The *Pseudomonas aeruginosa* CreBC Two-Component System Plays a Major Role in the Response to β-Lactams, Fitness, Biofilm Growth, and Global Regulation

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Pseudomonas aeruginosa is a ubiquitous versatile environmental microorganism with a remarkable ability to grow under diverse environmental conditions. Moreover, *P. aeruginosa* is responsible for life-threatening infections in immunocompromised and cystic fibrosis patients, as the extraordinary capacity of this pathogen to develop antimicrobial resistance dramatically limits our therapeutic arsenal. Its large genome carries an outstanding number of genes belonging to regulatory systems, including multiple two-component sensor-regulator systems that modulate the response to the different environmental stimuli. Here, we show that one of two systems, designated CreBC (carbon source responsive) and BlrAB (β-lactam resistance), might be of particular relevance. We first identified the stimuli triggering the activation of the CreBC system, which specifically responds to penicillin-binding protein 4 (PBP4) inhibition by certain β-lactam antibiotics. Second, through an analysis of a large comprehensive collection of mutants, we demonstrate an intricate interconnection between the CreBC system, the peptidoglycan recycling pathway, and the expression of the concerning chromosomal β-lactamase AmpC. Third, we show that the CreBC system, and particularly its effector inner membrane protein CreD, plays a major role in bacterial fitness and biofilm development, especially in the presence of subinhibitory concentrations of β-lactams. Finally, global transcriptomics reveals broad regulatory functions of CreBC in basic physiological aspects, particularly anaerobic respiration, in both the presence and absence of antibiotics. Therefore, the CreBC system is envisaged as a potentially interesting target for improving the efficacy of β-lactams against *P. aeruginosa* infections.

*P*seudomonas aeruginosa is a ubiquitous versatile environmental microorganism with a remarkable ability to grow under a variety of environmental conditions, including in soil and water and in human, animal, and plant hosts ([1]). Moreover, it is responsible for severe nosocomial infections in immunocompromised patients, as well as chronic lung infections in patients with cystic fibrosis ([2]). The genome of *P. aeruginosa* is relatively large (6.3 Mb) and carries a large number of genes belonging to regulatory systems ([3], [4]) and genes involved in the utilization of various carbon sources, anaerobic energy metabolisms through arginine catabolism ([5]), pyruvate fermentation ([6]), and denitrification in the presence of nitrogen oxides ([7], [8]), which might contribute to the environmental adaptability of this bacterium ([8], [9]). A well-established environment for *P. aeruginosa* growth under anoxic conditions is the biofilm developed within the mucus of the cystic fibrosis (CF) lung ([8], [10]). The nitrate and nitrite levels in CF mucus, generated in part by the host inflammatory response to infection, are sufficient to support the anaerobic metabolism of *P. aeruginosa* ([8], [11]).

P. aeruginosa is also a paradigmatic example of antimicrobial resistance development. Given its extraordinary capacity to develop resistance to nearly all of the antibiotics currently used in therapy, it is frequently associated with multidrug resistance (MDR) phenotypes ([12], [13]). Particularly noteworthy is its β-lactam resistance, caused by the selection of a complex repertoire of chromosomal mutations ([12]–[14]), leading to the hyperproduction of the chromosomal cephalosporinase AmpC ([12], [15]), which causes resistance to penicillins, cephalosporins, and monobactams. It is also well established that the expression of this concerning β-lactamase is linked to the peptidoglycan recycling pathways

and therefore that blocking these processes is a promising strategy for overcoming β-lactam resistance ([16]).

Previous studies showed that *P. aeruginosa* has three muropeptide amidase genes (*ampD*, *ampDh2*, and *ampDh3*) and that their sequential inactivation leads to a stepwise upregulation of *ampC* expression, reaching full derepression with very high-level basal *ampC* expression in the triple mutant ([17]). Recent work showed, however, that one-step high-level resistance in clinical strains of *P. aeruginosa* frequently results from the inactivation of *dacB*, which encodes the nonessential penicillin-binding protein 4 (PBP4) ([15]). The inactivation of PBP4 was shown to give rise to a complex β-lactam resistance response, triggering a transcriptional regulator AmpR-dependent overproduction of the chromosomal β-lactamase AmpC and specific activation of the CreBC (carbon source responsive) (BlrAB [β-lactam resistance]) two-component regulator, evidenced through the induction of the expression of the inner membrane protein CreD ([12], [15]). A high similarity was

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previously noticed between CreBC from *Escherichia coli* and its BlrAB counterpart of *Aeromonas* spp., which plays an important role in the regulation of β-lactamases in these species lacking AmpR (18–20). BlrA acts as a regulator connected to a sensor kinase, BlrB. Although the mechanism by which this system drives the induction of expression in *Aeromonas* species is still unknown, a clear association has been established between BlrAB and the expression of different β-lactamases (18–20).

On the other hand, CreBC from *E. coli* is a transcriptional regulator that modulates the expression of up to 8 different genes in response to changes in the composition of nutrients in the medium (20, 21). Proteins from the CreBC system present a homology of 70% with those from BlrAB. Moreover, it has been demonstrated that the transcriptional regulator CreBC of *E. coli* is capable of modulating the expression of *Aeromonas* β-lactamases (18).

Although it is clear that a PBP4 mutation specifically activates the CreBC transcriptional regulator (15, 22) and that this is key to high-level resistance to β-lactam antibiotics, the underlying mechanism is still uncertain. Two-component sensor-regulator systems modify the expression of multiple genes in response to environmental stimuli (such as antibiotics) and are capable of interacting with other related metabolic pathways. In this sense, the CreBC regulon in *P. aeruginosa* might play an important role in *ampC* regulation/induction but also in other broader physiological responses to the presence of β-lactams and their effects on the cell wall. Indeed, CreB (BlrA) might perceive changes in the peptidoglycan and act as a transducer signal for these responses, including the expression of β-lactamases (18, 23, 24). In order to gain insights into these potential major regulatory roles, we performed a comprehensive analysis of the *creBC* and *creD* mutants of *P. aeruginosa* strain PAO1 to elucidate the role of the CreBC system of *P. aeruginosa* in the response to β-lactams, bacterial fitness, biofilm growth, and global regulation.

MATERIALS AND METHODS

Bacterial strains and plasmids. The complete list and description of the laboratory strains and plasmids used in this study are shown in Table 1. *P. aeruginosa* single or multiple knockout mutants involving *ampD*, *ampR*, *ampC*, *ampG*, *creBC*, *creD*, *dacB*, or *nagZ* were constructed according to well-established procedures (15, 17, 25) based on the Cre-lox system for gene deletion and antibiotic resistance marker recycling in *P. aeruginosa* (26). The previously constructed plasmids were used as the donor for the generation of knockout mutants by conjugational transfer to the corresponding *P. aeruginosa* strains (15, 25). For competition experiments, the *P. aeruginosa* PAO1, PAΔ*CreBC*, and PAΔ*CreD* strains were tagged at the *att* intergenic neutral chromosomal locus with two mini-Tn7 constructs harboring fluorescent and antibiotic resistance markers (green fluorescent protein [GFP]-gentamicin [Gm] and yellow fluorescent protein [YFP]-streptomycin [Str]) (Table 1), as previously described (27, 28).

Quantification of basal and induced gene expression. The relative mRNA levels of *ampC* and *creD* (*blrD*) were determined by real-time reverse transcription-PCR (RT-PCR), according to previously described protocols (15, 17). For induction experiments, the strains were incubated in LB broth for 3 h in the presence of 1/4 (0.25 µg/ml) and 1/2 (0.5 µg/ml) MICs of ceftazidime and imipenem, and in 50 µg/ml of cefoxitin as a control. Total RNA was obtained from these cultures with an RNeasy minikit (Qiagen, Hilden, Germany). Fifty nanograms of purified RNA was then used for one-step reverse transcription and real-time PCR using a QuantiTect SYBR green reverse transcription-PCR kit (Qiagen, Hilden, Germany) in a SmartCycler II apparatus (Cepheid, Sunnyvale, CA), according to previously described conditions and primers (see Table S1 in

the supplemental material) (15, 17, 29). The *rpsL* housekeeping gene was used to normalize the expression levels, and the results were always referred to the basal level of expression for PAO1. All RT-PCRs were performed in duplicate, and the mean values of mRNA expression resulting from three independent experiments were considered in all cases.

