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Regulation of Carbon and Nitrogen Utilization by CbrAB and NtrBC Two-Component Systems in *Pseudomonas aeruginosa*▽†

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The global effect of the CbrAB and NtrBC two-component systems on the control of carbon and nitrogen utilization in *Pseudomonas aeruginosa* was characterized by phenotype microarray analyses with single and double mutants and the isogenic parent strain. The tested compounds were clustered based on the growth phenotypes of these strains, and the results clearly demonstrated the pivotal roles of CbrAB and NtrBC in carbon and nitrogen utilization, respectively. Growth of the *cbrAB* deletion mutant on arginine, histidine, and polyamines used as the sole carbon source was abolished, while growth on the tricarboxylic acid (TCA) cycle intermediates was sustained. In this study, suppressors of the *cbr* mutant were selected from minimal medium containing L-arginine as the sole carbon and nitrogen source. These mutants fell into two groups according to the ability to utilize histidine. The genomic library of a histidine-positive suppressor mutant was constructed, and the corresponding suppressor gene was identified by complementation as an *ntrB* allele. Similar results were obtained from four additional suppressor mutants, and point mutations of these *ntrB* alleles resulting in the following changes in residues were identified, with implications for reduced phosphatase activities: L126W, D227A, P228L, and S229I. The Ntr systems of these *ntrB* mutants became constitutively active, as revealed by the activity profiles of glutamate dehydrogenase, glutamate synthase, and glutamine synthetase. As a result, these mutants not only regain the substrate-specific induction on catabolic arginine and histidine operons but are also expressed to higher levels than the wild type. While the Δ*cbrAB ntrB*(Con) mutant restored growth on many N-containing compounds used as the carbon sources, its capability to grow on TCA cycle intermediates and glucose was compromised when ammonium served as the sole nitrogen source, mostly due to an extreme imbalance of carbon and nitrogen regulatory systems. In summary, this study supports the notion that CbrAB and NtrBC form a network to control the C/N balance in *P. aeruginosa*. Possible molecular mechanisms of these two regulatory elements in the control of arginine and histidine operons used as the model systems are discussed.

Pseudomonads are known for their metabolic versatility, being able to utilize a wide range of organic compounds as carbon and/or nitrogen sources. Expression of catabolic enzymes for the assimilation of these compounds is subjected to carbon catabolite repression (CCR) of unknown mechanisms in these organisms. In contrast to what occurs in *Escherichia coli* and *Bacillus subtilis*, glucose does not play the central role in CCR in pseudomonads in many aspects. In *Pseudomonas aeruginosa*, the presence of succinate was reported to repress enzymes of hexose catabolism (35). In this organism, glucose uptake is not carried out by a phosphoenolpyruvate-dependent phosphotransferase system (21). Besides, the levels of cyclic AMP (cAMP) do not change in response to the presence or absence of glucose, and the cAMP-cAMP receptor protein complex plays no role in CCR in *P. aeruginosa* (18, 27, 35, 37, 38).

The Crc protein was first reported as a player in CCR of catabolic genes for sugars and aromatic compounds in *P. aeruginosa* and *Pseudomonas putida* (7, 12, 13, 19, 39). Crc exhibits sequence similarity to endo- and exonucleases, but the proposed nuclease or DNA-binding activity has not been demonstrated, despite extensive efforts (18). Recent reports suggested that Crc may regulate the expression of the affected genes posttranscriptionally as a component of a hypothetical signal transduction pathway in CCR (13, 40).

The CbrAB two-component system was discovered in *P. aeruginosa* as important regulatory elements for the expression of several catabolic pathways (23), and utilization of a variety of organic compounds as the sole carbon source was abolished or severely retarded in the mutant strains of *cbrAB* (23). Sequence comparison revealed that CbrAB belong to the NtrBC family. While the signal of the CbrAB system remained unknown, this system was suggested to function coordinately with NtrBC to maintain the carbon-nitrogen balance (23).

Similar to that in enteric bacteria (28), ammonia assimilation in *P. aeruginosa* is mediated by an NADPH-dependent glutamate dehydrogenase (GDH) when ammonia supply is high and by a combined action of glutamine synthetase (GS) and glutamate synthase (GOGAT) when ammonia supply is limited (5, 11, 16, 28). Except *glnB* and *nac*, all genes in the nitrogen regulatory cascade of *E. coli* have their functional homologues annotated in the finished genomic project of *P. aeruginosa*; however, none of them have been characterized to confirm the proposed physiological function.

