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Choline catabolism, σ^{54} factor and NtrC are required for the full expression of the *Pseudomonas aeruginosa* phosphorylcholine phosphatase gene

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Abstract

Choline favors the pathogenesis of *Pseudomonas aeruginosa* because hemolytic phospholipase C and phosphorylcholine phosphatase (PchP) are synthesized as a consequence of its catabolism. The experiments performed here resulted in the identification of the factors that regulate both the catabolism of choline and the gene coding for PchP. We have also identified and characterized the promoter of the *pchP* gene, its transcriptional organization and the factors that affect its expression. Deletion analyses reveal that the region between −188 and −68 contains all controlling elements necessary for *pchP* expression: a hypothetical −12/−24 promoter element, a consensus sequence for the integration host factor (−141/−133), and a palindromic sequence resembling a binding site for a potential enhancer binding protein (−190/−174). Our data also demonstrate that choline catabolism and NtrC (nitrogen regulatory protein) are necessary for the full expression of *pchP* and is partially dependent on σ^{54} factor.

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1. Introduction

When *Pseudomonas aeruginosa* is cultured in choline, glycine betaine (GB), dimethylglycine (DMG) (Lisa et al., 1994, 2007) or carnitine (Lucchesi et al., 1995) in the presence of high or low Pi concentrations, phosphorylcholine phosphatase (PchP) is synthesized. PchP catalyzes the hydrolysis of phosphorylcholine to choline and Pi. Because choline correlates directly with the production of phospholipase C (PlcH) in high or low Pi concentrations, we previously proposed that choline could be a factor that favors the pathogenesis of *P. aeruginosa* (Lisa et al., 1994, 2007). Based on the determination of enzymatic activities of PlcH and PchP, we have reported that the synthesis of these proteins was subject to catabolic repression control (Lisa et al., 1983, 1994). The *pchP* gene has been identified in the *P. aeruginosa* genome (Massimelli et al., 2005), cloned and subsequently expressed to study biochemical and biophysical properties of PchP (Beassoni et al., 2006, 2008, 2010; Otero et al., 2010). NtrC and CbrB are the corresponding response regulators of the two component systems, NtrBC and CbrAB, that control the expression of genes involved in the use of different substrates that provide carbon and nitrogen sources in *P. aeruginosa* and other bacteria (Nishijyo et al., 2001; Li and Lu, 2007; Zhang and Rainey, 2008). Alternatively, Wargo et al. (2009) reported that GbdR, a transcription factor of the AraC family, is required for the induction of *pchP*. Considering our previous results and those described above, we focused our experiments on the following key point: to investigate the global effect of the CbrB and NtrC on the control of choline utilization as carbon and nitrogen sources, and the possible roles of these global regulators in the controlling the transcription of *phcP*, that is induced by choline.

2. Material and methods

2.1. Bacterial strains and plasmids

Strains and plasmids are shown in Table 1. *Escherichia coli* and *Pseudomonas* strains were cultured in Luria–Bertani or high phosphate basal salt medium (HPi-BSM) (Lisa et al., 1994) at 37 °C. Carbon and nitrogen sources were added at 20 mM. Antibiotics were used at the following concentrations, listed in $\mu g\,mL^{-1}$: *E. coli* were treated with ampicillin (Ap), 100 or 150, and gentamycin (Gm), 15; the concentration used for *P. aeruginosa* were: Gm, 30; carbenicillin (Cb), 100–200.

2.2. Strain construction

The Δ*ntrC* (PAO160), Δ*cbrB* (PAO161), Δ*gbdR* (PAO164) and the double Δ*ntrC*Δ/Δ*cbrB* (PAO165) deletion mutant strains were obtained following methods described previously (Choi and Schweizer, 2005). Gene-specific fragments (*gene*-Up and *gene*-Dwn) were amplified using the primers. To obtain PAO1-Δ*ntrC*, the following primers were used: *ntrC*-UpF-GWL, *ntrC*-UpR-Gm, *ntrC*-DwnF-Gm, and *ntrC*-DwnR-GWL. To obtain PAO1-Δ*cbrB*, the following primers were used: *cbrB*-UpF-GWL, *cbrB*-UpR-Gm, *cbrB*-DwnF-Gm, and *cbrB*-DwnR-GWL. To obtain PAO1-Δ*gbdR*, the following primers were used: Gw-Up-F-5380, Gm-Up-R-5380, Gm-Dn-F-5380 and Gw-Dn-R-5380.