***In vitro* competition experiments.** Competition experiments were performed between strains *P. aeruginosa* PAΔ*creBC*Str and PAΔ*creD*Str versus PAO1Gm and PAΔ*creBC*Gm and PAΔ*creD*Gm versus PAO1Str to override any effect that the markers might have on fitness. Exponentially growing cells in the broth of the corresponding mutant and wild-type strains were mixed in a 1:1 ratio and diluted in 0.9% saline solution. Approximately 10^3 cells from each of the mixtures were inoculated into eight (four for each combination of markers) 10-ml LB broth flasks containing no antibiotics or 1/4, 1/2, and 3/4 MICs of ceftazidime (0.25, 0.5, and 0.75 µg/ml, respectively) imipenem (0.25, 0.5, and 0.75 µg/ml, respectively) or cefoxitin (250, 500, and 750 µg/ml, respectively). Ciprofloxacin at 1/2 MIC (0.03 µg/ml) was used as a non-β-lactam control in the competition assays. The cultures were grown at 37°C and 180 rpm for 20 h, corresponding to approximately 20 generations. Serial dilutions were then plated in duplicate onto LB agar (LBA) with 10 µg/ml of gentamicin and LBA with 100 µg/ml of streptomycin in order to determine the number of mutant and wild-type CFU after overnight incubation at 37°C. The competition index (CI) was defined as the CFU mutant-to-CFU PAO1 ratio (30). The CI values were calculated for each of the eight independent competition experiments, and the median values were recorded. Statistical analysis of the distribution of the CI values was performed using the Mann-Whitney U test. *P* values of <0.05 were considered to be statistically significant. To assess the growth rates under noncompetitive conditions, the doubling times of exponentially growing cells in LB broth at 37°C and 180 rpm were determined by plating serial 10-fold dilutions on LBA at 1-h intervals. Three independent experiments were performed for each of the mutants, and the results were compared with those for PAO1 using Student's *t* test.

Biofilm growth. Biofilm assays were performed according to previously described procedures (31). Briefly, 10^5 cells in stationary phase were inoculated into each of four wells per strain of a microtiter plate containing 100 µl of LB medium supplemented or not with 50 µg/ml of cefoxitin. After incubation for 24 h or 48 h at 37°C, the microtiter plates were gently rinsed with tap water. After removing all planktonic cells, the plates were air-dried and stained with 125 µl of a 0.1% crystal violet solution per well for 10 min. Next, the plates were gently rinsed with water and air-dried again. The dye was solubilized with 200 µl of 30% acetic acid for 25 min at room temperature. Once mixed by pipetting, 125 µl from each well was individually transferred to a clear flat-bottom 96-well plate. Finally, the absorbance was measured at 590 nm. All experiments were repeated on three independent occasions, and the final results were expressed as the mean values (± standard deviation) of 12 determinations (from three quadruplicate experiments).

Analysis of whole-genome gene expression. Three independent replicates of PAO1 and PAΔ*creBC* were grown at 37°C to an optical density at 600 nm (OD_{600}) of 1 in vigorously shaken 50-ml baffled flasks containing 10 ml of LB broth without antibiotics or supplemented with 50 µg/ml cefoxitin or 0.5 µg/ml ceftazidime. The cells were collected by centrifugation (8,000 × *g* for 5 min at 4°C), and total RNA was isolated using the RNeasy minikit (Qiagen), according to the manufacturer's instructions. RNA was dissolved in water and treated with 2 U of Turbo DNase (Ambion) for 30 min at 37°C to remove contaminating DNA. The reaction was stopped by adding 5 µl of DNase inactivation reagent. Ten micrograms of the total RNA was checked by running on an agarose gel prior to cDNA synthesis. cDNA synthesis, fragmentation, labeling, and hybridization were performed according to the Affymetrix GeneChip *P. aeruginosa* genome array expression analysis protocol. An expression analysis was performed as described previously (15, 32, 33). Only transcripts showing ≥2-fold increases or decreases were considered to be differentially expressed. In all cases, the posterior probability for differential expression

TABLE 1 Strains and plasmids used or constructed in this work

Strain or plasmid	Genotype/relevant characteristic(s)[a]	Reference or source
P. aeruginosa strains		
PAO1	Reference strain completely sequenced	9
PAΔD	PAO1 Δ*ampD*::lox; AmpD is an *N*-acetyl-anhydromuramyl–L-alanine amidase involved in peptidoglycan recycling; negative regulator of AmpC expression	17
PAΔdacB	PAO1 Δ*dacB*::lox; *dacB* encodes the nonessential penicillin-binding protein 4	15
PAdacBΔD	PAdacB Δ*ampD*::lox	15
PAΔDDh2Dh3	PAO1 Δ*ampD*::lox Δ*ampDh2*::lox Δ*ampDh3*::lox; AmpDh2 and AmpDh3 are the two additional AmpD homologues of *P. aeruginosa*	17
PAΔR	PAO1 Δ*ampR*::lox; AmpR is a LysR-type transcriptional regulator required for *ampC* induction	15
PAΔC	PAO1 Δ*ampC*::lox; *ampC* encodes the chromosomal β-lactamase AmpC	15
PAΔDΔC	PAO1 Δ*ampD*::lox Δ*ampC*::lox	15
PAdacBΔC	PAdacB Δ*ampC*::lox	15
PAΔDDh2Dh3ΔC	PAO1 Δ*ampD*::lox Δ*ampDh2*::lox Δ*ampDh3*::lox Δ*ampC*::lox	30
PAΔnZ	PAO1 Δ*nagZ*::lox; *nagZ* is a β-*N*-acetylglucosaminidase that processes GlcNAc-1,6-anhydromuropeptides to generate 1,6 anhydromuropeptides	34
PAΔDnZ	PAO1 Δ*ampD*::lox Δ*nagZ*::lox	34
PAΔdBnZ	PAO1 Δ*dacB*::lox Δ*nagZ*::lox	34
PAdacBΔDnZ	PAdacB Δ*ampD*::lox Δ*nagZ*::lox	34
PAΔDDh2Dh3nZ	PAO1 Δ*ampD*::lox Δ*ampDh2*::lox Δ*ampDh3*::lox Δ*nagZ*::lox	34
PAΔG	PAO1 Δ*ampG*::lox; AmpG encodes an inner membrane permease for GlcNAc-1,6-anhydromuropeptides	25
PAΔDG	PAO1 Δ*ampD*::lox Δ*ampG*::lox	25
PAΔdBG	PAO1 Δ*daB*::lox Δ*ampG*::lox	25
PAdacBADG	PAdacB Δ*ampD*::lox Δ*mpG*::lox	25
PAΔcreBC	PAO1 Δ*creBC*::lox; CreBC is a two-component response regulator	15
PAΔcreD	PAO1 Δ*creD*::lox; inner membrane protein of unknown function known to be regulated by the CreBC system	15
PAO_GFP	PAO1 tagged with eGFP-Gm in a mini-Tn*7* construct	This work
PAO_EYFP	PAO1 tagged with eYFP-Str in a mini-Tn*7* construct	This work
PAΔcreBC_GFP	PAΔcreBC tagged with eGFP-Gm in a mini-Tn*7* construct	This work
PAΔcreBC_EYFP	PAΔcreBC tagged with eYFP-Str in a mini-Tn*7* construct	This work
PAΔcreD_GFP	PAΔcreD tagged with eGFP-Gm in a mini-Tn*7* construct	This work
PAΔcreD_EYFP	PAΔcreD tagged with eYFP-Str in a mini-Tn*7* construct	This work
Plasmids		
Mini-Tn*7*-gfp2	Plasmid based on pUCP19 (Amp^r) with eGFP mini-Tn*7*, Gm^r	27
Mini-Tn*7*_PA1/04/03-eyfp-a	Plasmid based on pUCP19 (Amp^r) with eYFP mini-Tn*7*, Str^r	28
Mini-Tn*7*_PA1/04/03-ecfp-a	Plasmid based on pUCP19 (Amp^r) with eCFP mini-Tn*7*, Gm^r	28

[a] eGFP, enhanced green fluorescent protein; eYFP, enhanced yellow fluorescent protein; eCFP, enhanced cyan fluorescent protein; Amp^r, ampicillin resistant; Gm^r, gentamicin resistant; Stm^r, streptomycin resistant.