Arginine serves as a good carbon and nitrogen source for *P.*

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or description[a]	Source or reference
Strains		
E. coli		
DH5α	F$^-$ ϕ80d*lac*ΔM15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17*(r_K^- m_K^+) *supE44* λ$^-$ *thi-1 gyrA96 relA*	Bethesda Research Laboratory
SM10	*thi-1 thr leu tonA lacY supE recA*::RP4-2-Tc::Mu (Km^r)	8
P. aeruginosa		
PAO1	Wild type	10
PAO1-Sm	Spontaneous Sm^r of PAO1	25
PAO4460	*rpoN*::Km	23
PAO5100	*cbrAB*::Gm	17
PAO5122	PA5122::Tc	This study
PAO5125	*ntrBC*::Tc	This study
PAO5126	*cbrAB*::Gm, *ntrBC*::Tc	This study
Arg8	*cbrAB* suppressor mutant of PAO5100	This study
Arg9	*cbrAB* suppressor mutant of PAO5100	This study
Arg10	*cbrAB* suppressor mutant of PAO5100	This study
Arg24	*cbrAB* suppressor mutant of PAO5100	This study
Arg18	*cbrAB* suppressor mutant of PAO5100	This study
Arg34	*cbrAB* suppressor mutant of PAO5100	This study
Plasmids		
pUCP18	*Escherichia-Pseudomonas* shuttle vector	34
pRTP1-M	Ap^r; conjugation vector	36
pHIS	Ap^r; pUCP18 derivative carrying the 6.5-Kb *glnA-ntrC* fragment of Arg18	This study
pHIS1	Ap^r; derived from pHIS with a deletion of 4.1-Kb ClaI fragment covering *glnA*-PA5123	This study
pNTR18	Ap^r; pUCP18 derivative carrying the 1.4-Kb *ntrB* fragment of Arg18	This study
pQF52	Ap^r; *lacZ* translational fusion vector	26
pSA500	*glnA*::*lacZ* translational fusion of pQF52	This study
pST500	*aotJ*::*lacZ* translational fusion of pQF52	24
pSU500	*hutU*::*lacZ* translational fusion of pQF52	This study
pCBR	Ap^r; pUCP18 derivative carrying the 7.0-Kb EcoRI *cbrAB* fragment of PAO1	This study

[a] Antibiotic resistance phenotypes: Ap^r, ampicillin for *E. coli* and carbenicillin for *P. aeruginosa*; Gm^r, gentamicin; Km^r, kanamycin; Sm^r, streptomycin; Tc^r, tetracycline.

aeruginosa, as reflected by the existence of four catabolic arginine pathways (9, 15). Under aerobic conditions, arginine is transported into cells via an ABC transporter system encoded by the *aotJQMP* genes (24) and is converted into glutamate via the arginine succinyltransferase pathway encoded by the *aruCFGDBE* operon (15). Both the *aot* and the *aru* operons are induced by the presence of exogenous arginine. ArgR, the arginine-specific regulatory protein belonging to the AraC/XylS family of transcriptional regulators, is essential for the activation of the *aot-argR* and *aru* operons by arginine (24, 25).

Histidine is another good source of carbon and nitrogen of *P. aeruginosa*. The σ^{54}-dependent *hutUHIG* operon encodes enzymes for degrading histidine into glutamate and formamide (32). In *P. aeruginosa* and *P. putida*, the *hut* operon is repressed by HutC through its interactions with an operator proximal to the *hut* promoter, and binding of urocanate produced from histidine catabolism to HutC removes this repression effect (14).

To further understand the molecular mechanism of CbrAB in CCR, in this study spontaneous suppressors of the *cbrAB* mutant were isolated and analyzed to identify additional players that may interact with CbrAB in the control of nutrient utilization. Using catabolism of arginine and histidine as two models, here we present evidences to support that CbrAB and NtrBC two-component systems work coordinately to control the carbon and nitrogen metabolic flows in *P. aeruginosa*.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Luria-Bertani (LB) and 2× tryptone-yeast extract media (33) were used for strain construction with the following supplements as indicated: ampicillin at 100 μg/ml, carbenicillin at 200 μg/ml, gentamicin at 50 μg/ml, tetracycline at 10 μg/ml for *E. coli* or 100 μg/ml for *P. aeruginosa*, and 5-bromo-4-chloro-3-indolyl-β-D-galactoside at 0.03% (wt/vol). Minimal medium P (MMP) supplemented with indicated carbon sources at 20 mM and nitrogen sources at 5 mM was used for the growth of *P. aeruginosa* (10).

Growth phenotype analysis. Phenotype microarray (PM; Biolog Co.) plates were used to analyze utilization of carbon, nitrogen, sulfate, or phosphate sources. Ammonium was provided as the sole nitrogen source when carbon sources were tested, and succinate was provided as the sole carbon source when nitrogen sources were tested. PM experiments were conducted according to the manufacturer's instruction. In short, fresh overnight cells from an agar plate were suspended in the inoculating fluid and the turbidity was adjusted to 85%. The cells were inoculated into PM plates and incubated at 37°C for 24 h for carbon plates and 48 h for nitrogen plates. Cell growth was reflected by the development of purple coloration as monitored and recorded by OmniLog PM Station and PM Kinetic (Biolog). Further information on the compounds tested with the PM kit can be found at the Biolog website.

Isolation of the suppressor mutants. Cells were grown in the LB medium overnight and harvested by centrifugation at 3,000 × *g* for 10 min. The cell pellet was washed twice, suspended, and spread onto MMP plates supplemented with

