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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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Abbreviations: AHL, acyl-homoserine lactones; Cho, choline; DRs, direct repeat sequences; IHF, integration Host Factor; MU, Miller Units; N, nitrogen; Pi, orthophosphate; polyP, inorganic polyphosphate; Ppx, exopolyphosphatase; TSSs, transcriptional start sites; 5´RACE, rapid amplification of cDNA5´ends; S, succinate; X-gal, 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside

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Summary

The exopolyphosphatase (Ppx) of *Pseudomonas aeruginosa* is encoded by PA5241 gene (*ppx*). Ppx catalyzes the hydrolysis of inorganic polyphosphates (polyPs) to orthophosphate (Pi). In the present work we identified and characterize 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. On the other hand, under Pi limitation the expression was controlled from a σ^{70} promoter, activated by PhoB. Results obtained from the *ppx* null mutant demonstrate 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 indicate that *P. aeruginosa* consistently maintains proper levels of Ppx regardless of environmental conditions. The precise control of *ppx* expression appears to be essential for the survival of *P. aeruginosa* and the ocurrence of either acute or chronic infection in the host.

INTRODUCTION

Inorganic polyphosphates (polyPs) are linear polymers consisting of tens to hundreds orthophosphate (Pi) residues linked by energy-rich phosphoanhydride bonds. There are numerous reports indicating that polyP is essential for the growth of microorganisms, their responses to stresses and stringencies, and the virulence of pathogens (reviewed in Rao *et al*., 2009). PolyP is synthesized by polyphosphate kinases (Ppks) that catalyze the reversible transfer of the terminal phosphate (γ) of ATP to the polyP chain (Kornberg *et al*., 1999). The polymer can be hydrolyzed by the exopolyphosphatase (Ppx) that processively cleaves Pi 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, humans included. PolyPs and Ppks are clearly related to virulence of *P. aeruginosa* and other pathogens since both are essential for swimming, swarming and twitching motilities, biofilm development and quorum sensing (Rashid & Kornberg, 2000; Rashid *et al*., 2000 a, b). PolyP and Ppks in particular, are also involved in the adaptation of microorganisms to changes in their surroundings such as phosphate (Pi) deficiency or nitrogen (N) starvation. In various bacteria, including numerous pathogens, *ppk* gene is part of the PHO regulon and is upregulated in response to a low external Pi 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*., 2006; Silby *et al*., 2009). It was also reported that in *E. coli*, under N-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 that played by Ppx in the physiology of harmless or pathogenic bacteria. It was reported that Ppx is essential for the pathogenesis of *Mycobacterium tuberculosis* (Thayil *et al*., 2011), *Bacillus cereus* (Shi *et al*., 2004), *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 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 choline 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 choline metabolism is controlled by the intracellular balance between carbon and N and consequently regulated by the global regulators NtrC and CbrB (Massimelli *et al.*, 2011). Preliminary studies carried out in our laboratory suggested that choline 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 choline, a N-limited source, and with Pi-deprivation conditions.

In this report, we provide 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 acyl homoserine lactones (AHL). We also demonstrated that *ppx* expression is mediated by both σ^{54}- and σ^{70}-dependent promoters, activated by NtrC under N limitation and by PhoB under conditions of low Pi availability, respectively. Our results highlight 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 Pi basal salt medium (HPi-BSM) (Lisa *et al*., 1994). The low-Pi medium previously described (Lucchesi *et al*., 1989) was modified by adding no exogenously Pi, and termed BSM(-Pi). Pi (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 N 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 ampicillin (Ap) 150 μg ml^{-1}. Liquid cultures were incubated at 37℃ with shaki ng. The primers UpF-*ppx*-Gw, UpR-*ppx*-Gm, DwnF-*ppx*-Gm, DwnR-*ppx*-Gw, and UpF-*phoB*-Gw, UpR-*phoB*-Gm, DwnF-*phoB*-Gm and DwnR-*phoB*-Gw (Supplementary Table S1) were used to construct Δ*ppx* and Δ*phoB* strains (Choi & Schweizer, 2005), respectively. To achieve complementation of the Δ*ppx* strain, *ppx* gene was amplified by PCR using the primers $P1_{(500pb)}$ and *ppx*-Dwn (Supplementary Table S1). The obtained amplicon was cloned into pUC18-mini-Tn7T-Gm plasmid by 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 (2005) 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 of LB medium (OD_{600}=0.5) and incubated with shaking for 24 h at 37℃. 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 the dye excess. Biofilm formation was quantified by solubilization of crystal violet with 1ml of 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℃ 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 AHL 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 (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside).

Pyocyanin and pyoverdine production assays. Cells were grown in LB broth for 24 h at 37℃ 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).

Rhamnolipid production. For detection of this biosurfactant, *P. aeruginosa* cells were grown 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 *et al*. (1972).