(PPDE) was between 0.999 and 1. The expression of 3 selected representative genes was further analyzed using real-time RT-PCR according to the protocols described above using the primers listed in Table S1 in the supplemental material to confirm the microarray results.

Data analysis. The GraphPad Prism 5 software was used for graphical representation and statistical analysis. Quantitative variables were compared using the Mann-Whitney U test or Student's *t* test, as appropriate. Categorical variables were compared using the χ^2 test. A *P* value of <0.05 was considered statistically significant.

Microarray data accession number. The microarray data for this study can be found under GEO accession no. GSE58758.

RESULTS AND DISCUSSION

The CreBC (BlrAB) system specifically responds to PBP4 inhibition by AmpC inducers. In a previous work, we showed that the CreBC system is responsive to cefoxitin (a strong AmpC inducer and PBP4 inhibitor), as evidenced by a marked induction of *creD*

expression in the presence of this cephalosporin (15). Moreover, we demonstrated that the inactivation of *dacB*, which encodes PBP4, leads to a constitutive upregulation of *creD*. These observations led us to generate the hypothesis that CreBC may specifically respond to PBP4 inhibition. To test this hypothesis, we determined *ampC* and *creD* induction in parallel in the presence of the cephalosporin ceftazidime (a weak AmpC inducer/weak PBP4 inhibitor) and the carbapenem imipenem (a strong AmpC inducer/ strong PBP4 inhibitor), and cefoxitin was additionally used as a control. As shown in Table 2, in contrast to cefoxitin, ceftazidime had only a marginal effect on *ampC* and *creD* expression, whereas imipenem was a very potent *ampC* and *creD* inducer. Therefore, these results strongly suggest that the capacity of β-lactams to activate the CreBC system does not depend on structural aspects (cefoxitin and ceftazidime are both cephalosporins, in contrast to imipenem) but rather on cell target aspects, since imipenem and

TABLE 2 Level of expression of *ampC* and *creD* in the presence of cefoxitin, ceftazidime, and imipenem subinhibitory concentrations in *P. aeruginosa* strain PAO1

β-Lactam[a]	Concentration (μg/ml)	Relative mRNA level (mean ± SD)[b]	
		ampC	*creD*
CAZ	0.25 (1/4 MIC)	1.29 ± 0.1	1.7 ± 0.7
	0.5 (1/2 MIC)	1.26 ± 0.3	2.5 ± 1.4
IMP	0.25 (1/4 MIC)	193 ± 20.2	34 ± 10
	0.5 (1/2 MIC)	269 ± 73.1	70 ± 8
FOX	50	78 ± 34	24 ± 9.1

[a] CAZ, ceftazidime; IMP, imipenem; FOX, cefoxitin.
[b] Relative amount of *ampC* and *creD* mRNA compared to PAO1 basal levels (set at 1).

cefoxitin, in contrast to ceftazidime, share the single property of being potent PBP4 inhibitors. Thus, *ampC* and *creBC* regulatory pathways seem to essentially sense the same signals, which are directly or indirectly driven by the inhibition of PBP4 by certain β-lactams (AmpC inducers).

Interplay between the CreBC (BlrAB) system and the AmpC regulatory pathway. In order to gain insights into the role of the CreBC system in β-lactam resistance and its interplay with *ampC* regulation, a comprehensive analysis of *ampC* and *creD* expres-

sion under basal and cefoxitin-induced conditions was performed with a large collection of single and multiple mutants, including all the genes known to be involved in the AmpC regulatory pathway (described in Table 1), and the results are presented in Fig. 1A to D. As indicated in Fig. 1, the presented results include all available expression data, including those generated for the present work and those reported in our previous studies (15, 25, 34) for comparative purposes. The previous studies showed that inactivation of *dacB* leads to the specific activation of the CreBC system, as evidenced by the constitutive overexpression of *creD*. Moreover, we demonstrated that the CreBC system plays a major role in the high-level β-lactam resistance phenotype of the *dacB* mutant; while the ceftazidime MIC increased from 1 to 32 μg/ml in PAO1 after *dacB* inactivation, the *dacB-creBC* double mutant showed an MIC of only 4 μg/ml despite the fact that *ampC* expression was not modified (i.e., still overexpressed). Furthermore, the inactivation of the CreBC system in PAO1 dramatically decreased the spontaneous emergence of ceftazidime-resistant DacB mutants (15). On the other hand, inactivation of the CreBC system or CreD in wild-type PAO1 did not have any direct impact on β-lactam MICs (15). In the present work, through a complete analysis of the collection of PAO1 mutants involved in the AmpC regulatory pathway, we demonstrate that of all the single mutants analyzed, only *dacB* leads to a significant constitutive upregulation of *creD* (Fig. 1C). Interestingly, we also show in the present work that the inactiva-