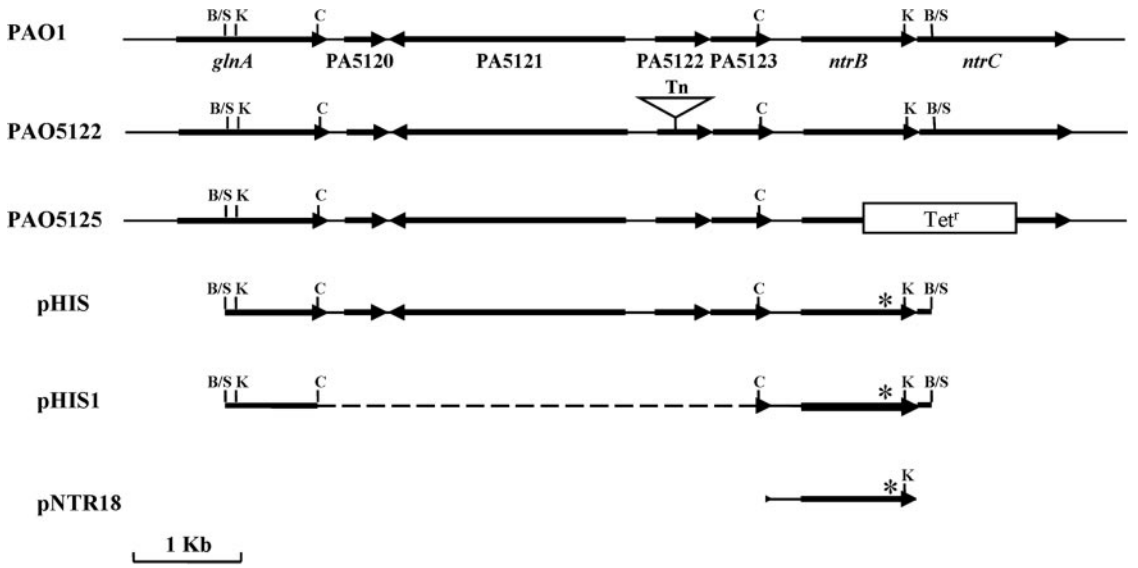

FIG. 1. *glnA-ntrC* loci of PAO strains and plasmids. The PA gene numbers are based on annotations of the Pseudomonas Genome Project (http://www.pseudomonas.com/). Drawings for genes and transposon cassettes (Tn) are symbolic and not scaled. Dashed lines denote regions of deletion. Thick arrows represent genes and open reading frames with orientations. Strain PAO5122 carries a tetracycline resistance Tn inserted in PA5122 that was used as the source of PCR to make the *ntrBC* deletion mutant PAO5125. Strain PAO5125 has an antibiotic resistance (Tet^r) cassette in place of the deleted *ntrBC* region. Plasmids pHIS, pHIS1, and pNTR18 carry an *ntrB*(Con) allele from Arg18 (Table 1); the asterisks depict the relative locations of the point mutations carried on the *ntrB* genes of these constructs. Only relevant restriction sites are indicated: B, BamHI; C, ClaI; K, KpnI; S, Sau3AI.

20 mM arginine or histidine as the sole source of carbon and nitrogen. The suppressor mutants that showed visible colony sizes after incubation at 37°C for 48 h were isolated.

Genomic library construction and screening. Genomic DNA of *P. aeruginosa* Arg18 was subjected to partial digestion with Sau3AI, and the DNA fragments of 4 to 20 kb were size fractionated by agarose gel electrophoresis and subjected to cloning into the dephosphorylated BamHI site of pUCP18 (34). After transformation into *E. coli* DH5α, the genomic library was further enriched by agarose gel electrophoresis to separate recombinant plasmids from the vector. The library was then introduced into PAO5100 by transformation (6), and the transformants were screened onto MMP plates supplemented with nutrients as indicated.

Construction of *lacZ* fusions. DNA fragments containing the *glnA* and *hutU* promoter regions were PCR amplified from the genomic DNA of PAO1 with the following synthetic oligonucleotide primers: 5′-ACGAT AAGCTT CAT GCG AGG GAC GCGAA-3′ and 5′-CTT GTA CGA CAT GCT GTC CTC CAG GAGG-3′ for *glnA*; 5′-GAC TAC AAGCTT CAG GCG CAG GGC TGG-3′ and 5′-CGG GGT GGT CAC GGC AGG CTC CTG GGC-3′ for *hutU*. The PCR products were purified from a 1% (wt/vol) agarose gel, digested by restriction endonuclease HindIII, and ligated to the HindIII and SmaI sites of the translational fusion vector pQF52 (26). The resulting plasmids, pSA500 and pSU500, contain the entire upstream intergenic sequence of *glnA* and *hutU* and the 5′ end of the coding sequence fused in-frame to the eighth codon of the *lacZ* gene in the vector. The nucleotide sequences of the resulting constructs were verified by nucleotide sequence determination.

Construction of mutant strains and plasmids. To make the *ntrBC* mutant, the DNA fragment containing the N-terminal part of *ntrB* was amplified by PCR from the genomic DNA of PAO5122 (Fig. 1) with a transposon-specific flanking primer containing synthetic oligonucleotides designed to generate the HindIII or BamHI restriction site (underlined): 5′-AGGG AAGCTT CGC GCT TGG TGA ACG GATGT-3′. The C-terminal DNA fragment of *ntrC* was PCR amplified from the PAO1 genomic DNA with the following two primers: 5′-TGGA AAGCTT GGT GCG CGA CGG CAA GTTC-3′ and 5′-GTC GGATCC ATC GAC GTT CAT CCC CAG CTC-3′. The PCR products were purified from a 1% (wt/vol) agarose gel, digested by appropriate restriction endonucleases, and assembled onto the EcoRI and BamHI sites of pRTP1-M (36). The Tet^r cassette of EZ::TN<TET-1> was gel purified after HindIII digestion of a transposon-inserted plasmid and ligated to the HindIII fusion site of the NtrB-NtrC hybrid as described above. For gene replacement, the resulting transposon insertion plasmid in *E. coli* SM10 was mobilized into the recipient strains by biparental mating (8). Following incubation at 37°C overnight, transconjugants were selected on LB plates supplemented with tetracycline (100 μg/ml) and streptomycin (500 μg/ml). The correct gene replacements were confirmed by PCR with the following two primers flanking the *ntrBC* locus: 5′-GGT CAG CGC GAT AGA GCC AAG CGA-3′ and 5′-GTT GGG TCG CTC ATG GGA GCA GTG GCG CTT-3′.

