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Cloning and Characterization of *argR*, a Gene That Participates in Regulation of Arginine Biosynthesis and Catabolism in *Pseudomonas aeruginosa* PAO1

SEUNG-MOON PARK, CHUNG-DAR LU, AND AHMED T. ABDELAL*

Department of Biology, Georgia State University, Atlanta, Georgia 30303

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Gel retardation experiments indicated the presence in *Pseudomonas aeruginosa* cell extracts of an arginine-inducible DNA-binding protein that interacts with the control regions for the *car* and *argF* operons, encoding carbamoylphosphate synthetase and anabolic ornithine carbamoyltransferase, respectively. Both enzymes are required for arginine biosynthesis. The use of a combination of transposon mutagenesis and arginine hydroxamate selection led to the isolation of a regulatory mutant that was impaired in the formation of the DNA-binding protein and in which the expression of an *argF*::*lacZ* fusion was not controlled by arginine. Experiments with various subclones led to the conclusion that the insertion affected the expression of an arginine regulatory gene, *argR*, that encodes a polypeptide with significant homology to the AraC/XylS family of regulatory proteins. Determination of the nucleotide sequence of the flanking regions showed that *argR* is the sixth and terminal gene of an operon for transport of arginine. The *argR* gene was inactivated by gene replacement, using a gentamicin cassette. Inactivation of *argR* abolished arginine control of the biosynthetic enzymes encoded by the *car* and *argF* operons. Furthermore, *argR* inactivation abolished the induction of several enzymes of the arginine succinyltransferase pathway, which is considered the major route for arginine catabolism under aerobic conditions. Consistent with this finding and unlike the parent strain, the *argR*::Gm derivative was unable to utilize arginine or ornithine as the sole carbon source. The combined data indicate a major role for ArgR in the control of arginine biosynthesis and aerobic catabolism.

Arginine metabolism is of considerable significance in *Pseudomonas aeruginosa*, which can utilize this amino acid as a good source of carbon, nitrogen, and energy (16). The significance of arginine as a nutrient to *P. aeruginosa* is reflected in its being one of the strongest chemotactic attractants for this organism (10). This significance is also reflected in the presence of four catabolic pathways for the utilization of arginine (Fig. 1) (16): the arginine deiminase pathway, the arginine succinyltransferase (AST) pathway; the arginine dehydrogenase pathway, and the arginine decarboxylase pathway.

Recent work by Haas and coworkers has elucidated the role of *anr* (for anaerobic regulation) in induction of the arginine deiminase pathway by low oxygen tension (15). The arginine deiminase pathway functions to provide *P. aeruginosa* with energy in the absence of appropriate terminal electron acceptors (43). The *anr* gene of *P. aeruginosa* appears to be a close relative of *fnr* of *Escherichia coli* (49); in fact, the two genes can replace each other in heterologous systems (15). In contrast to the current understanding of the molecular basis for control of the arginine deiminase pathway, analogous information is lacking regarding the AST pathway (Fig. 1). This pathway, which converts arginine to glutamate, is considered the major route for catabolism in *P. aeruginosa* under aerobic conditions (16). Enzymes of the AST pathway catalyze the conversion of arginine to glutamate and are strongly induced by exogenous arginine (16). The encoding genes have been mapped in the *aru* (arginine utilization) cluster in the 66-min region of the chromosome (19). Arginine also induces the catabolic NAD-dependent glutamate dehydrogenase (NAD-GDH), which is required for the utilization of glutamate and regeneration of succinyl coenzyme A (succinyl-CoA) through the citric acid cycle (16). Interestingly, the transamination steps in both the anabolic (*argD*) and catabolic (*aruC*) pathways in *P. aeruginosa* (Fig. 1) are catalyzed by a single protein, which is inducible by arginine (24, 42).

Similarly, limited information is available regarding the regulation of genes encoding the arginine biosynthetic genes in *P. aeruginosa*. Expression of *argF*, encoding the anabolic ornithine carbamoyltransferase (aOCT) (20, 23) and the *car* operon, encoding carbamoylphosphate synthetase (CPS) (27, 29), is negatively controlled by arginine. However, the relevant arginine regulatory gene has not been identified, and the available data indicate that the product of such a gene lacks homology to *argR* of enteric bacteria: sequences homologous to the Arg box that characterizes *arg* and *car* operators of enteric bacteria are absent in *P. aeruginosa* (16, 27); in a heterologous system, *P. aeruginosa argF* is not controlled by the *argR* repressor of *E. coli* (9).

Previous studies from other laboratories (22, 47) reported the isolation of regulatory mutations that simultaneously abolish repression of the biosynthetic gene *argF* and induction of the catabolic AST pathway. These studies led to the proposal that regulation of arginine biosynthesis and catablism in *P. aeruginosa* have common regulatory elements (22, 47). We have recently reported (34) preliminary data regarding a putative arginine regulatory gene, designated *argR*, that encodes a DNA-binding protein of the AraC/XylS family. This paper reports the cloning and characterization of this gene. Inactivation of *argR* by gene replacement abolishes arginine repression of the biosynthetic *argF* and *car* operons. The *argR* inactivation also abolishes the induction of enzymes of the catabolic AST pathway.

* Corresponding author. Mailing address: College of Arts and Sciences, Georgia State University, P.O. Box 4038, Atlanta, GA 30302-4038. Phone: (404) 651-1410. Fax: (404) 651-4739.