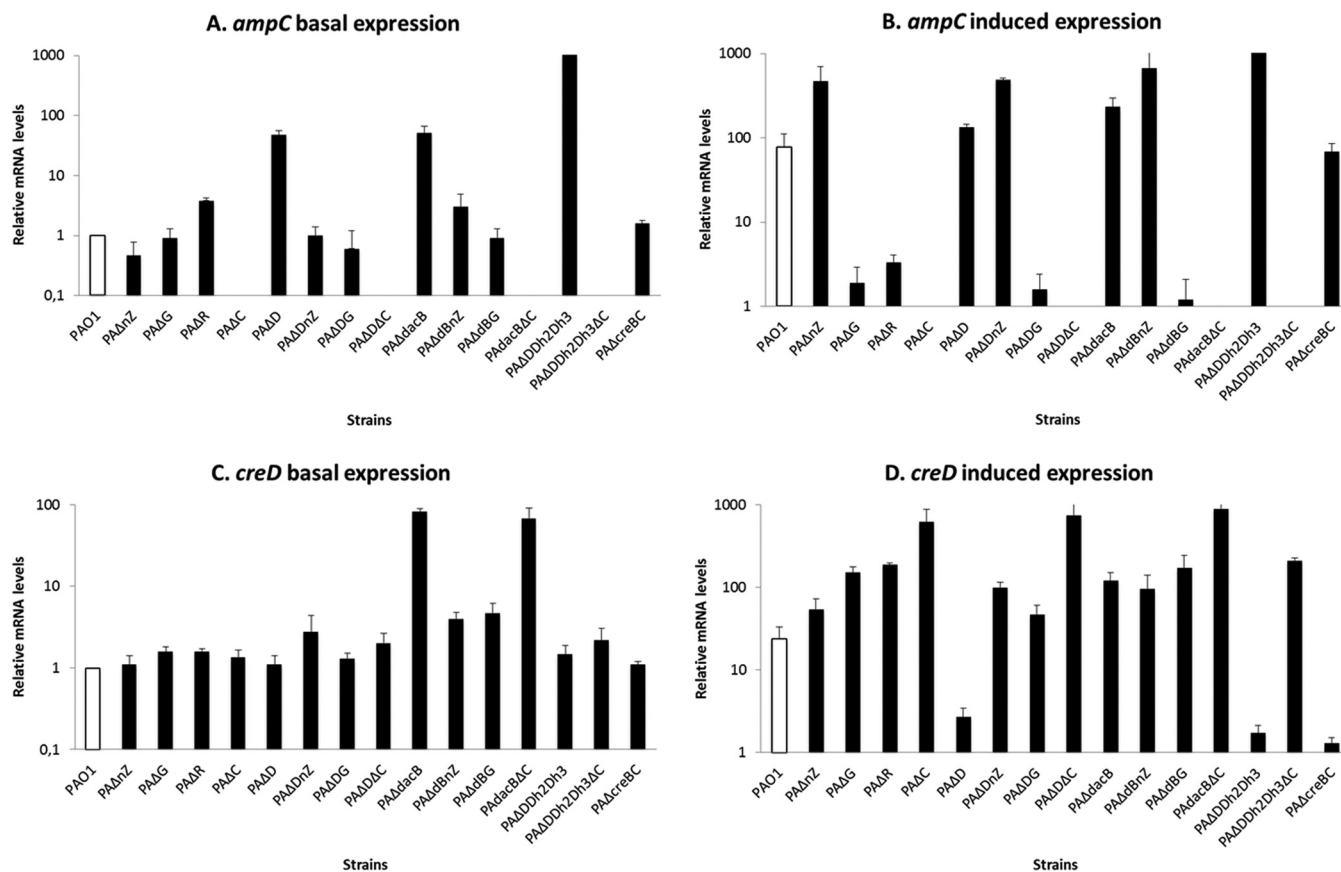**FIG 1** *ampC* and *creD* basal (A and C) and induced (B and D) expression in the collection of mutants in the different components of AmpC regulatory pathways. The *ampC* and *creD* expression data for PAΔR, PAΔD, PAΔcreBC, PAΔDDh2Dh3 (15), and *ampC* expression data for *nagZ* (34) and *ampG* (25) mutants were obtained in previous works and included for comparative purposes. Data shown are means plus standard deviations.
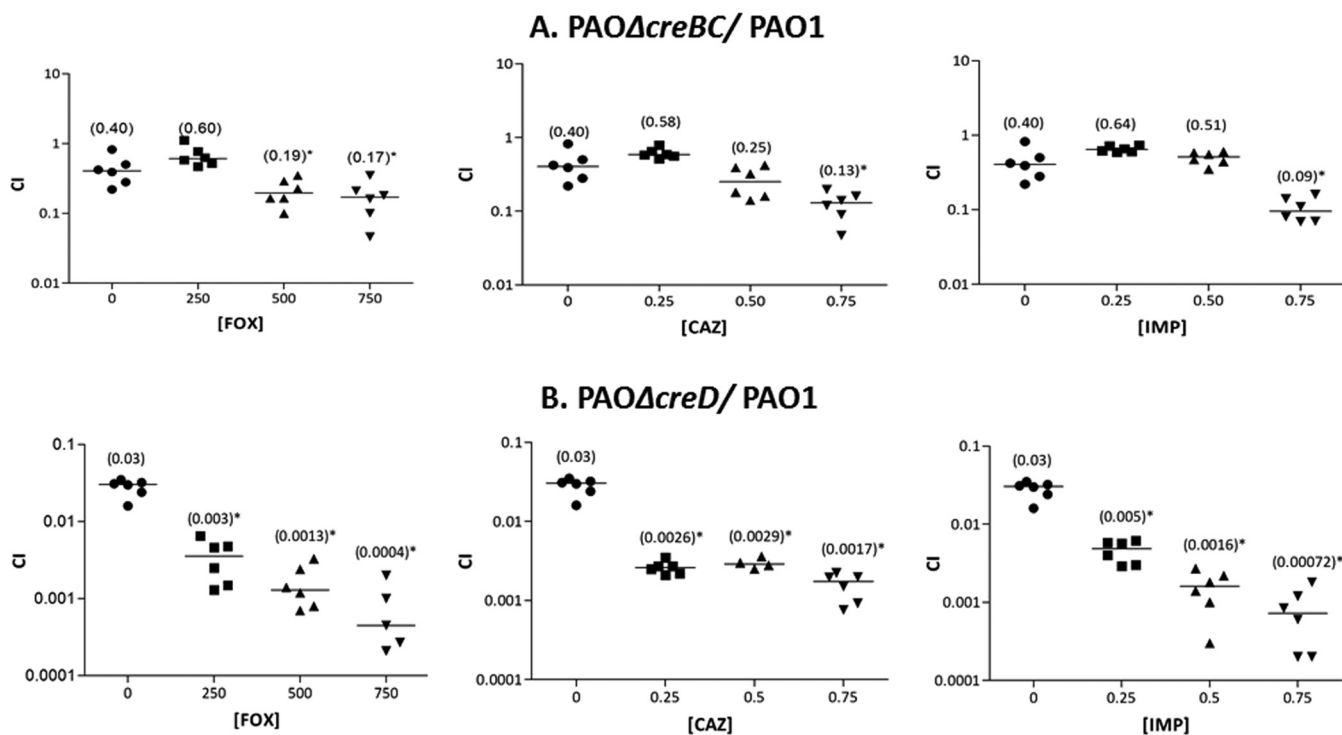
A. PAOΔcreBC/ PAO1

B. PAOΔcreD/ PAO1

FIG 2 Results of *in vitro* competition experiments of PAOΔcreBC versus PAO1 (A) and PAOΔcreD versus PAO1 (B) in the presence of β-lactams. *In vitro* competitions were performed using LB broth flasks containing 1/4, 1/2, and 3/4 MICs of ceftazidime (CAZ) (0.25, 0.5, and 0.75 μg/ml, respectively), imipenem (IMP) (0.25, 0.5, and 0.75 μg/ml, respectively), or cefoxitin (FOX) (250, 500, and 750 μg/ml, respectively). The CI values obtained for each of the eight independent experiments are plotted, and the median values are shown in parentheses. *P* values of <0.05 for the comparison of the CIs in the presence and absence of antibiotics are indicated with an asterisk.

tion of either *nagZ* or *ampG* in the *dacB* mutant determines a major attenuation of *creD* basal expression levels (Fig. 1C).

As shown in Fig. 1D, a major impact of the *ampC* regulatory components on *creD* inducibility in the presence of cefoxitin was also evidenced. First, the inactivation in PAO1 of any of the components that need to be functional for *ampC* induction or constitutive overexpression, including *ampG*, *nagZ*, *ampR*, and *ampC* itself, leads to significantly enhanced *creD* inducibility. Second, as previously described (15), the inactivation of *ampD* blocked *creD* inducibility, but, interestingly, we show here that the inactivation of *nagZ*, *ampG*, or *ampC* fully restored *creD* inducibility in this mutant (Fig. 1D). Finally, the results of the present work show that the inactivation of *ampC* but not of *nagZ* or *ampG* in the *dacB* mutant also further increased *creD* inducibility (Fig. 1D).

Although the results presented here certainly highlight the complexity of the interplay between the CreBC and AmpC regulatory pathways, several conclusions can be reached. A first obvious conclusion is that *ampC* expression itself greatly affects *creD* inducibility. The explanation for this finding also appears to be obvious; the higher the expression of *ampC*, the greater the hydrolysis of cefoxitin, which avoids *creD* induction. Indeed, AmpC hydrolysis of cefoxitin as a feedback mechanism to tune down *creD* expression might explain many of the findings, including the increased *creD* inducibility in PAO1 *ampG*, *nagZ*, *ampR*, and *ampC* mutants and the restoration of *creD* inducibility in the *ampD* mutant after *nagZ*, *ampG*, or *ampC* inactivation. On the other hand, as previously suggested (15), the inhibition (by cefoxitin or other PBP4 inhibitors, such as imipenem) or inactivation of

the peptidoglycan carboxypeptidase DacB directly triggers the activation of the CreBC system, leading to *creD* upregulation. Moreover, we show that through a still uncertain feedback mechanism, blocking the intracellular recycling of the produced cell wall metabolites through the inactivation of *ampG* or *nagZ* attenuates *creD* expression in the *dacB* mutant.