2.3. DNA and RNA manipulations

DNA manipulations and PCR amplifications were performed as described previously (Sambrook and Russel, 2001). Chromosomal and plasmid DNA purifications were performed using QIAamp DNA and QIAprep Mini-spin kits (Qiagen), respectively. The transcriptional fusion between *lacZ* and the different fragments (called P_{1-6}) belonging to a section of PA5292, the intergenic region and a section of PA5293, and their subsequent insertion into the bacterial chromosome, were performed as described (Choi and Schweizer, 2005; Choi et al., 2006). P_{1-6} fragments were PCR-amplified from genomic DNA templates using the following pairs of primers: for P1, P*pchP*-UP2 and P*pchP*-DN; for P2, P*pchP*-UP2 and P*pchP*-DN2; for P3, P*pchP*-UP and P*pchP*-DN2; for P4, P*pchP*-UP5 and P*pchP*-DN2; for P5, P*pchP*-UP2 and P*pchP*-DN4; for P6: P*pchP*-UP4 and P*pchP*-DN. The PCR fragments were cloned into pCR2.1 (P_1, P_2, P_3, P_6) or into pCR2.1-TOPO (P_4, P_5) to yield the following plasmids: pPS1972, pPS1974, pPS1996, pPS1975, pPS2000ar and pPS1999ar, respectively. These plasmids were digested and then ligated into pUC18-mini-Tn*7*-Gm-*lacZ*, to produce p$P_{(1-6)}$::*lacZ*. These fusion plasmids and pTNS2 were co-transformed into *P. aeruginosa* by electroporation (Choi et al., 2006). Colony PCR using the primers P_{Tn7R} and $P_{glmS\text{-}down}$ was used to verify chromosomal Tn*7* insertions; the Gm marker was excised as described (Choi and Schweizer, 2005). β-Galactosidase assays were performed as in Miller (1972). For site-directed mutagenesis, promoter regions were mutated using the Quickchange mutagenesis kit (Stratagene). The following primers were employed: for −80TTG−78 deletion, 12.a; for −79T/G substitution, 12.b; for −74TA−73 deletion and −69T/C substitu-

Table 1. Bacterial strains and plasmids.

Strain or plasmid	Genotype and/or description	Reference or source
Strains		
E. coli		
HPS1	F$^-$ Δ(*lac-proAB*) *endA1 gyrA96 hsdR17 supE44 relA1 recA1 thi* Rif^r *zzx*::mini-Tn*5*Lac4	Schweizer (1994)
Top10F′	F′ {*lacI*^{q} Tn*10*(Tet^R)} *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Δ80*lacZ*ΔM15 Δ*lacX74 deoR recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL* (Str^R) *endA1 nupG*	Invitrogen
P. aeruginosa		
PAO1-MV	Prototrophic wild-type strain	
PAO1-P$_{1-6}$::*lacZ*	*P. aeruginosa* PAO1 with a chromosomal integration of mini-Tn*7T* carrying the fusions pP $_{(1-6)}$::*lacZ*	This study
PAO160	PAO1 Δ*ntrC*	This study
PAO161	PAO1 Δ*cbrB*	This study
PAO6358	PAO1 Δ*rpoN*	Heurlier et al. (2003)
PAO6359	PAO1 Δ*rpoN* (PAO6358) with a chromosomal integration of mini-Tn*7T* carrying the fusion pP$_1$::*lacZ*	This study
PAO6360	Δ*rpoN att* Tn*7*::*rpoN*′$^+$ Gm^r	Heurlier et al. (2003)
PAO6361	Δ*rpoN att* Tn*7*::*rpoN*′$^+$ Gm^r (PAO6360) with a chromosomal integration of mini-Tn*7T* carrying the fusion pP$_1$::*lacZ*	This study
PAO162	PAO160 with a chromosomal integration of mini-Tn*7T* carrying the fusion pP$_1$::*lacZ*	This study
PAO163	PAO161 with a chromosomal integration of mini-Tn*7T* carrying the fusion pP$_1$::*lacZ*	This study
PAO164	PAO1 Δ*gbdR*	This study
PAO165	PAO1 Δ*ntrC*/Δ*cbrB*	This study
Plasmids		
pCR2.1	Ap^r; TA cloning vector	Invitrogen
pCR2.1-TOPO	Ap^r; TA cloning vector	Invitrogen
pUC18-mini-Tn*7T*-Gm-l*acZ*	Gm^r on mini-Tn*7T*; *lacZ* transcriptional fusion vector	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)
pPS1972	Ap^r; P1 promoter fragment of 282 bp cloned in pCR2.1	This study
pPS1974	Ap^r; P2 promoter fragment of 188 bp cloned in pCR2.1	This study
pPS1975	Ap^r; P6 promoter fragment of 176 bp cloned in pCR2.1	This study
pPS1996	Ap^r; P3 promoter fragment of 144 bp cloned in pCR2.1	This study
pPS1999ar	Ap^r; P5 promoter fragment of 157 bp cloned in pCR2.1-TOPO	This study
pPS2000ar	Ap^r; P4 promoter fragment of 126 bp cloned in pCR2.1-TOPO	This study
pP$_{1-6}$::*lacZ*	Gm^r, Ap^r; pUC18-mini-Tn*7T*-Gm-*lacZ* with a *Xho*I-*Nsi*I fragment from pPS1972, pPS1974, pPS1996, pPS2000ar, pPS1975	This study

tion, 10.a; for −66TTG−64 and −57G deletion, 12dwa; for −62T and −59TTG−57 deletion, 12dwb; and for substitutions of −141ACAACTCCT−133 to ACCCCTCCC, IHFmsd-a. RNA was extracted as described (Ochsner et al., 1999) and transcribed into cDNA using reverse transcriptase (Invitrogen or Promega) with random hexamers provided in the kit, or using primers RT-PCR-UP, P92 and JS1. Northern blot analyses were performed as described (Sambrook and Russel, 2001). Quantitative real-time PCR was performed using 5-fold dilutions of the cDNA obtained with reverse transcriptase and random hexamers (Invitrogen), as described above; the primers *pchP*-for-RT3 and *pchP*-rev-RT3 were used. The PCR reactions were performed in 96-well optical reaction plates (Applied Biosystems) using the Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen). Results were obtained using the software provided with the Applied Biosystems 7500 Real-Time PCR system cycler. The relative expression of *pchP* was determined using the $2^{-\Delta\Delta CT}$ (Livak) method. The *rpsL* gene was used as an internal control in relative quantification studies.