DNA methodology. Genomic and plasmidic DNA isolation was 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, Inc. (Gasan-dong Geumchen-gu, Seoul, Korea). For site-directed mutagenesis, promoter regions were mutated using the Quickchange mutagenesis kit (Stratagene). The primer 12.a was employed for −154 A/C substitution; to determine the transcriptional start sites (TSSs) of the *ppx* gene a modified 5´ RACE methodology was used as described by Mendoza *et al.* (2009), using two specific primers for *ppx* gene: *ppx*-tssA and *ppx*-tssB (Supplementary Table S1). For TSS1, a polyA tail was added at the 5´-RNA end and 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 harboring the putative promoter region of the ppx gene*: P1 to 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; Up-pho and Dwn-prom for P8 (Supplementary Table S1). The PCR products, $P1_{(500pb)}$, $P2_{(368pb)}$, $P3_{(355pb)}$, $P4_{(307pb)}$, $P5_{(191pb)}$,, $P6_{(219pb)}$, $P7_{(351pb)}$ and $P8_{(149pb)}$, were digested and then individually ligated into pUC18-mini-Tn*7*-Gm-*lacZ* to obtain P(1-8)::*lacZ*. These plasmids and pTNS2 were co-transformed 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 Gm 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 (Supplementary Table S1). Following overexpression of the protein in *E. coli* BL21-Codon Plus, histidine-tagged NtrC(S161F) was purified according to the manufacturer´s protocol (The QIA expressionist, Qiagen). The purity was visually estimated 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 independently incubated with 4 µgr of His6-NtrC(S161F) in 50 mM Tris-HCl (pH 8.0), 750 mM KCl, 2.5 mM EDTA, 0.5% (v/w) Triton-X100, 1 mM dithiothreitol, and 4% (v/v) glycerol, for 20 min at 28ºC as described by Leech *et al*. (2008). Electrophoretic mobility shift assays were conducted using a 5% no-denaturing polyacrylamide gel in Tris-HCl (pH 8.0) as upper phase and sodium acetate buffer (pH 5.0) as lower phase. Tris-borate-EDTA) pH 8.0 was used as running buffer, at 200 V for 2 h at 4ºC.

Statistical analysis. All our data were analyzed using Infostat software version 2.0 (Group Infostat, Argentine). Differences were considered significant at $p < 0.05$.

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 consensus 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, Δ*ppx*C

Survival in Pi-deficient medium. The PA01-WT and mutant strains grew similarly in LB and HPi-BSM. WT strain grew very poorly after 8 or 24 h of incubation in a culture medium without the external addition of Pi, BSM(-Pi) (cell density at initial time, T_0: 8.8 x 10^7 cfu ml^{-1} *vs* T_{24}: 3,5 x 10^8 cfu ml^{-1}). In this strain, polyP may be eventually used as a source of Pi. The Δ*ppx* mutant strain did no grow in this Pi-deficient medium, and the survival after 8 or 24 h declined ≈25-30% of the initial value, (T_0: 3.8 x 10^7 cfu ml^{-1} *vs* T_{24}: 2.3 x 10^5 cfu ml^{-1}). The behavior of Δ*ppx*C strain was similar to the WT.

Biofilm development and rhamnolipid production. Biofilm production was analyzed 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 WT strain (OD_{570} 1.93±0.22). The biofilm production was almost recovered in the Δ*ppx*C (Fig. 1a). The total amount of rhamnolipid determined in the culture supernatant of Δ*ppx* (1.88±0.2 mg ml^{-1}) was ≈34% of the value observed for WT (5.50±0.18 mg ml^{-1}), whereas in the Δ*ppx*C the value was ≈83% (4.53±0.35mg ml^{-1}) 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 the one of the WT (Fig. 1c). The Δ*ppx* strain also presented a decreased swarming motility: ≈65-80% respect to the WT. The Δ*ppx*C strain showed similar motility behavior as the WT strain (Fig. 1c and d).

*Extracellular blue/green pigments***.** Δ*ppx* strain presented only 10% of pyocyanin and 18% of pyoverdine of WT or Δ*ppx*C registered pigment values (data not shown).

Expression of *ppx* gene under various nutritional stress conditions

The above observations demonstrate that Ppx, similarly to PpK and polyP, is involved in the pathogenesis of *P. aeruginosa*. Then, we studied various nutritional conditions that the bacterium could be found in the host cell, such as carbon, N and Pi 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 *P. aeruginosa* PAO1-WT strain. This

fragment carried the intergenic region (183 nucleotides) of the divergent PA5241 (*ppx*) and PA5240 (*trxA*) genes plus 47 and 198 nucleotides 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 HPi-BSM with succinate (S) and NH_4^+((↑Pi)/S/NH_4^+), the preferred carbon and N sources.

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 favorable 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 N stress condition we replaced the preferential N source, NH_4^+, by the non-preferential N sources choline, histidine, nitrate (Figs. 3a and b), and also by arginine, betaine or dimethylglycine (data not shown). In all these conditions, the β-galactosidase activities were greatly increased. Briefly, after exhaustion of the intracellular N by growing the cells in (↑Pi)/S medium without addition of a external N source, the culture was divided and the non-preferential N 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 phase (≈7 h). Registered values were three-fold 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 choline, is due to the effect of N limitation rather than to the utilization of choline as carbon or N source as occurred with *pchP* expression (Massimelli *et al.,* 2011).

Effect of different Pi concentrations on ppx expression. Concentrations of Pi ≤0.2 mM in the growth media were defined as Pi limitation conditions. Therefore, we performed experiments using $P1_{(500)}$::*lacZ* cells grown in BSM(-Pi)/S/NH_4^+ medium with or without the addition of Pi at concentrations ranging from 0.1 to 5.0 mM (Fig. 3c). As expected, bacteria growth was proportional to the amount of Pi added and no growth occurred without the addition of Pi. The maximum level of β-galactosidase activity was observed after 2-3 h of incubation without adding Pi and declined as the concentration of Pi was increased (Fig. 3d). In the presence of 0.5 or 5.0 mM Pi, 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 (Figs. 3c and d).