Role of the CreBC (BlrAB) system in bacterial fitness. To assess the effect on bacterial fitness, *in vitro* competition experiments were performed between wild-type PAO1 and the corresponding mutants in the CreBC two-component regulator and its regulated inner membrane protein, CreD. As shown in Fig. 2, the inactivation of *creBC* produced a modest (CI, 0.4) but significant (*P* < 0.001) fitness cost. Moreover, a much higher fitness cost (CI, 0.03) was evidenced after the direct inactivation of *creD*. Interestingly, subinhibitory concentrations of all three β-lactams tested (therefore regardless of structure or whether they were potent AmpC inducers or not) significantly increased further the fitness cost of the mutants (Fig. 2). Nevertheless, the effect again was much more accentuated for the *creD* mutant than for the *creBC* mutant. Moreover, the effect was shown to be specific for β-lactams, since the CIs in the presence of 1/2 MICs of ciprofloxacin were not different from those without antibiotic for the CreBC versus PAO1 (0.49 ± 0.29 versus 0.57 ± 0.19) or CreD versus PAO1 (0.012 ± 0.008 versus 0.011 ± 0.013) competition experiments. All together, these results show that the CreBC system, particularly its regulated inner membrane protein CreD, plays an important role in bacterial fitness, especially in the presence of subinhibitory concentrations of β-lactams. The absence of major

TABLE 3 Duplication times of PAO1 and the corresponding CreBC and CreD mutants in the presence or absence of 1/2 MICs of ceftazidime or cefoxitin

Strain	Duplication time (mean ± SD) (min) under antibiotic conditions of:		
	No antibiotic	0.5 µg/ml ceftazidime	500 µg/ml cefoxitin
PAO1	29.8 ± 4.2	34 ± 1.6	30.4 ± 2.8
PAΔcreBC	29 ± 3.2	30.1 ± 4.0	33 ± 2.5
PAΔcreD	35 ± 1.2[a]	42.5 ± 5.6[a]	35.7 ± 2.9[a]

[a] Statistically significant differences (Student's *t* test) compared to PAO1 values.

fitness differences depending on whether the specific β-lactam induces (imipenem or cefoxitin) or does not induce (ceftazidime) *creD* may suggest that the basal expression of *creD* plays a greater role in fitness than the effect derived from the further induction or not of its expression.

Similar results were obtained when fitness was measured in terms of the growth rates in individual cultures (Table 3). While the duplication times were not sensitive enough to show the small fitness defect associated with CreBC inactivation in the competition experiments, they clearly evidenced the major cost associated with CreD inactivation and how it is significantly enhanced in the presence of subinhibitory concentrations of β-lactams (Table 3).

Role of the CreBC (BlrAB) system in global regulation and response to β-lactams. Using DNA microarrays, we compared the global expression profiles of PAO1 and PAOΔ*creBC*, in the absence of antibiotics, in the presence of subinhibitory (1/2 MIC [0.5 µg/ml]) concentrations of the weak AmpC inducer antipseudomonal cephalosporin ceftazidime, and in the presence of the strong AmpC inducer cephalosporin cefoxitin, which lacks antipseudomonal activity (i.e., the concentration used for AmpC induction, 50 µg/ml, is >20-fold lower than the MIC). This approach was therefore followed to evaluate the role of the CreBC system in (i) global regulation in the absence of antibiotics, (ii) the AmpC induction response, and (iii) β-lactam stress. The results are presented in Table 4 (genes showing modified expression in PAOΔ*creBC* compared to in PAO1 under the three evaluated conditions [no antibiotics, cefoxitin, and ceftazidime]), Table 5 (genes showing modified expression for each strain [PAO1 or PAOΔ*creBC*] in the presence or absence of cefoxitin or ceftazidime), and Fig. 3 (Venn diagram of differentially regulated genes).

As shown in Table 4, pairwise comparisons of the data sets of significantly differentially regulated genes (differential expression, ≥2-fold; *P* ≤ 0.01) identified a total of 53 genes showing modified expression in PAOΔ*creBC* with respect to that in PAO1 in either the absence of antibiotics (*n* = 24) or the presence of the AmpC inducer cefoxitin (*n* = 21) or the antipseudomonal cephalosporin ceftazidime (*n* = 24). Moreover, as shown in Table 5, the interstrain comparison identified a total of 23 genes differentially regulated in the presence of cefoxitin and 38 genes differentially regulated in the presence of ceftazidime. All together, these results clearly indicate that the CreBC system may act as a global regulator in *P. aeruginosa*, as was previously suggested for other Gram-negative bacteria (20).

To further address the interplay between *creBC*-dependent genes and antibiotic exposure, the differentially regulated genes were plotted in 4-way Venn diagrams (Fig. 3) and separated into

cefoxitin (Fig. 3A) and ceftazidime (Fig. 3B) differentially regulated genes. Indeed, genes that were differentially regulated due to a lack of *creBC*, to β-lactam antibiotic exposure, or both showed an intricate interconnection.

As shown in Fig. 3A and Table 5, there were 21 genes upregulated in PAO1 after cefoxitin exposure and 24 genes showing modified expression (23 upregulated and 1 downregulated) in the *creBC* mutant compared to in PAO1 in the absence of antibiotics (Fig. 3A and Table 4). Interestingly, 16 of these genes were upregulated under the two conditions, indicating a clear interconnection between the CreBC system and global gene expression profiles resulting from cefoxitin exposure. Those genes include *creD*, as expected, and also the whole gene cluster for dissimilatory nitrite reductase (NIR) and the nitric oxide synthase (NOS) gene cluster for nitrous oxide reduction, both of which play a major role in denitrification during anaerobic metabolism. However, as previously noted (15), a remarkable exception to the role of the CreBC system in the response to the presence of the AmpC inducer cefoxitin is the expression of *ampC* itself, which is independent of the CreBC system (Tables 4 and 5).

On the other hand, the transcriptional response of PAO1 to subinhibitory concentrations of the antipseudomonal cephalosporin ceftazidime included the modification of the expression of 17 genes, all but 1 of which were upregulated (Fig. 3B and Table 5), including *rpm*, *rpo*, *rps*, and *rpl* ribosomal proteins. In contrast, of the 22 genes that showed modified expression in the *creBC* mutant, 21 were downregulated. Indeed, the responses of PAO1 and PAOΔ*creBC* to ceftazidime exposure had only one gene in common (Fig. 3B). Moreover, 17 of the 21 downregulated genes in the presence of ceftazidime were upregulated in the *creBC* mutant compared to in PAO1 in the absence of antibiotics (Tables 4 and 5 and Fig. 3B), confirming an important role of the CreBC system in the response to β-lactam challenge.

Moreover, as shown in Table 4, genes from the entire denitrification cascade were upregulated in the absence of antibiotics when *creBC* was inactivated, including genes of the nitrogen regulatory pathway (N_2O reduction, [NOS], NO-2 reductase [NIR], and NO reductase [NOR], and nitrate/nitrite transport [NAR]) and MoaA1 MoaB1 (molybdate metabolism) (35, 36). Genes from the first step, catalyzed by the membrane-bound nitrate reductase, NAR, are required for anaerobic nitrate respiration of *P. aeruginosa* in CF sputum (37). The reduction of nitrate by NAR is coupled to quinol oxidation and consumes two protons from the cytoplasm, thereby contributing to the creation of a proton gradient across the membrane (38, 39).