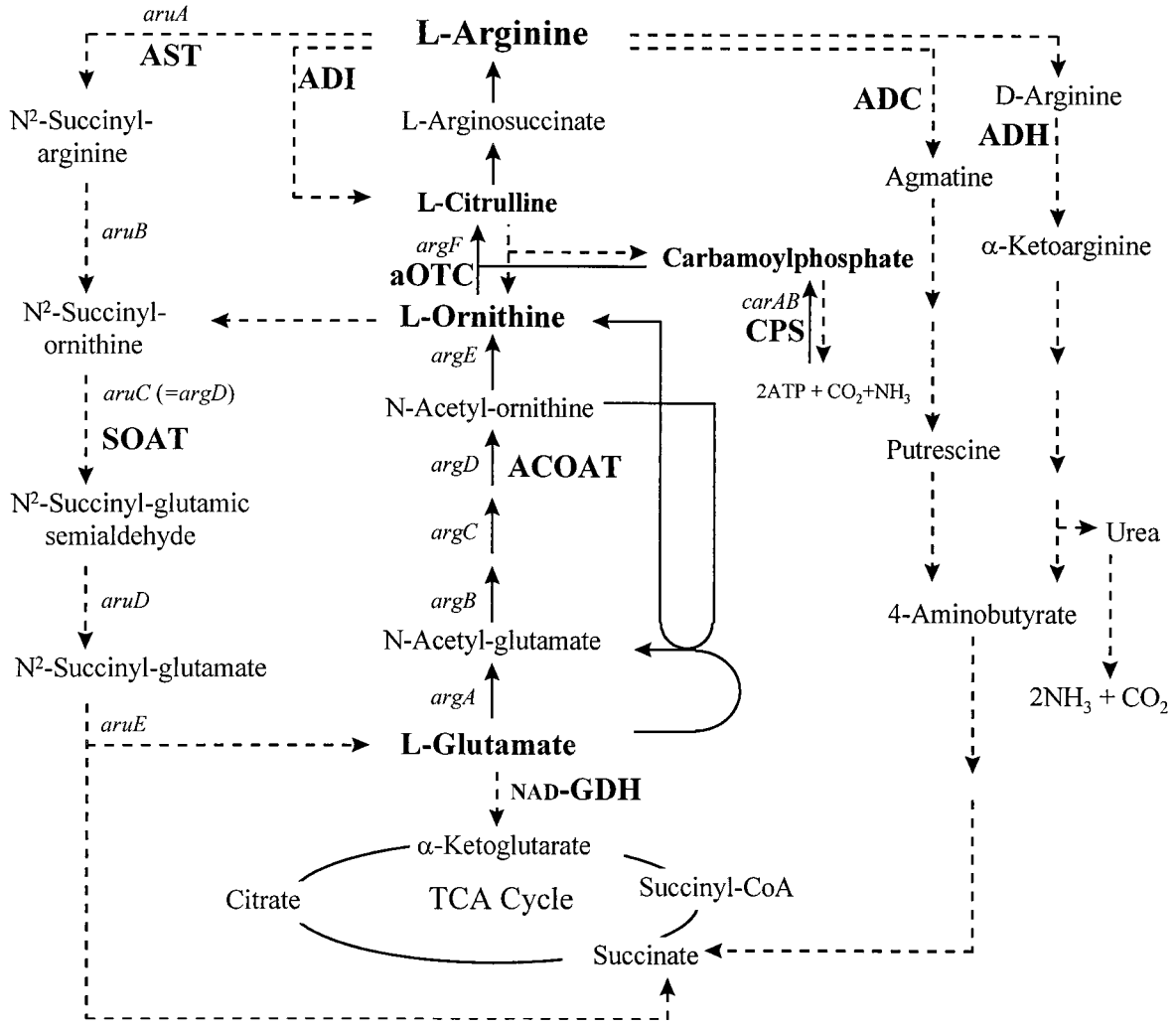

FIG. 1. Arginine metabolic pathways in *P. aeruginosa*. Only key intermediates are shown. The relevant genes and enzymes are given. Solid arrows denote anabolic reactions, and broken arrows denote catabolic reactions. ADI, arginine deiminase; ADC, arginine decarboxylase; ADH, arginine dehydrogenase; SOAT, succinylornithine transaminase; Gene symbols are designated as described by Holloway et al. (19).

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Luria-Bertani (LB) enriched medium (32) was used for strain construction with the following supplements as required: ampicillin, 50 μg/ml (*E. coli*); carbenicillin, 200 μg/ml (*P. aeruginosa*); gentamicin, 100 μg/ml; streptomycin, 500 μg/ml; kanamycin, 250 μg/ml; trimethoprim; 200 μg/ml; tetracycline, 25 μg/ml; arginine hydroxamate, 100 μg/ml; 5-bromo-4-chloro-3-indolyl-D-galactoside (X-Gal), 0.03%. Minimal medium P described by Haas et al. (17) and minimal medium E described by Vogel and Bonner (48) were used for the growth of *P. aeruginosa* and *E. coli*, respectively.

Construction of the *argF*::*lacZ* transcriptional fusion. A DNA fragment (nucleotides 1 to 380 of the *argF* promoter region [22]) was amplified from chromosomal DNA by PCR with two synthetic oligonucleotides: oligo-1 (5′-GCC GTGCCGGCGGCGCGGCGGCGA-3′) and oligo-2 (5′-CGAGAGAAAGTG CCGTACGCTCAT-3′). The PCR product was purified from a 1% agarose gel, treated with Klenow fragment to fill recessed ends, and ligated to the *Sma*I site of the broad-host-range *lacZ* transcriptional fusion vector, pQF50 (12). After transformation of *E. coli* DH5α, *lacZ*-positive clones were selected on plates containing X-Gal, and the nucleotide sequence of the chromosomal insert from one of the resulting plasmids, pKF52, was confirmed by double-stranded DNA sequencing.

For transformation into *E. coli* and *P. aeruginosa*, the method described by Chung et al. (8) was used for one-step preparation of competent cells.