2.4. Determination of the transcription start site (TSS)

The TSS of the *pchP* gene was determined with 5′ RACE as described (Mendoza et al., 2009; Olvera et al., 2009). Briefly, cDNA libraries of *P. aeruginosa* were generated using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, USA) using a random hexamer primer-adaptor B (5′-GCCTTGCCAGCCCGCTCANNNNNN-3′). PCR was performed using the program: 28 °C for 20 min, 45 °C for 40 min, 70 °C for 10 min. The final cDNA product was purified using the High Pure PCR product purification kit (Roche Indianapolis, USA). Purified cDNA libraries were labeled (tagged) at the 3′ terminal end by ligating a double stranded synthetic DNA molecule (5′-GCCTCCCTCGCGCCATCAGNNNNNN-3′, 3′-CGGAGGGAGCGCGGTAGTC-5′). Five microliters of purified cDNA and 35 pmol of the synthetic adaptor were incubated at 16 °C overnight with T4 DNA ligase (1 Weiss U/μL) in 1× reaction buffer and a final reaction volume of 25 μL. All reagents were purchased from Fermentas (St. Leon-Rot, Germany). The tagged cDNA library was amplified by PCR using primer adaptors A (5′-GCCTCCCTCGCGCCATCAG-3′) and B (5′-GCCTTGCCAGCCCGCTC-3′) under the following conditions: 1 cycle at 94 °C for 2 min; 35 cycles of 94 °C for 1 min, 59 °C for 45 s and 72 °C for 45 s; 1 cycle at 72 °C for 2 min. Both types of amplified samples were purified using the High Pure PCR product purification kit (Roche, Indianapolis, IN, USA). Primer-specific PCR amplification and nucleotide sequence determination for TSS identification was performed by using the cDNA pool as a template to selectively amplify the *pchP* gene, using the high fidelity PCR system (Fast Start High Fidelity PCR System, Roche USA). This PCR was performed with adaptor A (5′-GCCTCCCTCGCGCCATCAG-3′) and a primer that specifically anneals to the complementary strand of the *pchP* gene cDNA (5′-GATAGCTGGTGTTGTCCATGTC-3′). A sample of the PCR product was analyzed by 8% native polyacrylamide gel electrophoresis and the band was purified from the gel. Finally, the nucleotide sequences of the purified PCR products were determined using the *pchP* primer. Sequencing reactions were performed in an Applied Biosystems 3100 Genetic Analyzer/ABI PRISM device. The TSS was identified as the first nucleotide immediately adjacent to the adaptor.

2.5. Bioinformatics analysis

Promscan.pl Perl script (Studholme and Dixon, 2003) was used to identify σ^{54} promoters with a scoring matrix derived from a compilation described by Barrios et al. (1999). Gene context analysis was performed as described by Ciria et al. (2004).

3. Results

3.1. The physiological role of NtrC and CbrB in choline catabolism

To test the physiological role of NtrC and CbrB in choline utilization, growth phenotype analyses were performed with the mutants Δ*ntrC* (PAO160), Δ*cbrB* (PAO161), and the double mutant Δ*cbrB*/Δ*ntrC* (PAO165), cultured in medium supplemented with succinate/NH_4^+, choline, choline/NH_4^+ or choline/succinate (Table 2). The growth of the three mutants was comparable to the wild-type strain PAO1. The Δ*ntrC* (PAO160) mutant strain grew normally in choline or in choline/NH_4^+, but its growth was deficient in choline/succinate. In this latter case, there was not a problem in the change of the pH medium. It was a metabolic problem: in PAO160 the *ntrC* gene was deleted, but the synthesis of CbrB (the carbon regulatory protein) occurred because *cbrB* gene was intact. Therefore, PAO160 grow well in choline or choline/NH_4^+ because the enhancer protein CbrB is activated and as a consequence choline is metabolized

Table 2. Growth of different mutant strains on organic compounds as carbon and nitrogen sources.

Strains	Growth on			
	Succinate + NH_4	Choline	Choline + NH_4	Choline + succinate
PAO1 (wild-type)	1.2 ± 0.15	0.85 ± 0.10	0.90 ± 0.15	0.95 ± 0.10
PAO161 (Δ*cbrB*)	1.1 ± 0.20	0.19 ± 0.05	0.18 ± 0.04	0.79 ± 0.15
PAO160 (Δ*ntrC*)	1.1 ± 0.15	0.75 ± 0.01	0.80 ± 0.07	0.3 ± 0.02
PAO165 (Δ*ntrC*/Δ*cbrB*)	1.0 ± 0.22	0.10 ± 0.02	0.12 ± 0.01	0.10 ± 0.01
PAO164 (Δ*gbdR*)	1.0 ± 0,20	0.15 ± 0.03	0.10 ± 0.01	0.10 ± 0.03