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

Identification of functional motifs in the ppx regulatory region. To get insights on the molecular mechanisms responsible for N and Pi control of *ppx* expression, we performed *in silico* analyses of the regulatory *ppx* region (Fig. 2). Interestingly, two consensus promoter sequences were identified. First, a putative -24/-12 motif located between -168(**TC**G**G**G**ACG**N_4**TTG**$A_{\mathbf{A}}$)-153 nucleotides upstream of *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 -24 and -12 positions, respectively. There are some examples of functional promoters lacking these positions in *P. aeruginosa* and other bacteria (Wang & Gralla, 1998). Second, a putative σ^{70}-dependent promoter located at -44(**TTG**G**C**GN_{15}**TGG**C**A**GG**AT**)-15 nucleotides upstream of *ppx* (Fig. 1) presenting the tripartite delineation of this class of promoters ($^{-35}$TTGACA^{-30}/12-16bp/$^{-15}$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/-191; (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 (DRs) sequences (TTCAGCTTGC) from -347 to -338 (DR_1) and from -362 to -353 (DR_2) upstream of *ppx* and with unknown function, and (iv) a putative Pho binding site at -52((CTGCCGCN$_4$GCGACCC); underlined nucleotides match the consensus Pho binding site) -35 from the ATG start site (Fig. 2). In several microorganisms 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. 1) was overlapped with this *pho* box, as proposed by Makino *et al.* (1996). The -24/-12 region belong to a unique class of promoters that requires an activator protein for its expression. These activators, denominated Enhancer Binding Proteins, activate transcription by binding distant sites (enhancers), normally located more than 100 bp upstream of the σ^{54} promoter (Morett & Segovia, 1993). Activation takes place by direct interaction of the EBP with the RNA Polymerase-σ^{54} 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 N control directly exerted by the latter protein. On the other hand, 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 suggest that N and Pi control of *ppx* expression is exerted by the global regulators NtrC and PhoB acting upon two different promoters.

Transcription Start Site (TSS) mapping. To experimentally determine 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 at 8 and 140 nucleotides upstream of the ATG start codon were identified. TSS1 and TSS2 were located 6 and 13 nucleotides downstream of the putative σ^{70} and σ^{54} promoters described above (Fig. 2). Both TSSs were detected using at least two different strategies (Supplementary Fig. S1). Thus, the TSS mapping lend 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

Effect of different upstream sequences on ppx expression. 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) HPi-BSM/S/NH_4^+ ((↑Pi)/S/NH_4^+), a culture condition with all the nutritional requirements in which both PhoB and NtrC are inactive; (ii) (↑Pi)/S/Cho, a culture condition with an excess of Pi and a limiting N source in which PhoB is inactive but NtrC is active; and, (iii) BSM(-Pi)/S/NH_4^+, a Pi-limited condition with NH_4^+ in which PhoB is active but NtrC is inactive.

In (↑Pi)/S/NH_4^+) medium, β-galactosidase activity of the strains $P1_{(500)}$::*lacZ*, $P3_{(355)}$::*lacZ*, and $P8_{(149)}$::*lacZ* were 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 fulfill expression. Consequently we observed that in the strains $P6_{(219)}$::*lacZ* and $P7_{(351)}$::*lacZ* the β-galactosidase activities were reduced more than 75% (69±13, and 72±22 MU, respectively). The latter two strains have the putative -35/-10 σ^{70} promoter deleted. Thus, in high Pi and NH_4^+ *ppx* expression is mainly dependent 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* progressively decreased (982±80, 640±49, 631±52, 320±38, and 63±13 MU, respectively). P2::$lacZ_{(368)}$ (with only DR_1) and $P3_{(355)}$::*lacZ* (without DR_s) 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) conduced to the same mild effect on *ppx* expression. The activity of $P4_{(307)}$::*lacZ* strain (320±38 MU) was almost 67% less than the one of $P1_{(500)}$::*lacZ,* indicating that the putative NtrC binding site is required for full *ppx* expression in this N-limiting growth condition. It was confirmed by using the mutant strain Δ*ntrC* with $P1_{(500)}$::*lacZ* fusion since its reported activity (342±25 MU) was similar to that obtained with $P4_{(307)}$::*lacZ* strain (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 of purified His-NtrC were 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 (Supplementary Fig. S2). These results demonstrated not only the role of the NtrC protein but its DNA binding site in the expression *of ppx* under expression in N-limiting growth conditions.

Strain $P5_{(191)}$::*lacZ,* which does not carry the -12 σ^{54} promoter motif, retained only 9% of reporter activity in comparison with strain $P2_{(368)}$::*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 σ^{54} factor and in the fusion lacking the -12 σ^{54} promoter element was lower than those 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 other one of so many EBPs 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 and it finally resulted in about 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* in N-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 this condition *ppx* expression does not depend on the σ^{70} promoter.