Nitrite reductase catalyzes the second step of denitrification, the reduction of nitrite to NO, and the *nir* gene cluster, which is located in the periplasm (40). The reduction of NO to N_2O is catalyzed by NO reductase, which is encoded in the *norCBD* operon and is clustered with the *nir* genes for nitrite reductase in the genome of *P. aeruginosa*. NO reductase functions not only for anaerobic energy conservation as a respiratory enzyme but also for the detoxification of exogenous NO. The machinery for the detoxification of NO and its derivative reactive nitrogen species is necessary because the infected *P. aeruginosa* cells are subjected to nitrosative stress by attacks from the host immune system. The NO reductase-deficient mutant of *P. aeruginosa* shows a reduced survival rate in NO-producing macrophages (41). The final step of the denitrification pathway, the reduction of N_2O to N_2, is catalyzed by N_2O reductase. In *P. aeruginosa*, N_2O reductase is en-

TABLE 4 Gene loci showing modified expression (≥2-fold change) in the *creBC* mutant compared to the parental wild-type PAO1 strain under basal conditions and after cefoxitin or ceftazidime incubation as determined using Affymetrix GeneChips

Locus tag	Gene	Product	Basal	FOX	CAZ
			Fold change/condition under antibiotic conditions[a]:		
PA0515		Probable transcriptional regulator	**2.18**	−1.02	−1.09
PA0517	*nirC*	Probable *c*-type cytochrome precursor	**3.26**	1.07	1.08
PA0518	*nirM*	Cytochrome *c*-551 precursor	**3.43**	−1.05	1.04
PA0519	*nirS*	Nitrite reductase precursor	**3.35**	−1.02	−1.22
PA0523	*norC*	Nitric oxide reductase subunit C	**2.85**	1.17	−1.03
PA0524	*norB*	Nitric oxide reductase subunit B	**3.79**	1.25	1.05
PA0525		Probable denitrification protein NorD	**2.26**	1.08	1.26
PA1431	*rsaL*	Regulatory protein RsaL	**2.15**	1.68	1.91
PA1596	*htpG*	Heat shock protein HtpG	1.07	1.93	2.16
PA1901	*phzC2*	Phenazine biosynthesis protein PhzC	1.71	**2.03**	1.8
PA1903	*phzE2*	Phenazine biosynthesis protein PhzE	1.89	**2.06**	1.51
PA1905	*phzG2*	Probable pyridoxamine 5′-phosphate oxidase	**2.02**	**2.42**	2.06
PA2247	*bkdA1*	2-Oxoisovalerate dehydrogenase (alpha-subunit)	1.12	1.25	2.03
PA2248	*bkdA2*	2-Oxoisovalerate dehydrogenase (beta-subunit)	1.13	1.58	2.58
PA2300	*chicC*	Chitinase	1.5	**2.18**	1.49
PA3126	*ibpA*	Heat shock protein IbpA	1.04	1.91	**2.08**
PA3328		Probable FAD-dependent monooxygenase[b]	1.77	**2.42**	**2.1**
PA3329		Hypothetical protein	1.57	**2.14**	1.94
PA3330		Probable short-chain dehydrogenase	1.86	**2.61**	**2.27**
PA3331		Cytochrome P450	**2.11**	**2.65**	**2.2**
PA3332		Conserved hypothetical protein	1.83	**2.46**	1.88
PA3333	*fabH2*	3-Oxoacyl-(acyl-carrier-protein) synthase III	1.57	**2.21**	1.47
PA3334		Probable acyl carrier protein	1.72	**2.33**	1.75
PA3874	*narH*	Respiratory nitrate reductase beta chain	**2.93**	1.78	−1.05
PA3875	*narG*	Respiratory nitrate reductase alpha chain	**3.97**	**2.55**	−1.06
PA3876	*narK2*	Nitrite extrusion protein 2	**4.49**	**2.56**	1.04
PA3877	*narK1*	Nitrite extrusion protein 1	**5.35**	**2.95**	1.12
PA3392	*nosZ*	Nitrous oxide reductase precursor	**2.04**	−1.68	−1.32
PA3872	*narI*	Respiratory nitrate reductase gamma chain	**2.14**	1.43	1.01
PA3873	*narJ*	Respiratory nitrate reductase delta chain	**2.48**	1.46	1.04
PA3914	*moeA1*	Molybdenum cofactor biosynthetic protein A1	**2.19**	1.17	1.09
PA3915	*moaB1*	Molybdenum cofactor biosynthetic protein B1	**3.52**	1.65	1.1
PA4141		Hypothetical protein	**2.09**	**2.36**	**2.37**
PA4211	*phzB1*	Probable phenazine biosynthesis protein	**2.12**	**2.9**	**2.23**
PA4217	*phzS*	Flavin-containing monooxygenase	**2.43**	**3.23**	1.94
PA4386	*groES*	GroES protein	**2.21**	1.63	1.4
PA4762	*grpE*	Heat shock protein GrpE	1.13	1.98	**2.66**
PA5053	*hslV*	Heat shock protein HslV	1.14	**2.02**	**3**
PA5054	*hslU*	Heat shock protein HslU	−1.05	1.67	**2.24**
PA0465	*creD*	Inner membrane protein CreD	1.18	*−3.2*	1.15
PA0466		Hypothetical protein	1.18	*−2.02*	1
PA0887	*acsA*	Acetyl-coenzyme A synthetase	−1.55	1.23	*−2.34*
PA1123		Hypothetical protein	−1.32	−1.44	*−2.01*
PA1183	*dctA*	C4-dicarboxylate transport protein	−1.58	−1.62	*−2.3*
PA1557	*ccoN2*	Cytochrome *c* oxidase, cbb3-type, CcoN subunit	*−2.04*	−1.6	−1.68
PA3013	*foaB*	Fatty acid oxidation complex beta-subunit	−1.56	−1.72	*−2.16*
PA3040		Conserved hypothetical protein	−1.16	−1.31	*−2.07*
PA3049	*Rmf*	Ribosome modulation factor	−1.1	−1.78	*−2.66*
PA3284		Hypothetical protein	−1.35	−1.81	*−2.12*
PA3790	*oprC*	Putative copper transport outer membrane porin OprC precursor	−1.62	−1.72	*−2.23*
PA3841	*exoS*	Exoenzyme S	−1.29	−1.24	*−2.05*
PA4525	*pilA*	Type 4 fimbrial precursor PilA	−1.71	*−2.18*	*−2.83*
PA4587	*ccpR*	Cytochrome *c*551 peroxidase precursor	−1.37	−1.8	*−2.93*

[a] Bold type indicates increased gene expression (≥2-fold), and italics indicate decreased gene expression (≥2-fold).
[b] FAD, flavin adenine dinucleotide.

TABLE 5 Gene loci showing modified expression (≥2-fold change) in each of the strains tested under cefoxitin or ceftazidime incubation compared to the same strain under basal conditions as determined using Affymetrix GeneChips