Growth of each culture was tested on liquid HPi-BSM supplemented with the indicated carbon and nitrogen sources (20 mM), in rotary bath at 37 °C. The bacterial growth for 24 h and the absorbance at 660 nm was determined. Data are repetition of at least three independent experiments.

and the strain grew. In succinate/choline-culture medium, CbrB may not be activated because the presence of succinate inhibits its expression. The strain PAO161 (with a deletion of the *cbrB* gene and an intact *ntrC* gene) grew well when choline was the nitrogen source in the presence of a sufficient amount of succinate. Under this latter condition, the growth was proportional to its concentration in the culture medium, but if succinate was less than 2.5 mM growth ceased at an early stage (Fig. 1). The double mutant strain Δ*cbrB*/Δ*ntrC* (PAO165) did not grow in any culture condition where choline was present. However, the growth of PAO165 was similar to the wild-type strain PAO1 in succinate/NH_4^+ (Table 2), and it was logical because in this culture condition it was not necessary the presence of an active CbrB nor NtrC. Therefore the above results led us to consider that an intact *cbrB* gene might activate choline catabolic genes, and *ntr*C gene may have a role when choline was used as a sole nitrogen source. As GbdR has been described as a regulator required for choline catabolism (Wargo et al., 2008), the

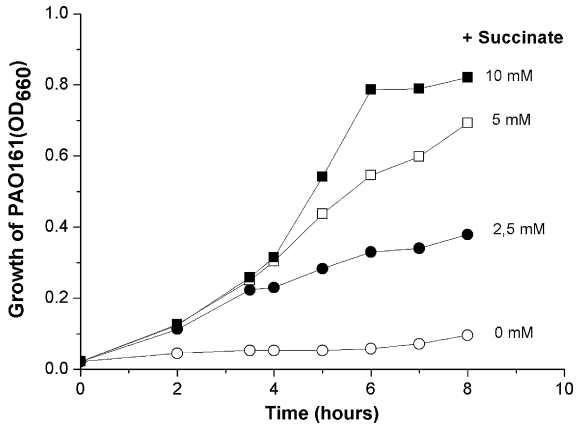

Fig. 1. Growth of mutant PAO161 (Δ*cbrB*) on HPi-BSM/20 mM choline supplemented with 0.0 (●), 2.5 (◯), 5.0 (■) or 10.0 mM (□) succinate. The results are the means of three independent experiments.

growth of mutant PAO164 (Δ*gbdR*) was examined in media containing choline, choline/NH_4^+ or choline/succinate (Table 2). Minimal growth was observed in the tested culture conditions for at least 24 h. Therefore, this latter result supports the previous one described by Wargo et al. (2008), who indicated a direct role of GbdR on choline catabolism genes. Additionally, with the experiments described here, CbrB and NtrC are also essential in the metabolism of choline. As choline is involved in the synthesis of PchP, and the genes of choline catabolism are regulated by NtrC and CbrB, our next goal was to understand if these global regulators were also involved in the expression of the *pchP* gene. The first step in accomplishing this goal was to discover the transcriptional organization of *pchP* gene.

3.2. Transcriptional organization of the *pchP* gene

First, we characterized the promoter and studied the transcriptional organization of *pchP* that is shown in Fig. 2a. The *pchP* gene (PA5292) is located adjacent to a putative transcriptional regulator of the LysR family (PA5293); both genes are transcribed in the same orientation. In our experimental conditions, the product of PA5293 gene did not interfere or was not significantly important with the expression of *pchP*, because in a deleted mutant (Δ*lysR*) the expression of *pchP* was in the same order than in the control. In choline medium, the β-gal activity in PAO1::lacZ and ΔPA5293 P1::lacZ was 3900 ± 150 and 4350 ± 90 Miller units, respectively. A putative choline/glycine betaine transporter is located on the opposite DNA strand in a convergent orientation. Northern blot analyses revealed that *pchP* is transcribed monocistronically; a band of approximately 1 kb, the expected size for the *pchP* transcript, was observed in samples from cells grown with choline (Fig. 2b). RT-PCR analy-