To analyze the nucleotide sequences of the histidine suppressor mutants, the *ntrB* and *ntrC* alleles were PCR amplified by *Pfu* polymerase from genomic DNA of the suppressor mutants and PAO5100 with the following synthetic oligonucleotides: for *ntrB*, 5′-GGT CAG CGC GAT AGA GCC AAG CGA-3′ and 5′-GAC GAT CCA GAC GGT CTC TGA TCG-3′; for *ntrC*, 5′-CCT GCC CCT GGA ACA AGG AGT GCA TTG ACC-3′ and 5′-GTT GGG TCG CTC ATG GGA GCA GTG GCG CTT-3′. The amplified fragments were purified from a 1% (wt/vol) agarose gel and ligated into the SmaI site of pUCP18 (34). To make the pCBR plasmid, the 7-kb EcoRI fragment covering the *cbrAB* operon was purified from a cosmid clone, which was identified from colony screening of a genomic library constructed in this laboratory, and subcloned into pUCP18 (34). The DNA sequences of the insert on the plasmid were confirmed by nucleotide sequencing.

Enzyme assays. GS was assayed as γ-glutamyltransferase activity in cetyltrimethyl ammonium bromide-treated cells (16). The GOGAT and anabolic GDH activities were assayed as described previously (11). β-Galactosidase activities were determined by the methods of Miller (22). Protein concentrations were determined by the method of Bradford (4) with bovine serum albumin as the standard.

RESULTS

Growth phenotype analysis of *cbrAB* and *ntrBC* mutants. The CbrAB two-component system was reported by Nishijyo et al. (23) to control the utilization of carbon/nitrogen sources in *P. aeruginosa*, and it was anticipated that the NtrBC system controls nitrogen utilization in this organism. To further understand the physiological function of CbrAB and NtrBC in carbon and nitrogen catabolism, we conducted extensive growth phenotype analyses by PMs with the wild-type strain

PAO1, the *cbrAB* mutant PAO5100, the *ntrBC* mutant PAO5125, and the *cbrAB ntrBC* double mutant PAO5126.

In comparison to the growth pattern of PAO1, that of the *ntrBC* mutant PAO5125 showed an expected deficiency on the utilization of N-only compounds (e.g., nitrite, nitrate, and urea) and specific C/N compounds (e.g., uridine and cytidine) as the sole nitrogen source (see Table S1 in the supplemental material), as has been reported for *E. coli* and many other enteric bacteria (20, 29). In contrast, the *cbrAB* mutant was defective in carbon utilization of a variety of compounds (24 out of 49), including C-only compounds (e.g., sugars, acetate, pyruvate, and lactate) as well as C/N compounds (e.g., arginine, histidine, and proline) (see Table S1 in the supplemental material). While the *cbr* mutant lost the capability to utilize many C/N compounds as the sole carbon source, almost all of these compounds could still serve as the sole nitrogen source in this mutant as well as in the *cbr ntr* double mutant, with few exceptions (e.g., L-histidine and L-serine). These results clearly delineate the pivotal role of CbrAB and NtrBC in regulation of carbon and nitrogen utilization, respectively; however, it was also intriguing to note that a *cbrAB ntrBC* double mutant retained the capability to utilize most C/N compounds as the sole nitrogen source.

Suppressors of the Δ*cbrAB* mutant. The spontaneous suppressors of PAO5100 (Δ*cbrAB*) were isolated from plates that restored growth on arginine used as the sole source of carbon and nitrogen. Among over 100 suppressors obtained, 90 of these mutants were randomly picked for further growth phenotype analyses of histidine and glucose, which can be utilized by PAO1 but not PAO5100. It was found that these suppressors can be divided into two groups: 5 mutants showing a histidine-positive, glucose-negative phenotype, as represented by Arg18, and 85 mutants exhibiting a histidine-negative, glucose-positive phenotype, as represented by Arg34.

To further explore the differences in catabolic capability, Arg18 and Arg34 were subjected to more extensive analyses by PMs. For carbon source utilization, Arg34 exhibited a pattern almost identical to that of the wild-type strain PAO1, except showing no growth on histidine, ornithine, and hydroxy-L-proline (see Table S2 in the supplemental material). Arg18 regained the ability to utilize most of the N-containing compounds but meanwhile lost the capability to utilize tricarboxylic acid (TCA) cycle intermediates (e.g., succinate) and most carbon sources containing no nitrogen moiety (see Table S2 in the supplemental material). For nitrogen source analysis, Arg34 had the same pattern as the wild-type strain PAO1. Arg18 was found surprisingly to show no growth on ammonium but grew normally on nitrate, urea, and other compounds (see Table S2 in the supplemental material). Since ammonium was used as the supplement in carbon source analysis and succinate was added as the carbon supplement in nitrogen source analysis, these results suggest that Arg18 might be deficient in the uptake or assimilation of ammonium per se.