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

Analysis of P1::*lacZ* to P8::*lacZ* strains in (-Pi)/S/NH_4^+ medium helped us to experimentally confirm 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 this culture condition. The activation of this promoter by PhoB was confirmed in cells of PAO1-WT and Δ*phoB* containing the insertion $P8_{(149)}$::*lacZ.* Under Pi-limiting growth condition (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 is 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 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 *Bacilus cereus* (Shi *et al*., 2004). In *Neisseria meningitidis*, a mutant lacking Ppx exhibit increased resistance to complement-mediated killing, and the authors 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 *Mycobacterium 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. The impairment in 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 chains 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*. Rhl controls the expression of genes responsible for the production of rhamnolipids, pyocyanin, and pyoverdine (Jimenez *et al*., 2012). In *P. aeruginosa ppx* null mutant obtained here, there was lower production of these factors, when compared with PAO1-WT strain. The Δ*ppx* strain was also impaired in 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 here, were related to a decrease in biofilm formation and swimming motility, both mechanisms required for attachment to abiotic surfaces. All the results obtained with Δ*ppx* mutant strain were reverted by the insertion of *ppx* gene into the bacterial chromosome of the mutant strain. Thus, the results obtained here demonstrate that, similarly 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 and biofilm formation).

The relationship between *P. aeruginosa* pathogenesis and the nutrient sources to sustain bacterial replication in infected tissues has been subject of many studies. For example,

Son *et al*. (2007) suggested that *P. aeruginosa* degrades amino acids (N depletion), and the principal lung surfactant lipid phosphatidylcholine (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 Pi depletion induces virulence systems in *P. aeruginosa* associated with quorum sensing and iron signaling. Here we studied how the *ppx* gene is transcriptionally regulated in response to various nutritional conditions, including preferential carbon and N sources, carbon and N limitation, and with or without the addition of Pi.

The N-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* gene. We confirmed the functionality of these motifs through physiological and molecular studies. We found that *ppx* expression was substantially reduced in the Δ*rpoN* and Δ*ntrC* strains containing the $P1_{(500)}$ DNA fragment, and the purified NtrC showed a direct binding with the DNA fragment ($P2_{(368)}$) that contains the putative upstream activation sequence for the transcriptional factor, NtrC.

The *ppx* expression was also dependent on Pi concentration in the culture medium. During Pi starvation, the Pho regulon is activated and regulates genes involved in Pi 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 *ppx* gene. It is reasonable to assume that full *ppx* expression is necessary to degrade the internal polyP and to allow bacteria to obtain Pi for their growth. Deletion of *phoB* confirmed that PhoB is the activator of σ^{70}-RNA polymerase in the expression of *ppx* gene. 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 *P. 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 activities 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, unidentified yet. 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 the *E. coli* enzyme. However, our results show that *ppx* expression is regulated at the transcriptional level under nutritional stress conditions, as N and Pi starvation. Based on data presented here and on recent observations made by Rao *et al*. (2009), Achbergerova & Nahalka, (2011) and Österberg *et al*. (2011), we performed a hypothetical model to explain the transcriptional regulation of *ppx* gene expression under Pi or N limiting conditions (Fig. 5). In bacteria under nutritional stress, levels of ppGpp increase, resulting in the recruitment of free RNAP in favor of formation of holoenzyme with alternative sigmas, as σ^{54} (Jishage *et al*., 2002; Österberg *et al*. 2011). Under N starvation, the Two-component NtrB-NtrC system is activated and ppGpp enables NtrC to active the expression of *ppx* promoter through the σ^{54}-RNAP (Fig. 5a). When *P. aeruginosa* is under Pi-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 hydrolyzed by the processive action of Ppx, yielding Pi, plus a shorter polymer. In this regard, it is important to consider that the PPK2 of *P. aeruginosa* is >100-fold induced at stationary phase, at which it preferentially catalyzes 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 evidences of the interrelationships between nutrient availability, polyP levels and the enzymes regulating its metabolism –particularly Ppx. Deregulated polyP-mediated signaling results in a deficient response to nutritional stress and might also impair the production of *P. aeruginosa* virulence factors.

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Figure legends

Fig. 1. Biofilm development, rhamnolipid amount, quorum-sensing and motility behavior 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). Rhammlipids amount was determined in culture supernatants in terms of rhamnose (mg ml^{-1}) (100%=5.57±0.30). The data represent means ± SD; n = 3. (b) Representative quorum-sensing bioassay results of PAO1-WT, Δ*ppx* and Δ*ppx*C strains. (c) Representative swimming phenotypes of PAO1-WT, Δ*ppx* and Δ*ppx*C on 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 swarm plate (LB medium plus 0.5% agar) after 72 h of incubation.

Fig. 2. DNA sequence of the 384 nucleotides upstream and 47 nucleotides downstream of the ATG start codon of the *ppx* gene of *P. aeruginosa* PAO1 strain. 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 boldface. The direct repeat sequences DR_2 and DR_1 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 transcriptional start sites 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 those to the right indicate positions relative to the ATG start codon of *ppx*.

Fig. 3. Effect of N and Pi starvation on *ppx* gene expression. (a) Growth of strain P1::*lacZ* cultured in (↑Pi)/S. At the time indicated by the arrow, the culture was divided into four subcultures, and 20 mM of NH_4^+ (○), Cho (●), histidine (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 Miller Units, MU). (b) Time course of β-galactosidase

activity during the growth of cells described in panel A. (c) Growth of strain P1::*lacZ* cultured in (-Pi)/S/NH_4^+. At the time indicated by the arrow, the culture was divided into five subcultures, which were then added with Pi concentrations of 0.0 mM (○), 0.1 mM (●), 0.2 mM (□), 0.5 mM (■), 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 panel C. The data represent means ± SD; n = 3.