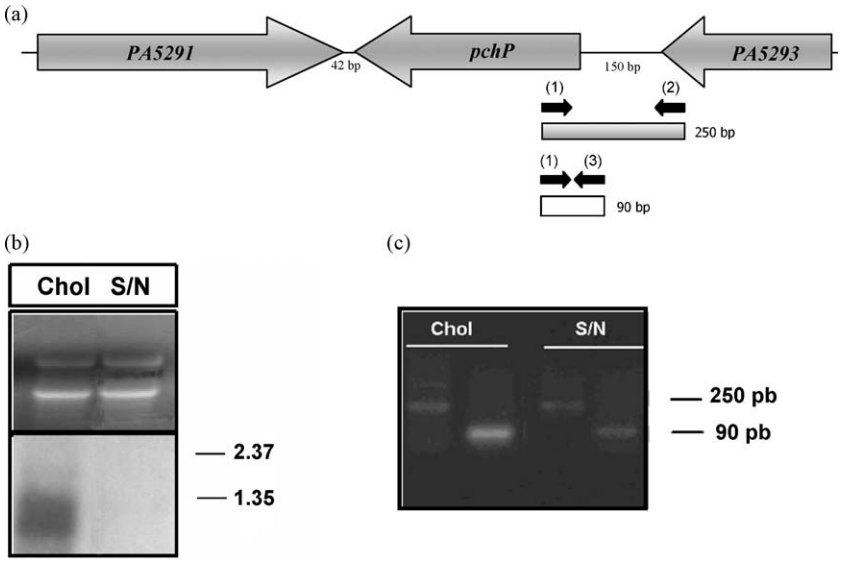

Fig. 2. (a) Genetic context of the *pchP* gene in the *P. aeruginosa* genome. Large arrows indicate ORFs, and genes are indicated inside the arrows. Primers used for RT-PCR are indicated by small arrows and numbered as follows: (1) RT-PCR-UP, (2) P92right, and (3) JS1. (b) Northern blot analyses. 7 μg RNA was obtained from wild-type *P. aeruginosa* cultured in HPi-BSM with 20 mM choline (Chol) or succinate plus NH_4Cl (S/N), applied to each lane and hybridized with a radio-labeled *pchP*-specific probe, obtained as described (Massimelli et al., 2005). RNA size markers (in kb) are indicated on the right. (c) Transcriptional analysis of *pchP* by RT-PCR. RNA from bacteria cultured in HPi-BSM supplemented with 20 mM choline (Chol), or succinate/NH_4Cl (S/N) was converted to cDNA; two PCR reactions, using the primers described in panel (a) were performed. The sizes of the expected PCR fragments (indicated in bp) were estimated by comparison to Hi-Lo molecular size markers, indicated on the right.

sis confirmed that *pchP* and PA5293 are transcribed independently. This experiment was conducted with RNA isolated from bacteria grown with choline or succinate/NH_4^+ and with primers complementary to sequences 1, 2, and 3 depicted in Fig. 2a. Two PCR products, 90 and 250 bp, were obtained (Fig. 2c). The 90-bp product was amplified more in cDNA derived from the choline-cultured cells compared to cDNA from succinate/NH_4^+-cultured cells (Fig. 2c). The 250-bp product was amplified at a basal level in cells grown under both culture conditions and apparently resulted from a low level of read-through from PA5293 (Fig. 2c).

3.3. Identification of the *pchP* promoter upstream region

Bioinformatic analyses of the putative regulatory region, comprised of 232 bp upstream and 58 bp downstream of the ATG initiation codon (Fig. 3a), revealed a sequence with high similarity to the −24/−12 promoter element for the σ^{54} factor, between −90(**GGC**GCA-N_4-**TTGC**)−77, with a score of 0.75. In position −141 to −133, a consensus sequence for the binding of the Integration Host Factor (IHF) was identified (Fig. 3a). A site with a 6 bp palindromic sequence with a 5 bp space (**G**GC**G**G**C**-N_5-**G**T**C**TG**C**) was found in position −190 to −174 (Fig. 3a), which could be a binding site for an Enhancer Binding Protein (EBP). Experiments with various fragments (P_1−P_6) of the *pchP* putative regulatory region fused to *lacZ* and integrated into the PAO1 chromosome indicated that PAO1-P_{1-3}::*lacZ* strains grown with choline expressed high levels of β-galactosidase activity (Fig. 3b). Cells carrying P_4, P_5 and P_6::*lacZ* fragments produced about 10-fold lower levels of activity (Fig. 3b). Therefore, regions −231 to −188 and −44 to −50 were dispensable, and the regions −74 to −44 and −188 to −170 were significant for full *pchP* expression. More tune experiments using site-directed mutations indicated that the minimal region required for *pchP* expression was located between −188 and −68 bp from the ATG start codon. This was supported by the following results: (i) deletion in the putative −12 element, −80(~~TTG~~)−78, reduced *pchP* expression by 95% (from 3360 ± 95 to 180 ± 25 Miller units); (ii) deletion and nucleotide substitution of −74(TAGGGT)−69 to −74(~~TA~~GGG**C**)−69 reduced *pchP* expression more than 90% (from 3360 ± 95 to 200 ± 36 Miller units); (iii) deletion of nucleotides downstream of position −69, such as −66(~~TTG~~)−64/−57(G)/−62(~~T~~), or −59(~~TTG~~)−57 did not affect *pchP* expression (3360 ± 95 in