Locus tag	Gene	Product	Fold change/condition under antibiotic conditions[a]:			
			PAO1 FOX	*creBC* FOX	PAO1 CAZ	*creBC* CAZ
PA0465	*creD*	Inner membrane protein CreD	**3.62**	*−3.2*	1.14	1.11
PA0466			**2.3**	*−2.02*	1.14	−1.03
PA0509	*nirN*	Probable *c*-type cytochrome	**2.1**	−1.3	−1.16	−1.74
PA0510		Hypothetical protein	**2.18**	−1.39	−1.44	*−2.18*
PA0511	*nirJ*		**2.17**	−1.2	−1.34	*−2.24*
PA0512		Conserved hypothetical protein	**2.43**	−1.22	−1.19	*−2.08*
PA0513		Probable transcriptional regulator	**2.42**	−1.12	−1.59	*−2.91*
PA0514	*nirL*		**2.25**	−1.13	−1.28	*−2.38*
PA0515		Probable transcriptional regulator	**2.46**	−1.01	−1.17	*−2.77*
PA0516	*nirF*		**2.74**	1.07	−1.33	*−2.66*
PA0517	*nirC*		**3.56**	−1.01	−1.22	*−3.66*
PA0518	*nirM*		**3.8**	−1.05	−1.28	*−4.57*
PA0519	*nirS*		**3.86**	−1.02	−1.21	*−4.94*
PA0523	*norC*		1.85		−1.18	*−3.45*
PA0524	*norB*		**2.43**	−1.25	−1.08	*−3.91*
PA0525		Probable denitrification protein NorD	1.62	−1.29	−1.15	*−2.07*
PA0918		Cytochrome b_{561}	**2.41**	−1	−1.14	*−1.78*
PA1557	*ccoN2*	Cytochrome *c* oxidase, cbb3-type, CcoN subunit	1.76	**2.25**	**2.08**	**2.54**
PA1582	*sdhD*	Succinate dehydrogenase (D subunit)	1.38	1.54	**2.02**	1.63
PA1588	*sucC*	Succinyl-CoA synthetase beta chain[b]	1.55	1.36	**2.16**	1.46
PA2742	*rpmI*	50S ribosomal protein L35	1.48	1.61	**2.03**	1.74
PA3205		Hypothetical protein	1.25	1.55	*−2.49*	−1.13
PA3392	*nosZ*	Nitrous oxide reductase precursor	**3.79**	−1.68	−1.53	*−4.1*
PA3393	*nosD*	NosD protein	**2.11**	−1.39	−1.28	*−1.87*
PA3394	*nosF*	NosF protein	**2.2**	−1.27	−1.24	*−1.80*
PA3395	*nosY*	NosY protein	**2.21**	−1.28	−1.01	−1.26
PA3872	*narI*	Respiratory nitrate reductase gamma chain	−1.00	−1.49	−1.24	*−2.61*
PA3873	*narJ*	Respiratory nitrate reductase delta chain	1.26	−1.34	−1.14	*−2.7*
PA3874	*narH*	Respiratory nitrate reductase beta chain	1.15	−1.43	−1.1	*−3.37*
PA3875	*narG*	Respiratory nitrate reductase alpha chain	1.19	−1.31	−1.13	*−4.73*
PA3876	*narK2*	Nitrite extrusion protein 2	1.09	−1.62	−1.17	*−5.08*
PA3877	*narK1*	Nitrite extrusion protein 1	1.18	−1.53	−1.11	*−5.34*
PA3915	*moaB1*	Molybdopterin biosynthetic protein B1	−1.03	−1.20	−1.15	*−3.67*
PA4110	*ampC*	β-Lactamase precursor	**5.16**	**4.17**	1.10	−1.09
PA4238	*rpoA*	DNA-directed RNA polymerase alpha chain	1.55	1.48	**2.01**	1.72
PA4242	*rpmJ*	50S ribosomal protein L36	1.41	1.23	**2.03**	1.41
PA4246	*rpsE*	30S ribosomal protein S5	1.46	1.72	**2.11**	1.91
PA4247	*rplR*	50S ribosomal protein L18	1.41	1.28	**2.03**	1.58
PA4254	*rpsQ*	30S ribosomal protein S17	1.68	1.43	**2.41**	1.88
PA4257	*rpsC*	30S ribosomal protein S3	1.78	1.51	**2.27**	1.92
PA4258	*rplV*	50S ribosomal protein L22	1.58	1.27	**2.01**	1.6
PA4260	*rplB*	50S ribosomal protein L2	1.49	1.24	**2.06**	1.44
PA4262	*rplD*	50S ribosomal protein L4	1.56	1.62	**2.21**	1.71
PA4266	*fusA1*	Elongation factor G	1.56	1.59	**2.1**	1.85
PA4268	*rpsL*	30S ribosomal protein S12	1.96	1.61	**2.47**	1.63
PA4272	*rplJ*	50S ribosomal protein L10	1.37	1.36	**2.08**	1.98
PA4944	*hfG*		1.88	**2.15**	1.79	1.97
PA5212		Hypothetical protein	**2.01**	1.55	−1.00	−1.09

[a] Bold type indicates increased gene expression (≥2-fold), and italics indicate decreased gene expression (≥2-fold).
[b] CoA, coenzyme A.

coded in the *nos* operon. It has been also proposed that the MoeA-catalyzed product, an activated form of molybdate, regulates the *nar* operon (42).

Role of the CreBC (BlrAB) system in biofilm growth. Considering the above results in light of previous findings revealing a role of the denitrification cascade in biofilm formation (37, 43), we evaluated the impact of the CreBC system in biofilm growth in the presence or absence of the AmpC inducer cefoxitin. As shown in Fig. 4, the inactivation of *creD* led to a significant reduction of biomass in both 24-h- and 48-h-old biofilms. Moreover, even at the low concentrations used (>20-fold below MICs), cefoxitin significantly reduced the biomass of 48-h biofilms from that in wild-type PAO1. Furthermore, the effect of cefoxitin on biofilm growth was dramatically enhanced in the *creBC* and *creD* mutants.
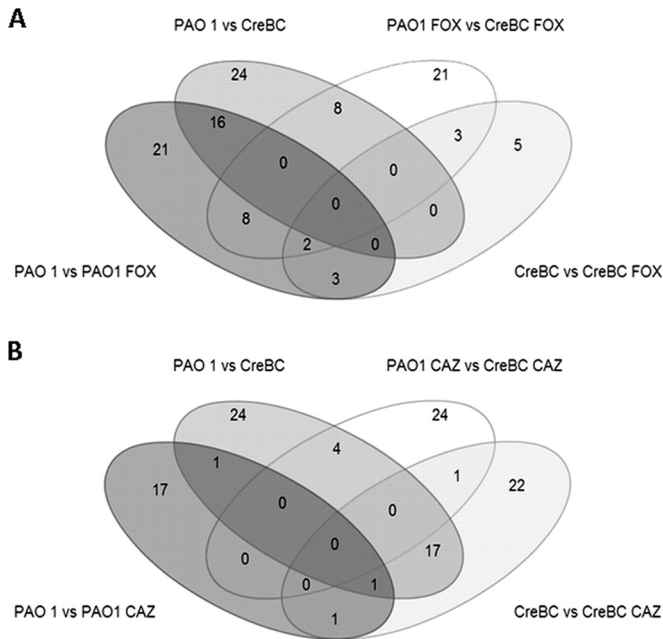
A

PAO 1 vs CreBC / PAO1 FOX vs CreBC FOX / PAO 1 vs PAO1 FOX

B

PAO 1 vs CreBC / PAO1 CAZ vs CreBC CAZ / PAO 1 vs PAO1 CAZ / CreBC vs CreBC CAZ

FIG 3 Venn diagrams of the differentially regulated genes. Shown are the distributions of significantly ($P < 0.01$) differentially regulated ($\geq$2-fold) genes in PAO1 and PAOΔcreBC treated or not with 50 μg/ml cefoxitin (A) or 0.5 μg/ml ceftazidime (B).

Therefore, our results indicate a clear connection between the CreBC system, AmpC induction pathways, and biofilm formation.

Identification of the cre-tag. In an attempt to identify if the genes that are differentially regulated by the CreBC system presented the CreBC-tag, a bioinformatics approach was applied to all CreBC-regulated genes from the microarray data for potential CreBC-binding sites. The criteria and consensus sequences described for *E. coli* (20, 21) and *Aeromonas* spp. (19, 20) from the CreBC-dependent genes were used. For a direct repeat to be classed as a cre-tag, it should be oriented in the same direction as a 3′-proximal gene and within 450 bp of the ATG initiation codon of that gene. Using this consensus sequence to search the upstream
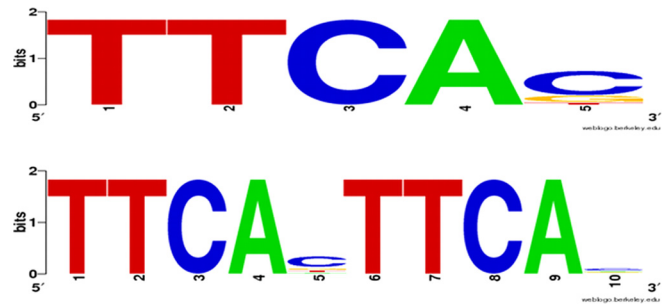

FIG 5 cre-tag analysis. The putative CreBC motif was found by analyzing the 500 bp upstream of differentially CreBC-regulated and CreBC-β-lactam-regulated genes/operons (*P* value, $\leq$0.01, $\geq$2-fold) (Tables 4 and 5) in PAOΔcreBC. The *Pseudomonas* Genome Database (35, 36) was used to identify the computationally predicted operons and the promoter-proximal TTCAC nnnnnnTTCAC cre-tag motifs. The output is represented using WebLogo (53) for the *creBC*-dependent genes, including the presence of the single tag (top) and the direct double repeats (bottom).

differentially regulated gene regions of the PAO1 genome led to the identification of the cre-tag in the upstream region of 10 of the 14 genes/operons analyzed (PA0515, *nirS*, *rsaL*, *phzA2*, PA3871, PA4141, *nosZ*, *moeA1*, *phzA1*, and *groeS*), with 50% of them being double direct repeats (Fig. 5). Moreover, an analysis of the upstream sequence of 100 randomly selected genes revealed that only 22 of them had the single tag, but no direct repeats were found, confirming the significant association between the presence of the cre-tag and experimental CreBC dependence.