Fig. 4. Schematic diagram of the *ppx* promoter region. The sizes of the P1 to P8::*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 in: (i) (↑Pi)/S/NH_4^+), (ii) (↑Pi)/S/Cho; or (iii) (-Pi)/S/NH_4^+. Cells grown under (↑Pi) condition were harvested after 7 h of growth. Cells grown under (-Pi) condition were collected after 2 h of incubation because no growth was detected. The data represent means ± SD; n = 3.

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

Supplementary Fig. S1: Transcription initiation mapping. (a) 5' RACE was carried out as described in Mendoza et al. 2009. A. TSS1 was located at 8bp upstream of the ATG initiation codon. (b) TSS2 was located at 140 bp upstream of the ATG initiation codon. For TSS1 polyA tail was added at the 5´-RNA end and for TSS2 both polyC and polyA (not shown) were added by terminal transferase, to identify the 5' mRNA end.

Supplementary Fig. S2: His-NtrC binds to the UAS of the *ppx* promoter *in vitro*. The gel retardation assays was performed at pH 5.0 as described in Material and Methods. DNA fragments $P4_{(307)}$ (Lanes 1 and 2) (without the NtrC binding site) and $P2_{(368)}$ (Lanes 3 and 4) (containing the potential NtrC binding site) were amplified by PCR and incubated (+) or not (-) with 4 µg of His-NtrC(S161F) for 20 min at 28ºC, as indicated.

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Fig. 1

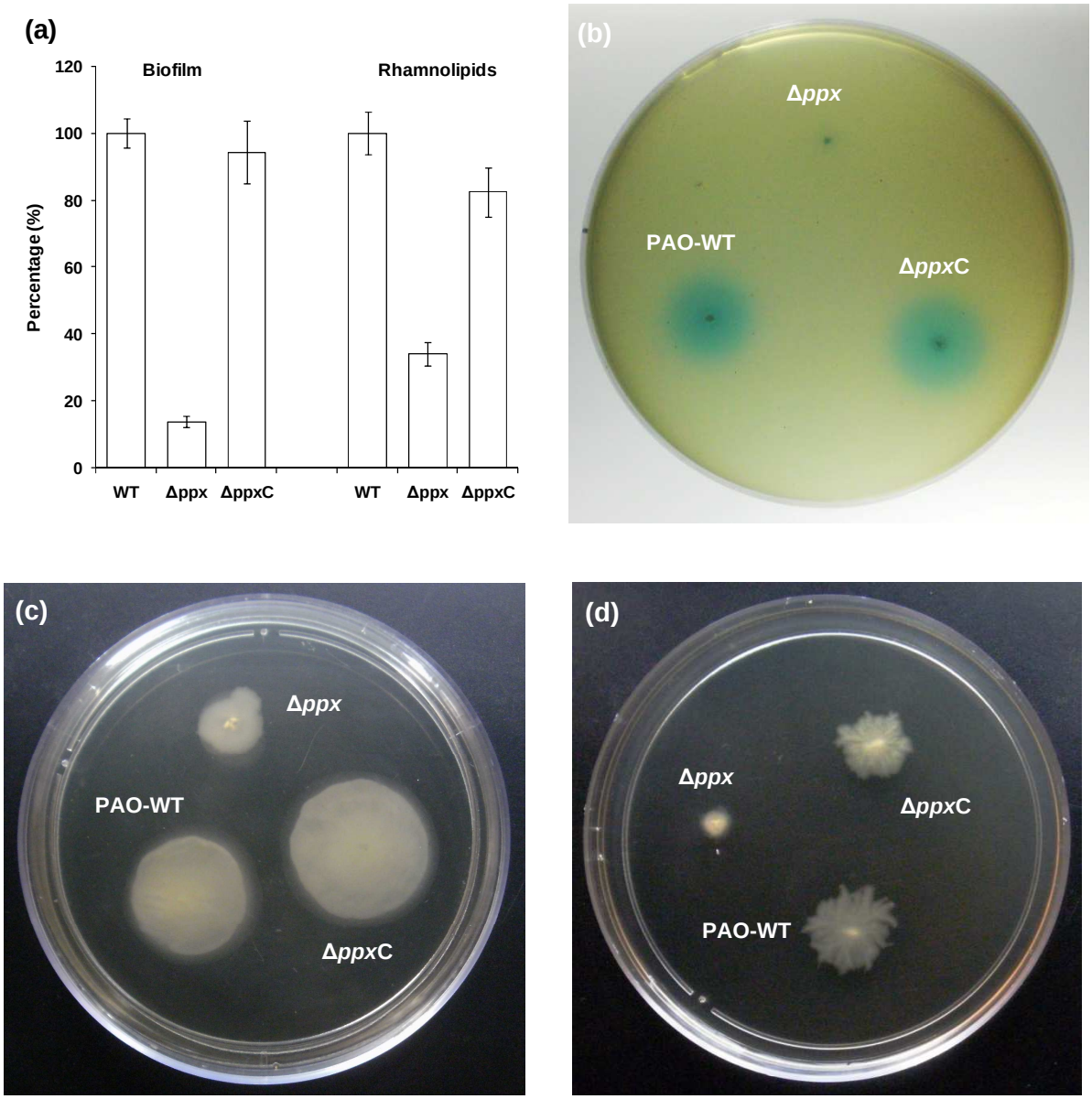

(a)
Biofilm
Rhamnolipids
Percentage (%)
120
100
80
60
40
20
0
WT
Δppx
ΔppxC
WT
Δppx
ΔppxC
(b)
Δppx
PAO-WT
ΔppxC
(c)
Δppx
PAO-WT
ΔppxC
(d)
Δppx
ΔppxC
PAO-WT