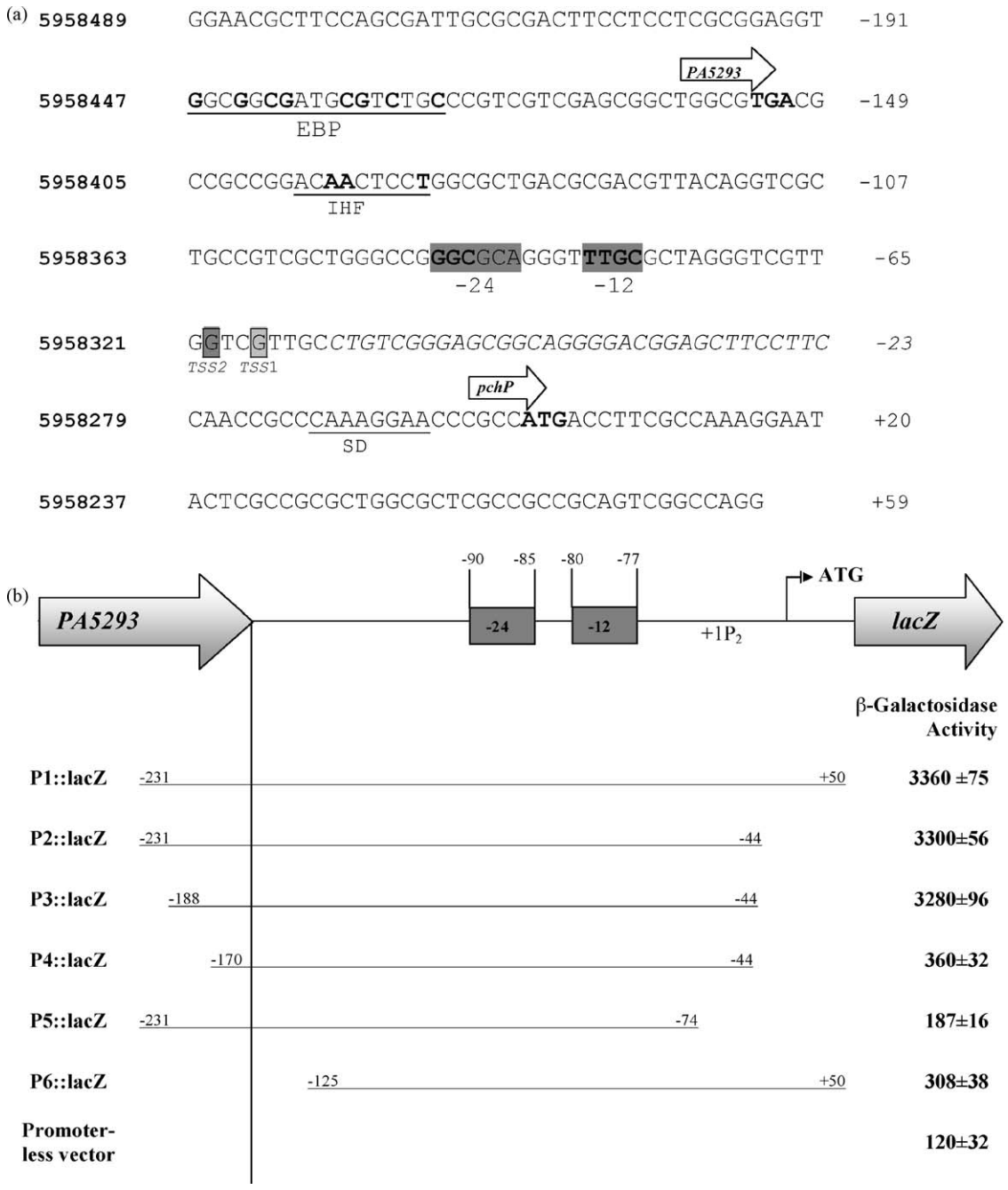

Fig. 3. Organization of *pchP* promoter region of *P. aeruginosa*. (a) Nucleotide sequence of the 290 nucleotides of the 3′ end of PA5293, the intergenic region and the 5′ region of the *pchP* gene in the *P. aeruginosa* genome. The conserved −24/−12 elements of the σ^{54}-dependent promoter are boxed. The consensus sequences of −24/−12, the stop codon (TGA) of PA5293, and the start codon (ATG) of the *pchP* gene are highlighted in boldface type. The putative IHF binding site, predicted Shine-Dalgarno (SD) sequences and the potential EBP binding site are underlined; consensus sequences are highlighted. The transcriptional start sites, as determined by 5′-RACE analysis are boxed and indicated by +1P2" and +1P1". The numbers to the left and right of the sequences denote nucleotide positions in the *P. aeruginosa* genome and relative to ATG, respectively. (b) Schematic representation of the *pchP* promoter region. P_{1-6}::*lacZ* constructs, indicated by lines, were integrated into the PAO1 chromosome, and the β-galactosidase activities (Miller units) were measured in cells grown in HPi-BSM supplemented with 20 mM choline. Results represent the average of three independent experiments.

PAO1-P1::*lacZ* vs. 3200 ± 130 and 3450 ± 112 Miller units in the mutations, respectively); (iv) the substitution (−79T/G) resulted in an almost 3-fold increase in *pchP* expression relative to the wild-type (3360 ± 95 and 8900 ± 170 Miller units, respectively); (v) mutations in the region resembling the IHF binding site −141(ACAACTCCT)−133 to −141(ACCCCTCCC)−133 significantly reduced *pchP* expression (3800 ± 120 and 450 ± 25 Miller Units in PAO1-P_1::*lacZ* and the mutant, respectively); (vi) deletion of the −190 to −174 region, as demonstrated by the comparison of β-galactosidase activities between P_3 and P_4::*lacZ* fragments (see Fig. 3), indicated that the putative EBP binding site was essential for full *pchP* expression.

3.4. Determination of the transcriptional start site (TSS)

In order to unambiguously locate the promoter controlling *pchP* expression, we carried out experiments to map the TSS using a modified 5′RACE methodology. Two very close initiation events originated at G residues located −60 and −63 bp upstream from the ATG start codon, TSS(*pchP*) + 1, TSS(*pchP*) + 2 in Fig. 3a. The presence of TSS(*pchP*)2 is 14 nucleotides downstream of the putative σ^{54} −12 element, providing further evidence that this promoter is transcribed by this sigma factor.