Transposon mutagenesis and isolation of *argR* mutants. The strategy for isolation of arginine regulatory mutants relied on the use of Tn*5* mutagenesis for inactivation of putative regulatory genes and the use of arginine hydroxamate for selection of the resulting mutants (33). The protocol for Tn*5-751* insertion mutagenesis of *P. aeruginosa* was a modification of the procedure described by Rella et al. (37). The recipient strain of *P. aeruginosa* PAO1-sm (a spontaneous streptomycin-resistant derivative of PAO1) used in this protocol harbors the *argF*::*lacZ* transcriptional fusion described above, pKF52. Following incubation in LB medium containing kanamycin and tetracycline, *E. coli* ED8654 (containing the temperature-sensitive suicide plasmid, pME9, bearing the Tn*5-751* transposon) and *P. aeruginosa* harboring pKF52 were mixed in equal amounts on LB agar and incubated for 4 h at 30°C. The cells were then washed from the LB agar with 1 ml of 0.9% NaCl; plated onto LB medium supplemented with kanamycin, trimethoprim, and streptomycin; and incubated at 30°C. This medium selects against *E. coli* ED8654. Colonies of *P. aeruginosa* containing Tn*5-751* were plated again on minimal medium P supplemented with 100 μg of arginine hydroxamate per ml. Fast-growing colonies on arginine hydroxamate plates were then spotted onto plates of minimal medium P containing X-Gal, with either L-glutamate or L-glutamate plus L-arginine (10 mM) as the sole carbon and nitrogen sources. Colonies that displayed strong and indistinguishable blue coloration on both plates were selected for further characterization.

Preparation of cell extracts. *P. aeruginosa* PAO1 and its derivatives were grown in minimal medium P in the presence of appropriate supplements. The cultures were grown to an optical density at 600 nm of 0.6, harvested, and resuspended in 20 mM potassium phosphate buffer (pH 7.6) containing 1 mM phenylmethylsulfonyl fluoride and 1 mM EDTA. The cell suspension was ruptured by passage through a French pressure cell at 600 lb/in^2, and the suspension was centrifuged at 48,000 × *g* for 30 min. The protein concentration was determined by the method of Bradford (5) with bovine serum albumin as the standard.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
E. coli		
DH5α	F$^-$ ϕ80d*lac* ΔM15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17*(r_K^- m_K^-) *supE44* λ$^-$ *thi-1 gyrA96 relA1*	Bethesda Research Laboratory
ED8654	Bearing the suicide vector pME9	37
SM10	Host strain for gene replacement	14
P. aeruginosa		
PAO1	Wild type	17
PAO1-sm	Spontaneous streptomycin-resistant mutant of PAO1	This study
PAO1214	Tn*5-751* insertion mutant of PAO1	This study
PAO501	*argR*::Gm	This study
Plasmids		
pQF50	*bla lacZ* promoter fusion vector; broad-host-range plasmid	12
pUC19	*bla lacZ*	45
pGMΩ1	*bla gen*; gentamicin cassette with omega loop on both ends	39
pRTP1	*bla* Str^s conjugation vector	41
pKA58	130-bp *Rsa*I-*Taq*I fragment of the *car* promoter region (27) cloned in the *Sma*I site of pUC19	This study
pKF52	*argF*::*lacZ* promoter fusion	This study
pTnR2	*argR* clone containing the right arm of Tn*5-751* in pUC19	This study
pSR21	*argR* subclone of pTnR2	This study
pSRH1	*argR* subclone of pSR21	This study
pSRS1	*argR* subclone of pSRH1	This study
pSRM1	*argR* deletion derivative of pSRS1	This study
pSRT1	*argR* subclone of pSR21	This study
pSM21	*argR* subclone of pSR21	This study

Gel retardation assays. DNA fragments carrying the promoter regions of the *car* and *argF* operons were used as probes. For the *car* probe, the 130-bp *Rsa*I-*Taq*I fragment carrying the *car* promoter region was obtained from pKA26 (27) and cloned into the *Sma*I site of pUC19. The 380-bp chromosomal insert of pKF52 was used as the *argF* probe. In either case, after suitable restriction endonuclease digestion, DNA was purified from a 1% low-melting-temperature agarose gel and labeled with [α-^{32}P]dATP or [α-^{32}P]dGTP by using the Klenow fragment.

The binding-reaction mixture (20 μl) contained 20 mM Tris (pH 8.0), 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol, 1 μg of carrier DNA (sheared salmon sperm DNA), and 3 to 5 μg of crude extracts. This reaction mixture was preincubated for 10 min at room temperature. The radioactively labeled DNA probe was added, and the reaction was continued for another 15 min. Aliquots (10 μl) were loaded onto a 5% polyacrylamide gel in 0.5× TAE buffer (32), and electrophoresis was carried out for 30 min at 200 V. The gel was dried and autoradiographed.

Gene replacement. A 1.6-kb *Sma*I fragment containing the gentamicin resistance (Gm) cassette was isolated from plasmid pGM1 (39) by digestion with *Sma*I and separation by agarose gel electrophoresis. The Gm cassette was cloned into the *Sca*I site of the *argR* gene on pSM21 (Table 1). A 3.0-kb *Sma*I fragment containing the *argR*::Gm region of the resulting plasmid was then ligated into the *Sca*I site of a conjugation vector, pRTP1 (41). The resulting gene replacement plasmid in *E. coli* SM10 was mobilized into a spontaneous streptomycin-resistant *P. aeruginosa* strain, PAO1-Sm, by biparental plate mating as described by Gambello and Iglewski (14). Following incubation at 37°C for 16 h, transconjugants were selected on LB plates supplemented with gentamicin and streptomycin.

Southern analysis. The 1.3-kb *Sma*I-*Sma*I fragment of pSM21 was purified after *Sma*I digestion and labeled with digoxigenin-11-dUTP by the random-primed method (Genius system; Boehringer Mannheim). The labeled DNA was hybridized for 16 h at 65°C to a Southern blot of *Sma*I-digested genomic DNA on a nylon membrane. The hybrids were detected by an enzyme-linked immunosorbent assay with anti-digoxigenin-alkaline phosphatase conjugate and subsequent color reaction as described by the manufacturer (Boehringer Mannheim). The sizes of the DNA fragments were determined by comparison with a *Hin*dIII digest of λ-DNA.

Enzyme assays. Acetylornithine aminotransferase (ACOAT) was assayed by the procedure described by Voellmy and Leisinger (46). The reaction mixture (0.5 ml) contained 100 mM Tris-HCl (pH 9.0), 15 mM acetyl ornithine, 15 mM α-ketoglutarate, and 0.075 mM pyridoxal-5′-phosphate. The reaction was initiated by the addition of enzyme, and the mixture was incubated at 37°C for 15 min. The reaction was terminated by the addition of 0.2 ml of 10.3 N HCl and incubation at 100°C for 45 min. After the reaction mixture was cooled at room temperature, 1.4 ml of 3.6 M sodium acetate and 0.2 ml of 33 mM *ortho*-aminobenzaldehyde were added, and the absorbance was read at 436 nm. A standard curve, made with pyrroline carboxylate, was used to determine product formation.