Fig. 2

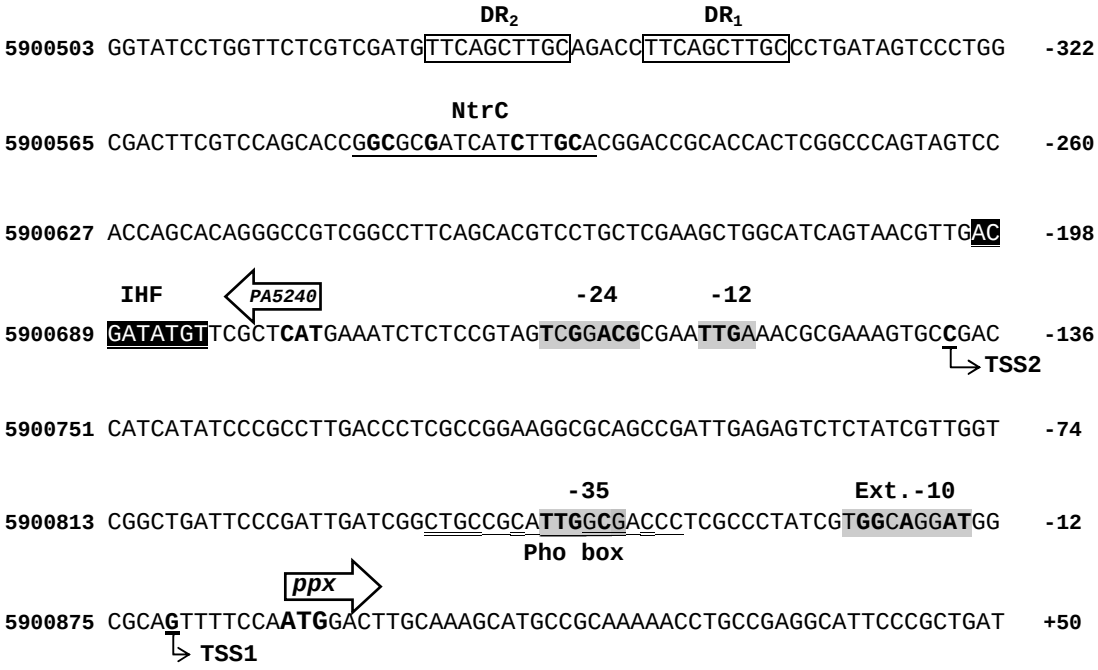

DR2
DR1
5900503 GGTATCCTGGTTCTCGTCGATGTTCAGCTTGCAGACCTTCAGCTTGCCCTGATAGTCCCTGG -322
NtrC
5900565 CGACTTCGTCCAGCACCGGCGCGATCATCTTGCACGGACCGCACCACTCGGCCCAGTAGTCC -260
5900627 ACCAGCACAGGGCCGTCGGCCTTCAGCACGTCCTGCTCGAAGCTGGCATCAGTAACGTTGAC -198
IHF
PA5240
-24
-12
5900689 GATATGTTCGCTCATGAAATCTCTCCGTAGTCGGACGCGAATTGAAACGCGAAAGTGCCGAC -136
TSS2
5900751 CATCATATCCCGCCTTGACCCTCGCCGGAAGGCGCAGCCGATTGAGAGTCTCTATCGTTGGT -74
-35
Ext.-10
5900813 CGGCTGATTCCCGATTGATCGGCTGCCGCATTGGCGACCCTCGCCCTATCGTGGCAGGATGG -12
Pho box
ppx
5900875 CGCAGTTTTCCAATGGACTTGCAAAGCATGCCGCAAAAACCTGCCGAGGCATTCCCGCTGAT +50
TSS1