3.5. The kinetics of the *pchP* gene in choline growth medium and the effect of NtrC, CbrB and RpoN on its expression

Choline-grown cells of PAO1-P_1::*lacZ* revealed that β-galactosidase activity progressively increased during the lag phase of bacterial growth and the maximal level of activity was at an OD_{660} ≅ 0.20−0.25 (≅6500 ± 500 Miller units). After this point the enzymatic activity decreased almost 50% (≅3200 ± 200 Miller units) and remained constant until the culture reached the stationary phase of growth (Fig. 4). When ammonium or succinate was added to choline-grown cells, at the beginning of the logarithmic phase (before an OD_{660} of 0.2), the growth patterns were similar in both culture conditions. The expression of the *pchP* promoter in both cases (not shown) was similar to the control (choline-grown cells, Fig. 4). When succinate and NH_4^+ were added to the choline-culture together, *pchP* expression decreased drastically (≅1000 ± 500 Miller units) and remained at a low level throughout bacterial growth. The higher expression in choline-relative to succinate/NH_4^+-grown cells was confirmed by quantitative real-time PCR. Relative expression of the *pchP* gene in choline medium was 8-fold higher relative to succinate/NH_4^+-cultured cells, using the *rpsL* gene as an internal control.

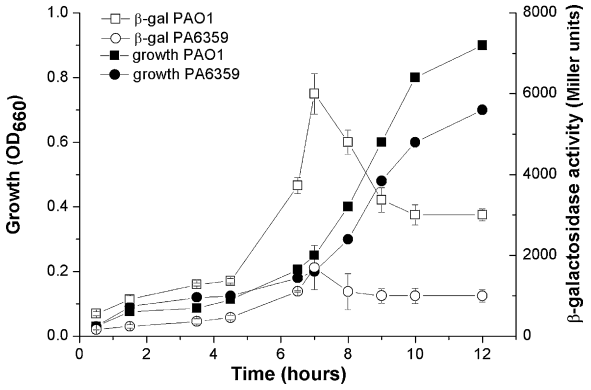

Fig. 4. Time course of β-galactosidase activity (expressed as Miller units) from wild-type *P. aeruginosa* PAO1 (□) and the PAO6359 strain (○), both bearing the P1::*lacZ* fusion integrated into the chromosome, and the growth of both strains *P. aeruginosa* PAO1 (■) and the PAO6359 strain (●) cultured in HPi-BSM/20 mM choline and inoculated from an overnight culture grown in LB (1:50). Results represent the average of three independent experiments.

To assess whether NtrC or CbrB are required for the activation of *pchP*, the P_1::*lacZ* construct was introduced into the bacterial chromosome of Δ*ntrC* (PAO160) strain and Δ*cbrB* (PAO161) strains to obtain the strains PAO162 and PAO163, respectively (Table 1). β-galactosidase activity of PAO162-P1::*lacZ* strain grown in choline was measured to be less than 35% of the activity obtained in PAO1-P1::*lacZ* (6500 ± 500 vs. 2200 ± 150 Miller units). The β-galactosidase activity of the PAO163-P1::*lacZ* strain grown in choline/succinate was similar (7000 ± 380 Miller units) to that observed for PAO1-P_1::*lacZ* (6500 ± 500 Miller units), measured in cells when maximal promoter expression occurred (OD_{660} ≅ 0.25). To determine whether *pchP* expression depends solely on the presence of activated NtrC, a condition that occurs in cells of *P. aeruginosa* grown in histidine (Li and Lu, 2007). PAO1-P_1::*lacZ* was grown with histidine/succinate or choline/succinate, and β-galactosidase activities were determined in both culture conditions (437 ± 80 Miller units, compared with the 4900 ± 105 Miller units observed in choline/succinate-grown cells). Therefore, it is possible to conclude that although the concentration of NtrC in histidine-cultured cells is high, the presence of choline is necessary for the expression of *pchP*.

As NtrC and CbrB are enhancers of σ^{54} promoters, strain PAO6358 (Δ*rpoN*) was also included in

this study. This mutant strain was able to grow with choline when used as a carbon and nitrogen source and showed no difference in growth rate compared to wild-type strain. To find out if σ^{54} was involved in the transcription of *pchP* promoter, the P_1::*lacZ* fusion was integrated into the chromosome of the PAO6358 mutant strain, giving rise, PAO6359 strain. In choline-grown cells, the wild-type and the PAO6359 strains reached stationary phase at similar levels (Fig. 4), but the β-galactosidase activity was highly reduced (≅75%) in the mutant compared to the PAO1-P_1::*lacZ* strain (Fig. 4). The complemented PAO6361 (with the chromosomal insertion P_1::*lacZ*) displayed almost a complete restoration of β-galactosidase activity, compared to the activity obtained in the wild-type strain at an OD_{660} of 0.5 (3500 ± 150 and 3800 ± 96 Miller units, respectively).