Arg18 possesses a mutation of nitrogen regulation in the *ntrB* gene. In order to uncover the suppressor mutation of Arg18, a genomic library of Arg18 was constructed and employed to conduct genetic complementation of PAO5100 on histidine utilization. By this approach, the recombinant plasmid pHIS was recovered from one clone. To substantiate the physiological function of pHIS, this plasmid was introduced into PAO5100, and the resulting recombinant strain of PAO5100 harboring pHIS exhibited the same growth phenotypes as Arg18.

The results of sequence analysis revealed that pHIS carries a 6.5-kb chromosomal fragment with five complete (PA5120 through *ntrB*) and two truncated (*glnA* and *ntrC*) open reading frames (Fig. 1). The *glnA*, *ntrB*, and *ntrC* genes encoding GS and the NtrBC sensor/response regulator of nitrogen regulation were annotated based on sequence analysis. PA5120 to PA5123, the four genes between *glnA* and *ntrB*, encoded hypothetical proteins with unknown functions. When the subclone pHIS1 (Fig. 1), carrying an intact *ntrB* and devoid of four upstream genes, was introduced into PAO5100, it exerted the same complementation effect as pHIS.

To define the nature of the mutation responsible for the Arg18 phenotype, the nucleotide sequence of the DNA fragment carried by pHIS was determined and subjected to sequence comparison to the finished genomic sequence of *P. aeruginosa* PAO1. Only a single T680G mutation, which led to the Asp227Ala substitution, was detected on the *ntrB* allele of pHIS. To confirm the authenticity of this identified mutation, the *ntrB* genes of PAO5100 and Arg18 were cloned after PCR and sequenced. Sequence analyses confirmed the same point mutation in *ntrB* of Arg18 as in pHIS. Furthermore, a complementation experiment demonstrated that only the plasmid containing the *ntrB* allele of Arg18 (pNTR18) (Fig. 1), but not the wild-type *ntrB* gene, was able to convert the growth phenotype of PAO5100 on carbon utilization to that of Arg18.

Analysis of four other histidine-positive suppressors of PAO5100. Like Arg18, four other suppressors of PAO5100 (Arg8, Arg9, Arg10, and Arg24) isolated from arginine plates that restored the ability to utilize histidine as the sole carbon and nitrogen source were subjected to genetic analysis. Based on the results of allele analysis of Arg18, we predicted that the suppressor genes of these four mutants might be either *ntrB* or *ntrC*. Accordingly, the *ntrB* and *ntrC* genes of these mutants were PCR amplified and cloned into pUCP18. The resulting constructs were subjected to DNA sequence analysis and complementation analysis. Each of these four mutants carried a point mutation in the *ntrB* gene which led to the following amino acid substitutions: L126W in Arg8, P228L in Arg9, and S229I in Arg10 and Arg24. Complementation of PAO5100 with these *ntrB* mutant clones also resulted in a growth phenotype identical to that of the suppressor mutants. These results were consistent to what was observed in Arg18 as described above.

Effect of CbrAB and NtrBC on histidine and arginine catabolism. To understand the molecular mechanism responsible for the growth phenotype of PAO5100 and Arg18, we selected histidine and arginine utilization as two model systems for further analysis. A *hutU*::*lacZ* translational fusion, pSU500, was constructed to investigate expression of the *hut* operon with histidine utilization in different strains (Table 2). The cells were grown in glutamate minimal medium in the presence and absence of histidine. In the wild-type strain PAO1, exogenous histidine induced the expression of the *hutU* promoter, and this induction effect was abolished in PAO5100 devoid of *cbrAB*. In Arg18, the *hutU* promoter exhibited a higher basal level of activity than in PAO1, and exogenous histidine could further induce this promoter over threefold higher than that of PAO1

TABLE 2. Effects of Cbr and Ntr on expression of β-galactosidase activities from the *PhutU*::*lacZ* and *PaotJ*::*lacZ* fusions

Strain	Genotype	Sp act (nmol/min/mg)[a] for:			
		pSU500		pST500	
		G	G+H	G	G+A
PAO1	Wild type	4	181	7,924	51,989
PAO5100	Δ*cbrAB*	2	9	6,036	10,391
Arg18	Δ*cbrAB ntrB*(Con)	30	633	35,077	164,784
PAO4460	*rpoN*	1	1	140	230

[a] Cells were grown in minimal medium P supplemented with 20 mM of glutamate (G), histidine (H), and arginine (A). Specific activities represent the averages for two measurements, with standard errors below 5%. For PAO4460, the medium was supplemented with 1 mM of glutamine.

TABLE 3. Effects of Ntr and Cbr on expression of enzymes in ammonium assimilation

Strain	Genotype	Nitrogen source	Sp act (nmol/min/mg)[a] for:			
			GDH	GOGAT	GS	GlnA-LacZ
PAO1	Wild type	Ammonium	427	34	28	3
		Nitrate	16	7	155	48
PAO5100	Δ*cbrAB*	Ammonium	339	33	20	2
		Nitrate	17	11	87	18
Arg18	Δ*cbrAB ntrB*(Con)	Ammonium	0.6	7	262	107
		Nitrate	9	12	141	50
PAO5125	Δ*ntrBC*	Ammonium	477	91	14	2
		Nitrate	389	81	19	2

[a] Cells were grown in minimal medium P with 20 mM of succinate as the carbon source and 5 mM of ammonium or nitrate as the nitrogen source. To grow Arg18 with ammonium and PAO5151 with nitrate, limiting amounts of glutamate (1 mM) and glutamine (0.4 mM), respectively, were added to support the growth until the optical density at 600 nm was ~0.5. GlnA-LacZ, β-galactosidase activity from the *PglnA*::*lacZ* fusion. Specific activities represent the averages for two measurements, with standard errors below 5%. Activities of GS were measured in the presence of 5 mM Mg^{2+} and are expressed in mg of dry weight, while those of GDH and GOGAT are given in mg of total protein.

