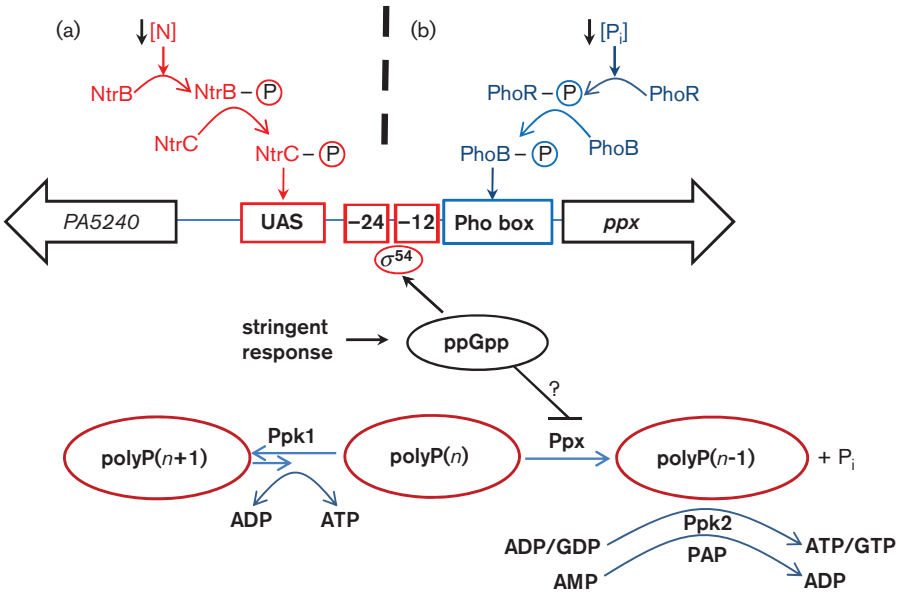


Fig. 5. Hypothetical scheme for *ppx* regulation. (a) ↓[N] (nitrogen starvation): the two-component system NtrB/NtrC is activated stimulating *ppx* expression through the σ^{54}-RNA polymerase holoenzyme, whose binding to the promoter is facilitated by ppGpp. The increase in this alarmone concentration is triggered by nitrogen starvation. (b) ↓[P_i] (P_i limitation): the two-component system PhoR/PhoB is activated, stimulating *ppx* expression through the interaction with the Pho box, which overlaps with the −35/−10 elements. As Ppx synthesis increases, the polyP is used to provide P_i and polyP(n−1). PolyP of shorter chains may be the substrate of Ppk2 to provide GTP or ATP, or polyP AMP-phosphotransferase (PAP) to provide ADP: the direct inhibition of Ppx by ppGpp is not reported in *P. aeruginosa*. UAS, Upstream activation sequence.

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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