4. Discussion

From the results shown here, it is reasonable to assume that the regulation of choline metabolism may reflect an adaptive response of *P. aeruginosa* to environmental conditions in which this compound is commonly found. CbrB is indispensable only when choline is the carbon source, and both CbrB and NtrC are crucial when choline is the nitrogen source. These results appear to be consistent with the model of Itoh et al. (2007) who showed (with histidine catabolism in *P. aeruginosa*) that the NtrBC system is activated by an excess of carbon caused by the utilization of succinate (>2.5 mM) which is responsible for increasing the cellular concentration of α-KG and ATP, cellular signals of either carbon excess or nitrogen depletion. Additionally, the experiments performed with different mutant strains using choline as carbon or as nitrogen source allowed us to conclude that the use of choline results in a similar pattern of regulation of certain amino acids *of P. fluorescens*, such as histidine, proline, leucine, isoleucine and valine (Zhang and Rainey, 2008), which are also controlled by CbrB and NtrC. Our results also confirm the direct relationship between the transcriptional regulator GbdR and the ability of *P. aeruginosa* to utilize choline as a nutrient for growth, as described by Wargo et al. (2008, 2009). In addition, we have also shown that *pchP* expression depends on choline catabolism, and NtrC is necessary for full *pchP* expression. If choline is used as the unique available nitrogen source, a different set of genes may be induced for its catabolism; perhaps *pchP* expression occurred to produce the PchP necessary to catalyze the hydrolysis of phosphorylcholine and render choline (to provide nitrogen and carbon needed for growth) and inorganic phosphate. Two other pieces of evidence reinforce the specific relationship between choline utilization and *pchP* expression. First, in the experiment with histidine/succinate, where activated NtrC (NtrC-P) is present (Li and Lu, 2007; Nishijyo et al., 2001), expression of *pchP* did not occur. Second, with the addition of succinate and NH_4^+ to choline-cultured cells, bacteria stopped utilizing choline and *pchP* was expressed at a basal level. Therefore, *pchP* is specifically expressed when choline is the substrate for bacterial growth.

Bioinformatics predictions, confirmed experimentally by site-directed mutagenesis and transcriptional fusion analyses, led us to the conclusion that full *pchP* expression depends on an upstream region located −188 to −68 bp from the ATG start codon; this region contains a putative σ^{54}-dependent promoter. Although this sequence has a score below the best prediction, when screened for similarity to σ^{54}-dependent promoters (Studholme and Dixon, 2003) it contains the conserved −24 GG and −12 GC elements (Barrios et al., 1999); these are the only nucleotides that have specific contacts with the σ^{54}-holoenzyme (Cannon et al., 1995). Site-directed mutagenesis of the TTG sequence in the −12 element, as well as deletion of *rpoN*, showed that the integrity of the promoter, as well as the presence of σ^{54}, is essential for full *pchP* expression. It is supported by different facts: some σ^{54} promoters are highly dependent on IHF and EBP for full expression (Kustu et al., 1991; Buck et al., 2000), and here, both regions were demonstrated because sequences resembling an IHF binding site and a potential EBP binding site were detected between −141/−133 and −190/−174 bp relative to the ATG, respectively. The IHF binding site has the consensus sequence suggested for *P. aeruginosa* (Wozniak, 1994), and the functionality of this element was confirmed by site-directed mutagenesis. The presence of an EBP element was initially suggested by the palindromic region at −190 to −174. Experiments with Δ*ntr*C mutant strain PAO162 and deletion analysis confirmed that *pchP* expression depended on the presence of activated NtrC, and the region between −190 and −174 bp was also essential for full *pchP* expression. This region showed sequence similarity to the NtrC-binding site consensus of bacteria belonging to different species of the *Pseudomonas* and *Enterobacteria* genera (Morett and Buck, 1989; Morett and Segovia, 1993; Ninfa et al., 1995; Itoh et al., 2007; Li and Lu, 2007; Hervas et al., 2008). With all these data in our hands we fell able to propose that the NtrC is the EPB of this system, and

considering statements made by different authors (Popham et al., 1989; Morett and Buck, 1989; Kustu et al., 1991; Morett and Segovia, 1993; Wozniak, 1994; Buck et al., 2000), we believe that is correct to conclude that the region between −188 and −68 contains all of the promoter elements necessary for *pchP* expression.

On the other hand, recently, Wargo et al. (2009), showed that the transcriptional regulatory protein GbdR is involved in *pchP* expression. Interestingly, they demonstrated that purified GbdR protein binds to a discrete region comprising part of what here we define as the −24/−12 promoter, the TSSs and the region up to approximately position +15. Thus, it is tempting to speculate that GbdR competes with σ^{54} for binding to the same DNA region. Therefore, the mutants we constructed in and around the −24/−12 promoter also affect the GbdR binding site. Even more, the expression analysis of *pchP* in the mutant *rpoN* strain revealed a 70−75% reduction in β-galactosidase activity. Therefore, the residual expression of *pchP* could be the result of a second promoter activated by GbdR. In addition, the unexpected increase in *pchP* expression in the −79T/G mutant promoter, which affects the conserved T proximal to the highly conserved −12 GC element, could be the result of an increased binding of GbdR activator protein but, alternatively, it could be the result of diminished binding strength of σ^{54} which will result in faster promoter clearance.

Although we have identified the promoter that drives the transcription of the *pchP* gene, noting that it is dependent on the σ^{54} factor, our results indicate that *pchP* expression is much more complex than previously anticipated. This suggests that more than a single form of the RNA polymerase and multiple transcriptional regulators could be involved.

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