(Table 2). A similar pattern of promoter activities was also observed when pST500, an *aotJ*::*lacZ* translational fusion (24), was used to investigate expression of the *aot* operon for arginine transport in the same set of strains in response to the presence and absence of exogenous arginine (Table 2).

Since NtrC and CbrB are enhancers of σ^{54} promoters, we also conducted measurements of *aotJ* and *hutU* promoter fusions in the *rpoN* mutant. As shown in Table 2, these two promoters were no longer subjected to the substrate-specific induction, and only low basal levels of expression can be observed in the *rpoN* mutant. These results strongly support the hypothesis that the CbrAB and NtrBC systems participate in regulation of genes for uptake or catabolism of histidine and arginine and possibly other C/N sources, as revealed by PM analysis.

The Ntr system in Arg18 is constitutively active. The *ntrB* mutation in Arg18 was anticipated to exert an effect on nitrogen regulation. To characterize the nature of this effect, the specific activities of GDH, GOGAT, and GS, three major enzymes in ammonium assimilation under the control of Ntr, were measured in PAO1 (wild type), PAO5100 (Δ*cbrAB*), PAO5125 (Δ*ntrBC*), and Arg18. The cells were grown in succinate minimal medium with ammonium or nitrate as the sole nitrogen source. For PAO5125 growing on nitrate and Arg18 on ammonium, limiting amounts of glutamine (0.4 mM) and glutamate (1 mM), respectively, were added to enable cell growth until the optical density at 600 nm reached 0.5.

In the wild-type strain PAO1, GDH and GOGAT of glutamate biosynthesis were induced by ammonium and repressed when nitrate was the nitrogen source (Table 3). As expected, the expression profile of GS in PAO1 was reciprocal to that of GDH and GOGAT, i.e., repression by ammonium and induction by nitrate. A similar expression pattern of these three enzymes was also observed in PAO5100, except that the level of GS in PAO5100 was about 50% of that in PAO1 when nitrate was provided as the nitrogen source (Table 3). These results suggested that CbrAB per se may not be essential for nitrogen regulation, consistent with the results of PM analyses.

In contrast, significant changes were observed when the Ntr system was disturbed. In PAO5125 devoid of *ntrBC*, both GDH and GOGAT activities were constitutively high regardless of the nitrogen sources while the levels of GS remained constitutively low (Table 3). These results strongly suggest that the Ntr system exerts a repression effect on expression of GDH and GOGAT and an activation effect on GS expression. In comparison, a completely reversed pattern of expression was observed in Arg18.

We also conducted experiments with pSA500, a *glnA*::*lacZ* fusion, to investigate the expression of the *glnA* gene at the transcriptional level in the same set of strains under the same growth conditions as those described above. As shown in Table 3, the resulting pattern of *glnA* promoter expression was basically identical to what was observed at the GS enzyme levels. Based on these results, we hypothesized that the *ntrB* allele of Arg18 makes the Ntr system constitutively active in this mutant.

An imbalanced act by *cbrAB* deletion and *ntrBC* activation in Arg18. As revealed by growth phenotype analyses, regaining the ability to utilize most of the N-containing carbon sources in Arg18 was compromised by losing the capability to utilize TCA cycle intermediates and most carbon-only sources when ammonium was the sole nitrogen source. To further dissect the possible contribution from Cbr deletion and Ntr activation in Arg18, PM analysis was conducted with Arg18 harboring pCBR, which contains and expresses functional CbrAB from the plasmid. The result showed that the pattern of carbon utilization by Arg18/pCBR was not different from that of PAO1, which strongly suggests that a constitutive Ntr system in Arg18/pCBR does not affect carbon utilization and that the unique growth phenotype of Arg18 might be the consequence of an extremely imbalanced act by Cbr deletion and Ntr activation.

DISCUSSION

The pleiotropic effect of CbrAB on carbon and/or nitrogen utilization was first reported by the work of Nishijyo et al. (23). In our current study, an extensive analysis of nutrient utilization in *cbrAB* and/or *ntrBC* mutants clearly established the physiological function of these regulatory systems in the control of carbon and nitrogen utilization in a coordinated manner. As shown in Table S1 in the supplemental material, all tested compounds used as the carbon and/or nitrogen sources