Fig. 3

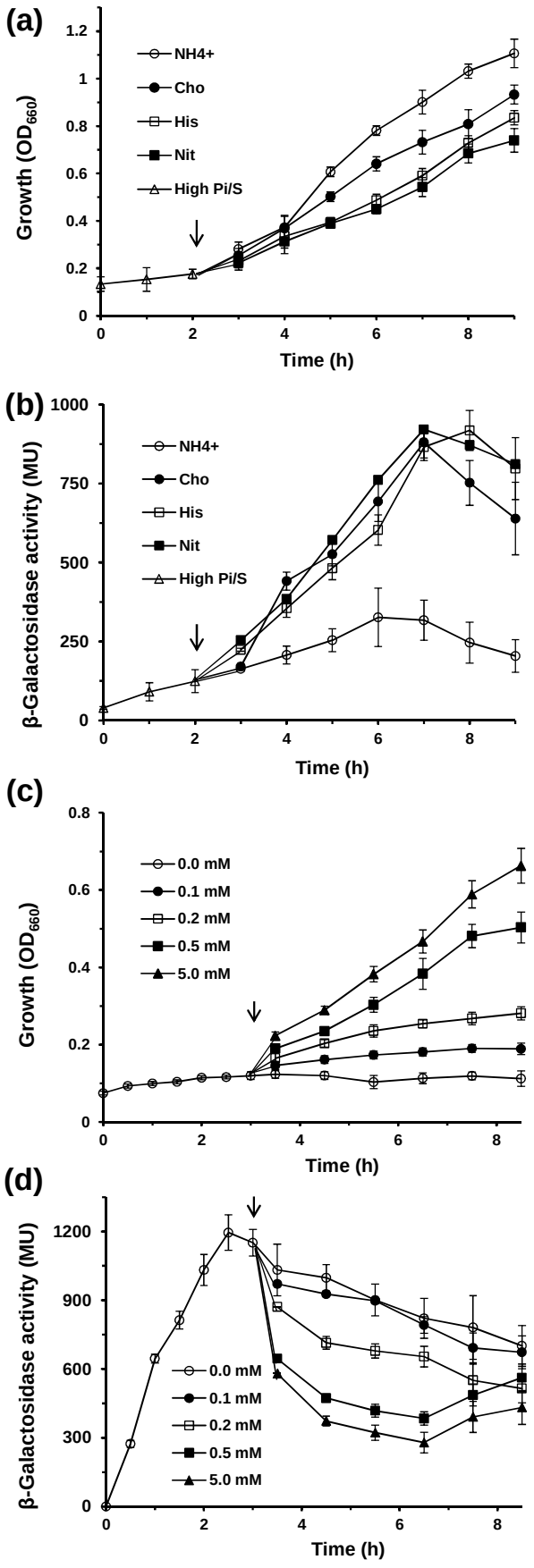
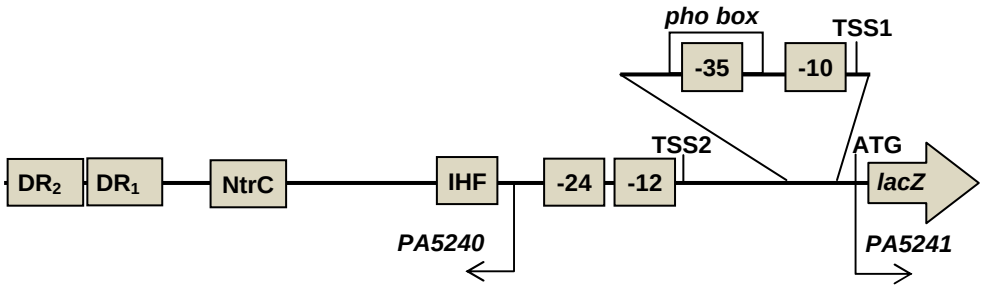

(a)
Growth (OD660)
Time (h)
NH4+
Cho
His
Nit
High Pi/S
(b)
β-Galactosidase activity (MU)
Time (h)
NH4+
Cho
His
Nit
High Pi/S
(c)
Growth (OD660)
Time (h)
0.0 mM
0.1 mM
0.2 mM
0.5 mM
5.0 mM
(d)
β-Galactosidase activity (MU)
Time (h)
0.0 mM
0.1 mM
0.2 mM
0.5 mM
5.0 mM

Fig.4



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 ———	ND	640 ± 49	1098 ± 99
P3(355)::*lacZ*	-337 ———	269 ± 46	631 ± 52	1181 ± 95
P4(307)::*lacZ*	-289 ———	ND	320 ± 38	1035 ± 98
P5(191)::*lacZ*	——— -159	ND	63 ± 13	103 ± 15
P6(219)::*lacZ*	——— -141	69 ± 13	619 ± 32	95 ± 13
P7(351)::*lacZ*	——— -131	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 ———	167 ± 59	103 ± 48	127 ± 71