AST was assayed by the procedure described by Vander Wauven and Stalon (44). The reaction mixture (1.0 ml) contained 100 mM Tris-HCl (pH 8.0), 0.2 mM succinyl-CoA, and 1 mM L-arginine. The reaction was initiated by the addition of arginine at 37°C. The decrease in succinyl-CoA concentration was monitored at an absorbance of 232 nm.

NAD-GDH was assayed by the procedure of Joannou and Brown (25). The reaction mixture (1.0 ml) contained 50 mM Tris-HCl (pH 8.0), 5 mM NAD^+, 10 mM L-glutamate, and 0.1% (vol/vol) Triton X-100. The reaction was initiated by the addition of glutamate, and the generation of NADH was monitored by measuring the absorbance at 340 nm.

aOTC was assayed as described by Haas et al. (17). The reaction mixture (1.0 ml) contained 0.3 M Tris-HCl (pH 8.7), 15 mM ornithine, and 10 mM lithium carbamoylphosphate. The reaction was initiated by the addition of lithium carbamoylphosphate, and the mixture was incubated for 15 min at 37°C. The reaction was terminated by the addition of 1 ml of the color mixture (antipyrine-sulfuric acid-diacetylmonoxime [36]). The mixture was placed in a 60°C water bath for 1 h, and the absorbance at 466 nm was measured. A standard curve, made with citrulline, was used to determine product formation.

CPS activity was assayed as described by Abdelal et al. (1, 2). The reaction mixture (0.5 ml) contained enzyme, 100 mM triethanolamine buffer (pH 8.0), 12 mM ATP, 16 mM $MgCl_2$, 10 mM $NaH^{14}CO_3$, 100 mM KCl, and 10 mM L-glutamine. The reaction was started by adding the enzyme and terminated by adding 50 μl of 1.2 M hydroxylamine hydrochloride and placing the mixture immediately in a boiling water both for 10 min. After the reaction mixture had cooled, 0.1 ml of 60% trichloroacetic acid was added and the mixture was shaken slowly at room temperature for 10 min to permit the liberation of excess $^{14}CO_2$. A 0.5-ml sample was placed in a vial, mixed with scintillation counting cocktail, and counted in a scintillation spectrometer.

Nucleotide sequence accession number. The sequence of *argR* and its flanking regions has been deposited in the GenBank database under accession no. AF008308.

RESULTS

Evidence for an arginine-inducible DNA-binding protein that binds to the regulatory regions of two biosynthetic operons. We have previously shown that the nonidentical subunits of *P. aeruginosa* CPS are encoded by the *car* operon (27, 29).

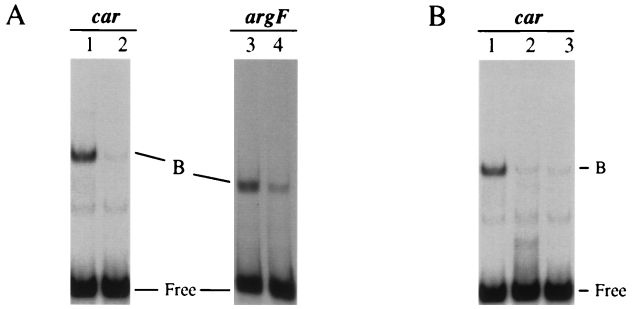

FIG. 2. Gel retardation experiments with extracts of wild-type *P. aeruginosa* and its Tn*5* derivative. Cells were grown in minimal medium P supplemented with 10 mM glutamate or with 10 mM glutamate plus 10 mM arginine as sources of carbon and nitrogen. Each lane contains 3 μg of cell extract. (A) Experiments with wild-type strain PAO1. The DNA probe contains either the *car* or *argF* regulatory region as indicated. Lanes 1 and 2, *car* DNA probe: lane 1, extract from cells grown with arginine and glutamate; lane 2, extract from cells grown with glutamate. Lanes 3 and 4, *argF* DNA probe; lane 3, extract from cells grown with arginine and glutamate; lane 4, extract from cells grown with glutamate. (B) Experiments with the Tn*5* derivative, PAO1214. The DNA probe was the *car* control region. Lanes: 1, control with extract from PAO1 grown with arginine and glutamate; 2, extract from PAO1214 grown with arginine and glutamate; 3, extract from PAO1214 grown with glutamate. B, protein-DNA complex; Free, free DNA probe.

Consistent with the physiological role of CPS in the biosyntheses of pyrimidines and arginine, expression of this enzyme, transcribed from a single promoter upstream of *carA*, is controlled by both pyrimidines and arginine. Previous work from this laboratory has shown that pyrimidine regulation is mediated by an attenuation-type mechanism, while arginine control is exerted by a *trans*-acting factor at the level of transcriptional initiation (27).

To investigate the presence of *trans*-acting factors that bind to the regulatory region of the *car* operon, gel retardation assays were performed with cell extracts of *P. aeruginosa* PAO1. The results (Fig. 2A) show the retardation of a DNA fragment containing the *car* regulatory region by extracts of cells grown in the absence (lane 2) or presence (lane 1) of exogenous arginine. Formation of the retarded DNA-protein complex was strongly induced by exogenous arginine.

To further characterize this arginine-inducible DNA-binding protein, the regulatory region of *argF* (23), encoding the aOTC of *P. aeruginosa*, was amplified from the chromosomal DNA by PCR and radioactively labeled as described in Materials and Methods. Gel retardation experiments with the amplified DNA fragment (Fig. 2A, lanes 3 and 4) similarly detected the presence of an arginine-inducible DNA-protein complex.