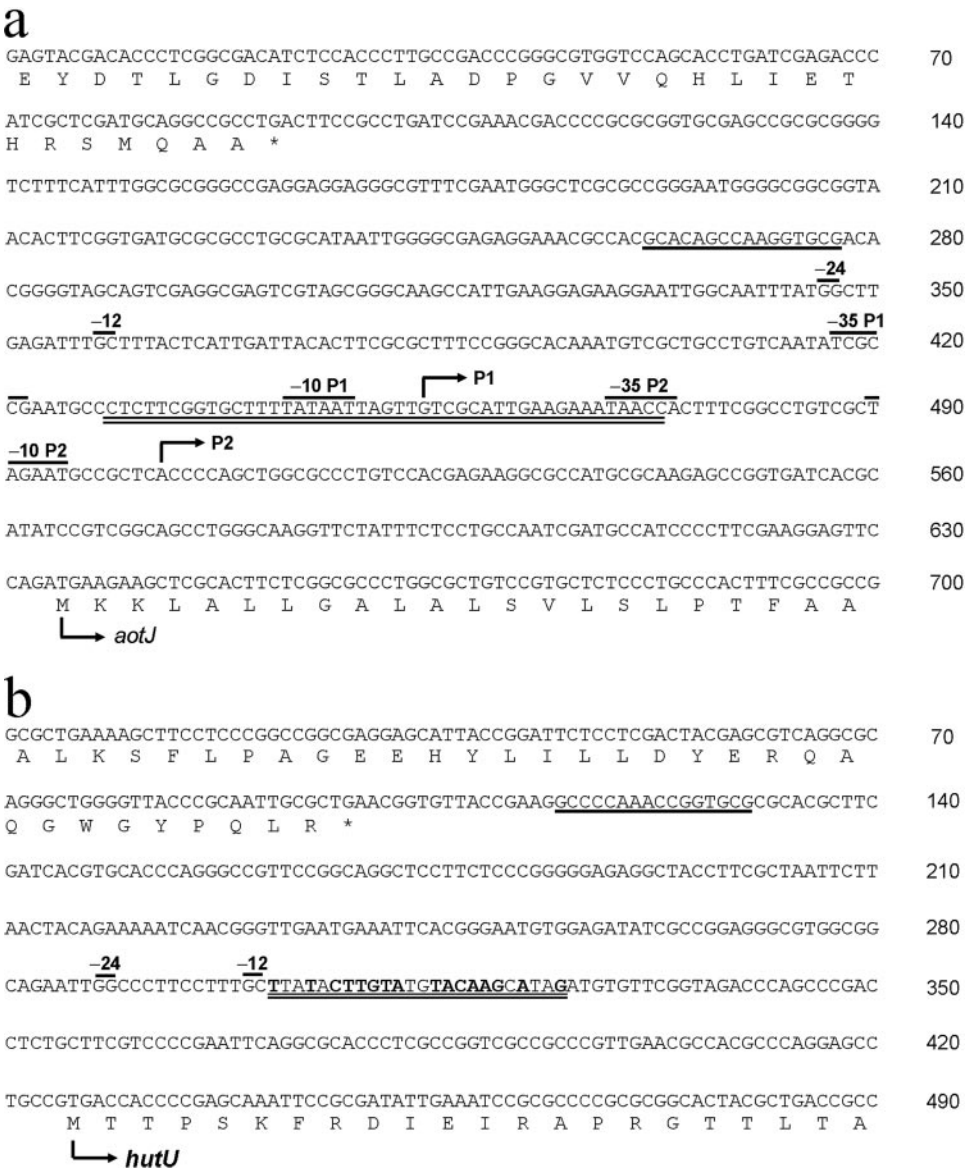

FIG. 2. Nucleotide sequences of the regulatory regions for the *aotJQMOP-argR* operon (a) and the *hutUHIG* operon (b). The −10 and −35 regions of each σ^{70} promoter and the −12 and −24 regions of a potential σ^{54} promoter are indicated accordingly. The reported transcriptional initiation sites of *aotJ* are marked by arrows above the nucleotides and labeled P1 and P2. The putative NtrC binding sites as identified by sequence similarities to the consensus sequence (30), TGCACCA-N3-TGGTGCA, are underlined. The reported ArgR binding site and the putative HutC binding site are double underlined.

can be categorized into different groups based on the effect of Cbr and/or Ntr. While utilization of many compounds as the carbon source was affected by CbrAB, it was noted that a specific group of compounds, including succinate, L-aspartate, glycerol, L-glutamate, L-asparagine, fumarate, α-ketoglutarate, and L-glutamine, was exempted from this regulation. One interesting common feature among these exempted compounds was their relationship to the TCA cycle. They are either TCA cycle intermediates or one or two catabolic steps away from the TCA cycle. It is intriguing to propose that perhaps these are the preferred carbon sources of *P. aeruginosa*, in accordance with the long-reported phenomenon of reversed CCR. Unlike in *E. coli* or *B. subtilis*, glucose is not the preferred carbon source in pseudomonads (39), and there are only two complete phosphoenolpyruvate-dependent phosphotransferase systems for fructose and *N*-acetylglucosamine uptake and utilization in *P. aeruginosa* PAO1 (31). Instead, *P. aeruginosa* preferentially utilizes succinate and other dicarboxylates of the TCA cycle (7, 39) over other compounds. While the molecular mechanism of CCR in pseudomonads is still unknown, it is not likely that CbrAB serves as the master regulator of CCR, but rather, it could be a direct target of CCR.