Fig. 5

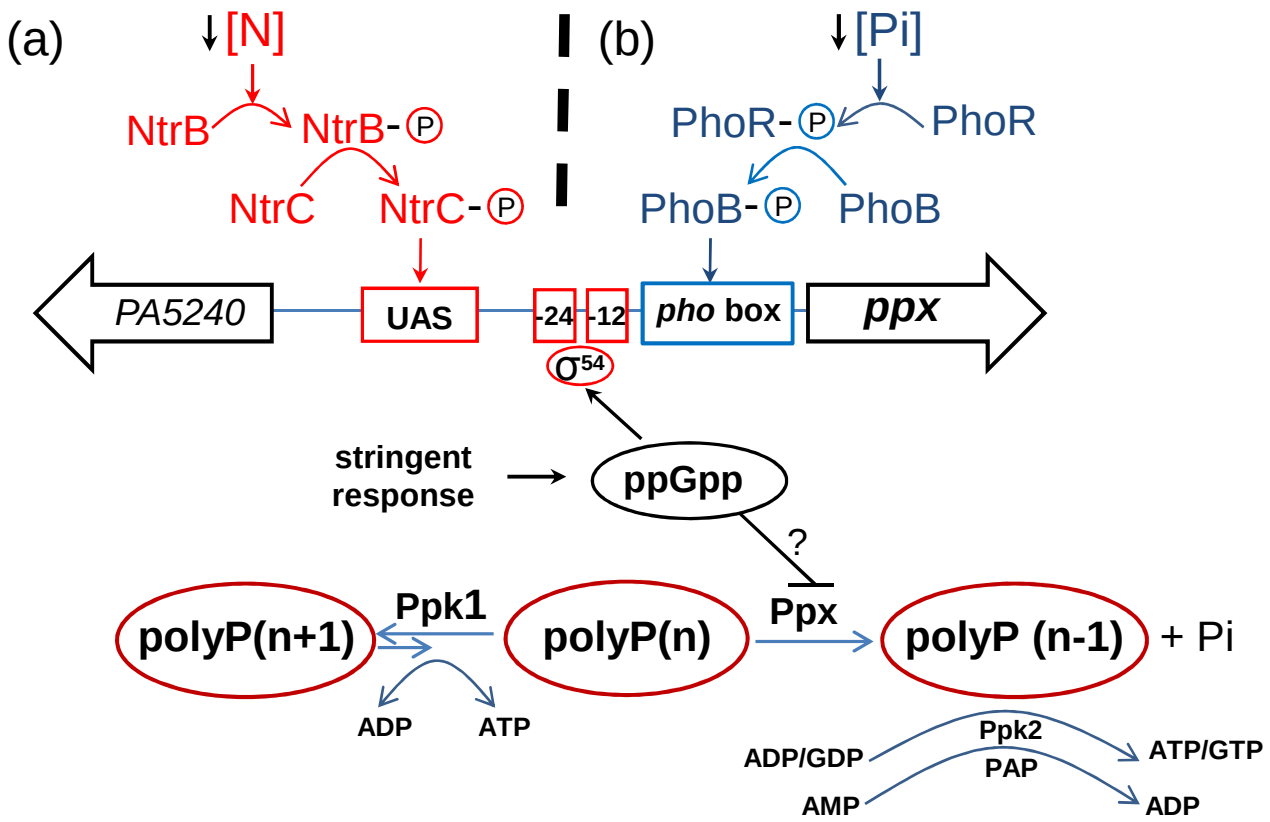

(a)
↓[N]
NtrB
NtrB-P
NtrC
NtrC-P
(b)
↓[Pi]
PhoR-P
PhoR
PhoB-P
PhoB
PA5240
UAS
-24
-12
pho box
ppx
σ54
stringent response
ppGpp
?
Ppx
Ppk1
polyP(n+1)
polyP(n)
polyP (n-1)
+ Pi
ADP
ATP
ADP/GDP
Ppk2
ATP/GTP
PAP
AMP
ADP

Table 1. Bacteria strains and plasmids used in this study

Strain or plasmid	Genotype and/or description	Reference or source
Strains		
E. coli		
XL10-Gold	Tet^r*D(mcrA) D(mcrCB-hsdSMR-mrr)173 end A1 suppE44 thi-1 recA1 gyrA96 relA1 lac Hte* (F$'$ *proAB lacZDM15 Tn10*(Tet^r)*Tn5*	Stratagene
BL21-CodonPlus (DE3)-RIPL	F$^-$*ompThsdS*($r_B^-m_B^-$) *dcm*$^+$Tet^r*gal*λ(DE3) *endA*The[*argUproL*Cam^r] [*argUileYleuW*Strep/Spec^r]	Stratagene
P. aeruginosa PAO1		
PAO1-WT	Wild-type strain	
P1-8::*lacZ*	*P. aeruginosa* PAO1 with a chromosomal integration of mini-Tn7*T* carrying the fusions P1-8::*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-Tn7*T* carrying the fusion pP1::*lacZ*	This study
Δ*rpoN* P1::*lacZ*	PAO1 Δ*rpoN* with a chromosomal integration of mini-Tn7*T* carrying the fusion pP1::*lacZ*	This study
Δ*ppx*	PAO1 Δ*ppx*	This study
Δ*ppx*C	PAO1 Δ*ppx* complemented with *ppx* gene	This study
Δ*phoB*-P1/P8::*lacZ*	PAO1 Δ*phoB* with a chromosomal integration of mini-Tn7*T* carrying the fusions P1 or P8::*lacZ*	This study
*A. tumefaciens*NTL4 (pZLR4)	This strain carries the plasmid pZLR4, which contains *atra*G::*lacZ*fusion and *traR*	Cha *et al*., 1998
Plasmids		
pUC18-mini-Tn7T-Gm-*lacZ*	Gm^r on mini-Tn7T; *lacZ* transcriptional fusion vector	Choi *et al*., 2005
pUC18-mini-Tn7T-Gm	Gm^r on mini-Tn7T	Choi *et al.,* 2005
pTNS2	Ap^r; helper vector encoding the site-specific Tn*7* transposition pathway	Choi *et al*., 2005
pFLP2	Ap^r; Flp recombinase-encoding vector	Choi *et al*., 2005
pDONR221	Km^r; Gateway entry or donor vector	Invitrogen
pEX18ApGW	Ap^r; gene replacement vector, compatible with Gateway system	Choi *et al*., 2005
pPS856	Gm^r; vector carrying Gm resistance gene	Choi *et al*., 2005
pP1 - 8::*lacZ*	Gm^r, Ap^r; pUC18-mini-Tn7T-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 pb *Eco*RI/*Nde*I fragment containing the *ntrC* gene cloned into pET-15b	This study
pUC18-*ppx*C	2018 pb *Spe*I/*Sac*I fragment containing the *ppx* gene plus 500pb upstream.	This study