Isolation and characterization of a mutant defective in the regulation of arginine metabolism. Previous work has shown that of the biosynthetic operons reported to be under arginine control, *car* (27) and *argF* (23), the latter is more sensitive to the presence of exogenous arginine in the growth medium. The level of aOTC (encoded by *argF*) in *P. aeruginosa* PAO1 is induced 10-fold by exogenous arginine (23). This significant difference in the level of aOTC was exploited in a strategy for isolation of mutants defective in arginine regulation. An *argF*::*lacZ* transcriptional fusion, pKF52, was constructed and introduced into PAO1 as described in Materials and Methods. When PAO1 carrying the *argF*::*lacZ* fusion was plated on minimal medium containing X-Gal, the induction of β-galactosidase expression in medium containing arginine as sole source of carbon and nitrogen was readily differentiated by the coloration. The strategy for isolation of arginine regulation mutants involved the use of Tn*5* mutagenesis for inactivation of putative regulatory genes and the use of arginine hydroxamate for selection of the resulting mutants. Arginine hydroxamate was successfully used by Mountain and Baumberg (33) for selection of arginine regulatory mutants of *Bacillus subtilis*. Since this arginine analog was reported to also inhibit the growth of *P. aeruginosa* (28), it appeared promising for use in the selection of mutants defective in arginine regulation in this organism.

P. aeruginosa PAO1 carrying pKF52 was subjected to transposon mutagenesis with a Tn*5* derivative, Tn*5-751*, that carries the trimethoprim resistance (Tp^r) determinant in addition to the kanamycin resistance (Km^r) marker of Tn*5*. Following transposon mutagenesis, cells were plated on glutamate minimal medium plates containing both antibiotics for transposon marker selection as well as arginine hydroxamate. Colonies resistant to trimethoprim, kanamycin, and arginine hydroxamate were further characterized on glutamate minimal medium plates containing X-Gal in the absence or presence of arginine. One mutant, PAO1214, with a strong blue coloration in both the absence and presence of arginine, was compared to the parent strain PAO1 with respect to arginine and ornithine utilization. The results (Table 2) show that unlike PAO1, PAO1214 did not grow in minimal medium with arginine or ornithine as the sole source of carbon or of carbon and nitrogen. The mutant grew when succinate was provided as the carbon source and arginine or ornithine served only as a nitrogen source.

Gel retardation experiments with cell extracts of PAO1214 and with DNA fragments carrying the *car* control region showed that formation of the arginine-inducible DNA-protein complex in PAO1214 was greatly impaired and was not induced by the presence of arginine in the growth medium (Fig. 2B). The position of this weak nucleoprotein complex was the same as that of the arginine-inducible nucleoprotein identified in Fig. 2A. These results indicate that the mutation of PAO1214 affects the expression of the DNA-binding protein observed in gel retardation experiments.

Cloning and identification of a putative *argR* gene for arginine regulation. To identify the gene affected by transposon insertion in PAO1214, the chromosomal DNA was isolated and used to clone the chromosomal regions flanking Tn*5-751*. By taking advantage of the presence of a single *Eco*RI site

TABLE 2. Growth phenotype of arginine regulatory mutants

Strain	Growth on[a]:									
	Glu + Arg	Arg	Orn	Suc + Arg	Suc + Orn	Suc + NH_4^+	NH_4^+ Arg	NH_4^+ + Orn	His	Pro
PAO1	+	+	+	+	+	+	+	+	+	+
PAO1214	+	−	−	+	+	+	−	−	+	+
PAO501	+	−	−	+	+	+	−	−	+	+

[a] Strains tested were grown on minimal medium P plates containing the indicated carbon and nitrogen source at 20 mM. Glu, glutamate; Arg, arginine; Orn, ornithine; Suc, succinate; His, histidine; Pro, proline; NH_4^+, ammonium chloride. +, growth in 24 h; −, no growth in 36 h.

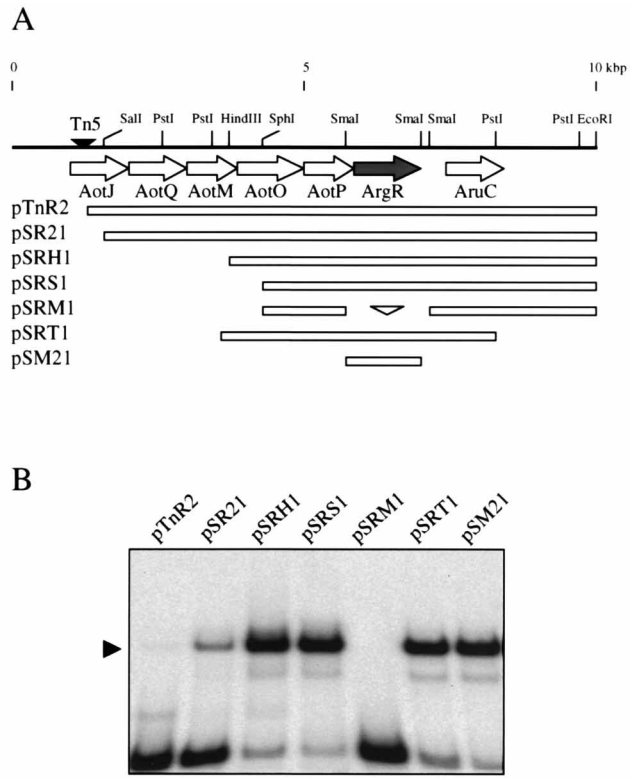

FIG. 3. (A) Partial restriction map and schematic drawing of subclones and genetic organization in the *aot-aru* locus. The gene order shown here was determined in this study as well as by Itoh (21). The transposon insertion site in PAO1214 is indicated. (B) Gel retardation experiments with extracts from *E. coli* DH5α harboring recombinant plasmids. Each lane contains 5 μg of cell extract. A DNA fragment containing the *argF* control region was used as the probe. The retarded ArgR-*argF* complex is indicated by a solid arrowhead.