In previous studies performed with *E. coli*, a direct analysis performed over the whole sequence led to the identification of eight genes that contained the cre-tag motif, *ackA*, *talA*, *radC*, *malE*, *trgB*, *creD*, *yidS*, and *yieI* (44). Nevertheless, our results reveal that none of those genes except for *creD* had cre-tag motifs or altered expression in *P. aeruginosa*. Zhou et al. (44) described in a systematical analysis of a CreBC mutant the differential expression of several open reading frames of unknown function, which were designated *cbrA*, *cbrB*, and *cbrC* (*creB*-regulated genes A, B, and C, respectively) (44). No phenotypic changes were observed for the multiple *creABCD* mutants analyzed, but significant differences were observed for the *cbrA* and *cbrBC* double mutants. In-
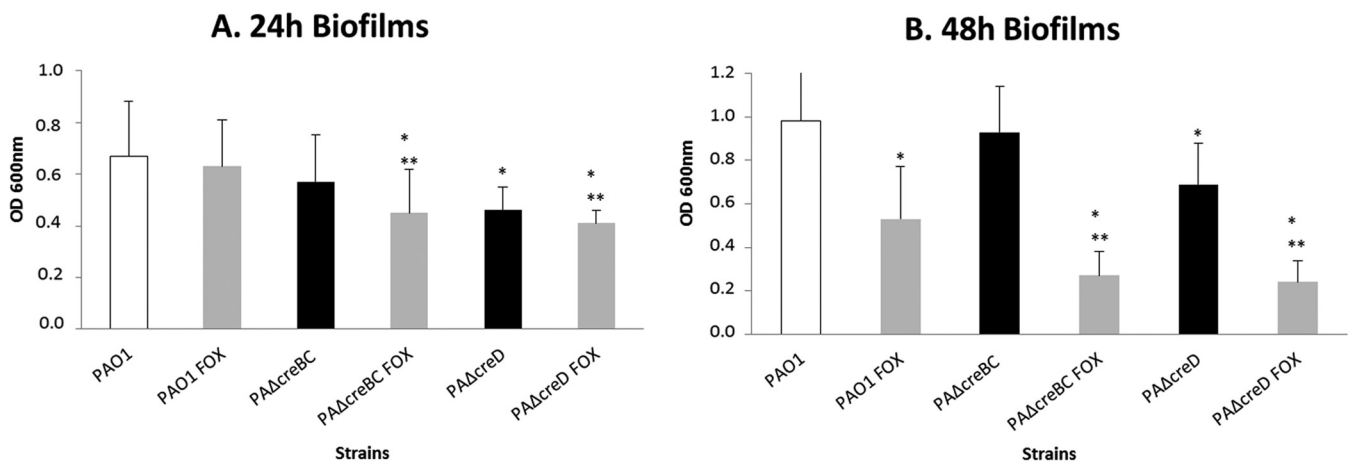

FIG 4 Biomass of 24-h (A) and 48-h (B) biofilms formed by PAO1, PAOΔcreBC, and PAOΔcreD strains exposed or not to 50 μg/ml cefoxitin (FOX). * and **, statistically significant differences for the comparisons with PAO1 and PAO1, respectively, exposed to cefoxitin. Data shown are means plus standard deviations.

terestingly, subinhibitory concentrations of colicin M induce the transcription of genes involved in adaptive responses, including the two-component CreBC system associated with increased resistance to some colicins (45). Recent studies have demonstrated that CbrA, being a nonessential protein, confers high resistance to colicin M and renders cells resistant to osmotic shock in a CreBC-dependent manner (46). On the other hand, the overproduction of YieJ (formerly CbrC) enhances the tolerance of colicin E2 (a role previously assigned to CreD). One role of the Cre regulon might be to cause modification of the cell envelope, since previously identified colicin E2 and colicin M tolerance mechanisms are associated with a reduction in the ability of both bacteriocins to cross the envelope. Although *P. aeruginosa* lacks orthologs of these two genes, there are clear implications of this system in the response to cell wall stress in the presence of β-lactams. It has also been reported that all of the β-lactamase genes known for *Aeromonas* spp. have at least one copy of the cre/blr-tag sequence, TTCAC. Indeed, *Aeromonas hydrophila* genes *ampH*, *cepH*, and *imiH* are in fact regulated by the CreBC (*blrAB*) system as well as the blr regulon gene, *blrD* (*creD*) (20).

We previously reported that in *P. aeruginosa*, a PBP4 mutation leads to AmpR-dependent *ampC* overexpression and CreBC-dependent *creD* overexpression (15), thus revealing an efficient mechanism of one-step high level β-lactam resistance. A link between PBP4, β-lactamase expression, and the BlrAB system was detected in *Aeromonas* spp. as well (47). Indeed, the *P. aeruginosa* CreBC system is also more functionally similar to the BlrAB system (15). Previous works showed that the regulatory system of *Aeromonas* spp. (BlrAB) is highly homologous to the *E. coli* CreBC system (20, 21). Furthermore, different studies suggested that the CreBC system of *E. coli* can regulate the expression of *Aeromonas* spp. β-lactamase when cloned into *E. coli* (43, 48).

In summary, the results obtained in this work evidence (i) the specific response of the CreBC system to AmpC inducer/PBP4 inhibitor β-lactams, (ii) an interplay between the CreBC system, peptidoglycan recycling, and the AmpC regulatory pathways, (iii) the important impact of the CreBC system in bacterial fitness and biofilm formation in the presence and absence of β-lactams, and (iv) broad regulatory functions of the CreBC system in basic physiological aspects. Therefore, this work demonstrates a clear role of the CreBC system in β-lactam resistance that is typical of the BlrAB system and a physiological/metabolic role typical of the *E. coli* CreBC system. Therefore, the CreBC/BlrAB pathways studied so far in *E. coli*, *Aeromonas* spp., and *P. aeruginosa* appear to be a single system, which responds to signals generated in the cell wall, and these pathways regulate the expression of a set of genes in response to specific environments and metabolic signals, including the presence of β-lactams.

We demonstrate the presence of the putative cre-tag motif in a high percentage of the genes regulated by this system (single or direct repeat) that turn CreBC into a global regulator. On one hand, they play a major role in high-level β-lactam resistance (the *blrAB* resemblance). On the other hand, they act as a global transcription control regulator (the *creBC* role) that exerts its function over several keystone components, such as *creD*, which plays a major role in fitness maintenance, or larger subsets of genes, like the NAR operon, a key factor for the development and maintenance of biofilms (37), as well as the NIR and NOS gene clusters. Indeed, the nitrate sensor-response regulator extends beyond the regulation of nitrate me-

tabolism and interferes in motility, biofilm formation, and virulence (49). Moreover, a recent work suggested that the activation of the nitrate respiratory chain in *P. aeruginosa* might be a response in order to compensate for the fitness cost associated with particular antibiotic resistance mechanisms (50). Therefore, our results complement previous studies performed with the AmpR mutant that point out a link between β-lactamase and global regulation in *P. aeruginosa* (3, 51, 52). Indeed, although the exact CreBC regulatory mechanisms are still unknown, we demonstrate the complexity and multiregulatory processes by which *P. aeruginosa* controls the expression of genes of diverse functions from β-lactam resistance to biofilm formation or anaerobic respiration. Therefore, the CreBC system is envisaged as a potentially interesting target for the development of new molecules for the treatment of *P. aeruginosa* infections.

ACKNOWLEDGMENTS

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