The results of PM analyses indicated that the *cbr ntr* double mutant retains the ability to grow on most C/N compounds used as the sole nitrogen source. At first sight, it seemed contradictory to the proposed pivotal role of CbrAB and

NtrBC in the control of carbon and nitrogen utilization. We proposed the following explanation based on a simple concept, supply and demand. It was well known that to support bacterial growth, the demand of nitrogen flow is much lower than that of carbon flow. Therefore, it might be that in the cases of most C/N compounds (e.g., arginine), the substrate-specific regulator per se can maintain levels of uptake and catabolism sufficient for sustaining growth as the nitrogen source, and the CbrAB system coordinates with the substrate-specific regulators to ensure a maximal supply when the demand as the carbon source is high. Depending on the regulatory circuit, the NtrBC system could play a significant role only in few cases (e.g., histidine).

The CbrB response regulator belongs to the NtrC family of transcriptional enhancers for the σ^{54}-dependent promoters (23). As in the Ntr system, it is conceivable that CbrB could activate the affected σ^{54} promoters of catabolic operons through the CbrA-CbrB phosphorelay in response to a signal(s) yet to be identified. This can be best demonstrated by the *hut* operon of histidine utilization. As shown in Fig. 2a, the *hutU* regulatory region of *P. aeruginosa* PAO1 contains one σ^{54} promoter (Y. Itoh, presented at the 9th International Congress on Pseudomonas, Quebec City, Quebec, Canada, 2003) which overlaps with a putative operator site of the HutC repressor ($HutC_{Pa}$). The helix-turn-helix DNA-binding domain sequence of the $HutC_{Pa}$ protein exhibits 85% identity to its counterpart in *Pseudomonas putida KT2440* ($HutC_{Pp}$), and the nucleotide sequence of this $HutC_{Pa}$ operator site is 94% identical to that of the reported $HutC_{Pp}$ site (14). In the wild-type strain PAO1, formation of the HutC-urocanate complex was proposed to release HutC from its operator, and expression of the *hutU*::*lacZ* fusion from a σ^{54} promoter was activated by either CbrB or NtrC, depending on the C/N status. As shown in Table 2, this histidine-dependent induction effect was abolished in the *cbr* mutant but was restored when a constitutive Ntr system was introduced. These data support the proposed model of Cbr/Ntr-dependent activation of σ^{54} promoters for catabolism of C/N compounds.

The model described above for the σ^{54}-dependent *hutU* promoter might not work properly for σ^{70} promoters, because structure-wide, σ^{54}-dependent transcriptional enhancers of the NtrC family do not activate directly on σ^{70} promoters, and no such case has been reported so far. For example, induction of the *aotJ* P2 promoter by exogenous arginine (Table 2) depends on the presence of ArgR, a transcriptional regulator of the AraC/XylS family. As shown in Fig. 2b, the reported *aotJ* promoters are presumably σ^{70} dependent, and it is unlikely that ArgR can serve as a transcriptional enhancer of σ^{54}-dependent promoters; however, Δ*cbrAB* and *ntrB*(Con) mutations were found to exert effects on the expression profile of the *aotJ*::*lacZ* fusion similar to those for the *hutU*::*lacZ* fusion (Table 2). It is possible that an additional regulatory protein that functions similarly to Nac in the Ntr system of *Klebsiella* and other enteric bacteria (1–3) could serve as a mediator of the Cbr/Ntr systems to extend the scopes of their control to σ^{70} promoters. On the other hand, a higher basal level of *aotJ*::*lacZ* expression in the $ntrB^c$ mutant implies the presence of a σ^{54} promoter yet to be identified in the *aotJ* regulatory region. Interestingly, a putative σ^{54} promoter (Fig. 2b) was predicted by computation analysis (http://www.promscan.uklinux.net/). More study is needed to verify this prediction and its possible contribution to Cbr/Ntr-dependent regulation of the *aotJQ-MOP-argR* operon.

With arginine utilization as the model system, two types of suppressors arose from the *cbr* mutant, as represented by Arg18 and Arg34. The genetic determinants were identified as *ntrB* alleles for suppression in Arg18 and several other strains in the same group. We proposed that mutations in these *ntrB* alleles resulted in a constitutively active Ntr system as supported by the expression profiles of GS, GOGAT, and GDH in Arg18 (Table 3). Mutations of the four *ntrB* alleles identified in this study are located in two regions of NtrB. Based on the reported characterization of *E. coli* NtrB, L126W is located in the central domain for the phosphotransferase/phosphatase activities whereas D227A, P228L, and S229I are clustered in a region at which the P_{II} protein interacts. The corresponding NtrB mutant forms of *E coli* exhibit impaired phosphatase activities, and thus, the NtrC protein remains phosphorylated and active.

In Arg18, although turning on the Ntr system enabled this mutant to regain growth on some C/N sources, including arginine and histidine, the trade-off was a compromised capability to grow on the "preferred" carbon sources (see description above) in the presence of ammonium. This unique growth phenotype of Arg18 was due to a combination of Δ*cbrAB* and *ntrB*(Con); complementation of the *cbrAB* deletion in Arg18 by a recombinant plasmid pCBR exhibited a pattern of C/N utilization not different from that of the wild type. Although currently we have no explanation of these observations at the molecular level, these data support the notion that CbrAB and NtrBC need to function in a balanced and coordinated manner in order to regulate the carbon and nitrogen metabolic flow in *P. aeruginosa*. One clue for solving this puzzle was buried in Arg34. It was intriguing that the growth phenotype of Arg34 was very much like that of the wild type except on a few compounds. The same approach of complementation was attempted but failed to identify the genetic determinant of Arg34, which suggest that Arg34 might carry a recessive suppressor mutation. Identification of the genetic determinant of Arg34 is currently in progress.

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