between the Km^r and Tp^r genes of Tn*5-751* (37), the chromosomal DNA of PAO1214 was digested to completion with *Eco*RI, followed by ligation, transformation into *E. coli* DH5α, and selection for clones carrying either antibiotic resistance determinant. Five kanamycin-resistant and two trimethoprim-resistant clones were obtained. Plasmid DNA of these recombinant transformants was isolated and subjected to DNA sequencing with a synthetic oligonucleotide primer (5′-CATGTT AGGAGGTCACATGGAAGTC-3′) based on the sequence determined for the IS element of transposon Tn*5* (GenBank accession no. V00617 [3]). This procedure permitted determination of the nucleotide sequences of the adjacent chromosomal regions in these plasmids. The nucleotide sequences obtained were identical for clones resulting from the same antibiotic selection. A GenBank search showed that the gene being disrupted by transposon insertion in PAO1214 encodes a polypeptide with very high homology to periplasmic binding proteins of the ATP-binding cassette transport systems (4). Itoh has presented preliminary data that this gene (designated *aotJ*) is the first gene of an operon of six genes for the transport of arginine and ornithine in *P. aeruginosa* (Fig. 3) (21). The indicated open reading frame (ORF) order was confirmed by nucleotide sequence determination (data not shown).

Although it is possible that inactivation of arginine uptake will result in the phenotype of PAO1214, this possibility was considered unlikely. The ability of PAO1214 to utilize arginine as the sole nitrogen source indicated that arginine uptake in the mutant could be mediated by another transport system, as is the case with *Pseudomonas putida* (17). Experiments on the uptake of labeled arginine by *P. aeruginosa* PAO1 and PAO1214 (not shown) showed that this organism is similar to *P. putida* (17) in having two arginine uptake systems, an arginine-specific system and a general basic amino acid transport system. Accordingly, we investigated the possibility that the phenotype of PAO1214 is the result of a polar effect for the transposon insertion in *aotJ* on the expression of a downstream regulatory gene. Gel retardation assays were carried out with cell extracts of *E. coli* transformants carrying various subclones of the Tp^r plasmid. The results (Fig. 3B) showed very strong expression of the DNA-binding protein in extract of cells transformed with pSM21, which carries a 1.3-kb chromosomal insert located downstream of *aotJ* (Fig. 3A). Based on these results, pSM21 was assumed to carry a gene that participates in the regulation of arginine metabolism; this gene was designated *argR*.

Nucleotide sequence of putative *argR* and flanking regions. Since transformants carrying pSM21 showed strong expression of the putative ArgR protein, the nucleotide sequence of the subcloned 1.3-kb chromosomal insert was determined. The result (Fig. 4) shows one truncated ORF and one complete ORF. The first ORF, truncated by the *Sma*I subcloning site, extends from nt 1 to 281, with a coding capacity of 93 amino acids. The derived amino acid sequence for this partial ORF showed a high degree of homology to that of the HisP protein of *Salmonella typhimurium*, which belongs to the family of ATP-binding cassette transport systems (4). The second ORF extends from an ATG triplet at position 317 and terminates with a TGA triplet at position 1304. It has a coding capacity of 329 amino acids, yielding a polypeptide with a estimated molecular mass of 37 kDa (Fig. 4). As will be pointed out below, the results of sequence comparison indicated that the putative polypeptide has features of a DNA-binding protein of the AraC/XylS family. Accordingly, this ORF was tentatively designated *argR*, for arginine regulator.

The complete nucleotide sequence for the HisP homolog was determined from the appropriate restriction fragment (pSRS1 [Fig. 3A]). The resulting sequence was identical to that of the fifth gene of the *aot* operon partially characterized by Itoh (21, 21a). The nucleotide sequence downstream of *argR* was also determined from pSRS1. The results (Fig. 4) showed that *argR* is followed by a 500-bp noncoding sequence and an ORF with 66% identity to acetylornithine aminotransferase of *E. coli* (18). This gene, designated *aruC* in *P. aeruginosa* (19, 42), is the first ORF in an arginine utilization operon (*aru*) encoding enzymes of the AST pathway (21a). Analysis of the 500-bp intergenic region between *argR* and *aruC* did not show any significant secondary structure resembling Rho-independent terminators of enteric bacteria.

Accordingly, as schematically shown in Fig. 3A, *argR* is the sixth and terminal gene in the arginine/ornithine transport operon and precedes by 500 bp the *aru* operon.

Construction and characterization of an *argR*::Gm insertion mutant of *P. aeruginosa*. The *argR* gene on pSM21 was inactivated by insertion of a Gm cassette as described in Materials and Methods. The resulting inactivated *argR* was transferred into the gene replacement vector pRTP1 and mobilized into a spontaneous streptomycin-resistant strain of *P. aeruginosa* by biparental conjugation. Transconjugants were plated on LB medium supplemented with gentamicin and streptomycin to select for organisms in which *argR* has been replaced in double-crossover events. The occurrence of gene replacement in one such transconjugant, PAO501 was confirmed by Southern blot analysis (data not shown). The hybridization pattern shows a shift from a 1.3-kb band for the wild type to a 3.0-kb band for

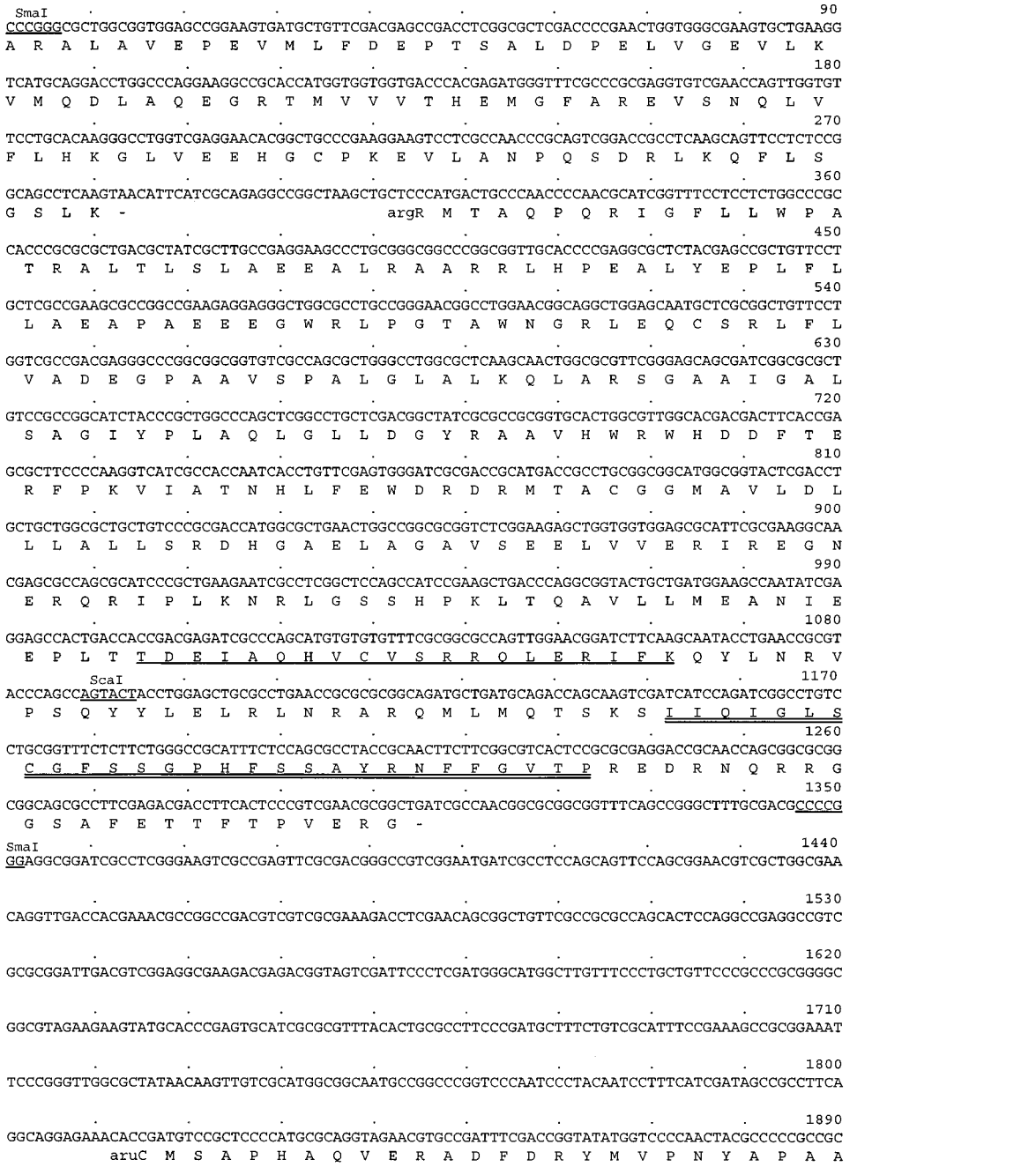

FIG. 4. Nucleotide and deduced amino acid sequences for *argR* and its flanking regions from *P. aeruginosa*. The deduced amino acid sequences are shown below the nucleotide sequence. The *Sma*I cloning sites in pSM21 and the *Sca*I site used for insertion of the genamicin cassette in PAO501 are underlined. A putative H-T-H DNA-binding motif is also underlined; this motif is in the same location as an H-T-H motif in AraC that was shown to be important for protein-DNA interactions (38). A sequence identical to the consensus sequence pattern of AraC/XylS family (13, 38) is doubly underlined.

PAO501, confirming that *argR* in this isolate has been replaced with the gentamicin-marked insertion.

Gel retardation assays were carried out with cell extracts of the *argR*::Gm derivative, using ^{32}P-labeled *argF* operator DNA as the probe. As shown in Fig. 5, the arginine-inducible nucleoprotein complex observed with PAO1 (lanes 1 and 2) was completely absent in PAO501 (lanes 3 and 4).

The growth behavior of strain PAO501 in minimal media supplemented with various amino acids as the sole carbon and/or nitrogen sources was examined. The results (Table 2) show that PAO501 can utilize arginine or ornithine as the sole nitrogen source but not as the sole carbon source. This phenotype for the inactivated putative *argR* gene in PAO501 is thus identical to that described above for the transposon insertion derivative, PAO1214. The ability to utilize histidine or proline as the sole source of carbon and nitrogen was not affected in either strain (Table 2), indicating that *argR* inactivation specifically affects expression of arginine catabolic genes.

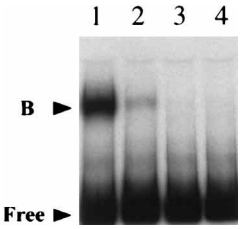

FIG. 5. Gel retardation experiments with extracts of *P. aeruginosa* and *argR*::Gm derivative. A ^{32}P-labeled *argF* DNA was used as the probe. Lanes: 1 and 2, extracts from the wild-type strain PAO1 grown with glutamate in the presence or absence of arginine, respectively; 3 and 4, extracts from an *argR*::Gm derivative, PAO501, grown with glutamate in the presence or absence of arginine, respectively. B, protein-DNA complex; F, free DNA probe.

Regulation of expression of a number of enzymes of arginine metabolism was compared in PAO1 and PAO501. Cultures were grown in minimal medium P supplemented with glutamate in the presence or absence of arginine, and cell extracts were used for determination of the specific activities of certain anabolic and catabolic enzymes. The results (Table 3) show that exogenous arginine repressed the biosynthetic enzymes, CPS and aOTC, in the parent strain, PAO1, but not in the *argR* derivative, PAO501. Similarly, arginine induced the catabolic enzymes, AST, ACOAT, and NAD-GDH, in the wild-type parent but not in the *argR* derivative.

DISCUSSION

Gel retardation experiments (Fig. 2) demonstrated the presence in *P. aeruginosa* cell extracts of an arginine inducible DNA-binding protein that interacts with the control regions for the *car* and *argF* operons of this organism. The use of a combination of transposon insertion for mutagenesis and arginine hydroxamate for selection led to the isolation of mutants with mutations in arginine regulation. One such mutant, PAO1214, exhibited a lack of arginine control of an *argF*::*lacZ* fusion and was greatly impaired in formation of the arginine-inducible DNA-protein complex relative to the parent strain. Furthermore, while the parent strain, PAO1, readily utilized arginine or ornithine as the sole source of carbon and/or nitrogen, the mutant derivative was able to utilize either amino acid only as a source of nitrogen. Accordingly, the mutation in PAO1214 simultaneously affects control of the biosynthetic gene, *argF*, as well as one or more genes of arginine catabolism.

Comparison of the DNA sequence of the chromosomal segments adjacent to the insertion element in PAO1214 with the GenBank library revealed that the disrupted gene (*aotJ*) (21) encodes a polypeptide with high homology to a periplasmic transport protein of the ATP binding family (4). While inactivation of a gene required for arginine transport could result in insensitivity to exogenous arginine, this is not the case here, since *P. aeruginosa*, like *P. putida* (28), possesses a general basic amino acid transport system in addition to the specific arginine transport system. Rather, gel retardation experiments with various subclones localized, on a 1.3-kb fragment downstream of *aotJ*, a gene encoding a DNA-binding protein, that interacts with the control regions for the *car* and *argF* operons. Determination of the DNA sequence for this 1.3-kb fragment (Fig. 4) showed that it contains only one complete ORF with a coding capacity of 329 amino acids, yielding a polypeptide with a molecular mass of 36,870 Da. A search of the GenBank protein database indicated that this protein has features characteristic of members of the AraC/XylS family. The complete ORF was designated *argR* (for arginine regulation). Determination of the nucleotide sequence for the flanking regions showed that *argR* is the sixth and terminal gene of the arginine/ornithine transport operon, which has been partially characterized by Itoh (21). Work to be reported elsewhere (33a) showed that the *aot* operon is also controlled by ArgR acting at a promoter upstream of the first gene in the operon, *aotJ*.

More than 50 members of the AraC/XylS family have been identified in a wide variety of bacteria (for a review, see reference 38). Extensive studies on the AraC protein in the laboratory of R. Schleif have provided the major contributions to our current understanding of the structure-function relationship of proteins in this family. Sequence alignment of the arboxyl-terminal 102 residues of ArgR with the carboxyl termini of 31 members of the AraC family by using the CLUSTAL program (data not shown) revealed the presence of the conserved features that characterize the DNA-binding domain of the AraC family (38). A sequence identical to the consensus sequence pattern of the AraC/XylS family (13), (L, I, V)-X2-(L, I, V)-X4-G-(I, F, Y)-X5-F-X3-(F, Y)-X7-P, is found at the C-terminal region of the ArgR protein (Fig. 4). In addition, a putative helix-turn-helix DNA-binding motif of ArgR of *P. aeruginosa* was detected by using the program in the PCGENE package (Fig. 4). This motif is at the same location as the H-T-H motif of the AraC protein that was shown to be important for protein-DNA interactions (6, 38). Based on this analysis, it is clear that the ArgR protein of *P. aeruginosa* identified in this study is also a member of the AraC family.

The function of ArgR in the regulation of arginine metabolism of *P. aeruginosa* was demonstrated by the phenotype of a gene replacement derivative (*argR*::Gm mutant; PAO501). While the growth phenotype of this derivative (PAO501) was identical to that of the transposon insertion derivative (PAO1214), no expression of *argR* was observed with PAO501 (Fig. 5), indicating complete inactivation by gene replacement. In contrast, weak expression was observed with PAO1214 (Fig. 2B) as a consequence of the polar effect of transposon insertion in *aotJ*. Experiments on regulation of the expression of the

TABLE 3. Effect of exogenous arginine on expression of arginine biosynthetic and catabolic enzymes in PAO1 and PAO501

Strain (genotype)	Growth condition[a]	Sp act[b] (nmol/mg/min) of:				
		CPS	aOTC	AST	ACOAT	NAD-GDH
PAO1 (wild type)	Glutamate	2.9 (0.1)	121 (2)	69 (2)	42 (2)	51 (2)
	Glutamate + arginine	1.8 (0.1)	39 (3)	448 (9)	245 (6)	470 (14)
PAO501 (*argR*::Gm)	Glutamate	5.8 (0.1)	268 (3)	54 (2)	40 (2)	18 (1)
	Glutamate + arginine	5.2 (0.2)	290 (6)	66 (3)	37 (2)	21 (1)

[a] Cell were grown at 37°C in minimal medium P supplemented with 20 mM amino acids as the carbon and nitrogen source. The doubling time for all cultures was determined to be 70 min at 37°C and was not affected by the supplements indicated.

[b] Each value shown is the mean of two or more determinations with standard errors indicated in parentheses.

first enzyme in the AST pathway (*aruA* [Fig. 1]) in the *argR*::Gm derivative showed that unlike the parent strain, this enzyme is not induced by arginine (Table 3). This is also the case for the third enzyme of the AST pathway (succinyl arginine aminotransferase; *aruC* = *argD*), as well as for the catabolic NAD-GDH that is required for conversion of glutamate to α-ketoglutarate (Table 3). Similar experiments with the biosynthetic enzymes, CPS and aOTC, showed that, again unlike the parent strain, these enzymes were not repressed by arginine. These results indicate that ArgR acts as a major element in the regulation of arginine biosynthesis and catabolism via the AST pathway. Studies with homogeneous ArgR showed that it binds specifically to control regions of a number of arginine biosynthetic and catabolic operons (35).

The ArgR protein of *P. aeruginosa* identified in this work does not exhibit any significant sequence homology to the arginine regulatory proteins characterized from enteric bacteria (30, 31) or *B. subtilis* (7, 11). The ArgR protein of enteric bacteria and the AhrC protein of *B. subtilis* exhibit 27% identity in amino acid sequences and are very similar in size (17,000 Da) and subunit composition (hexameric). These proteins bind to operators (Arg boxes) that overlap promoters of *arg* biosynthetic genes as well as their own structural genes. Previous work (40) has shown that AhrC can substitute for ArgR of *E. coli* in repression of *arg* genes. In contrast, in a heterologous system, the *argF* of *P. aeruginosa* is not controlled by the ArgR repressor of *E. coli* (8) and no sequences homologous to the Arg boxes of enteric bacteria are present in sequences upstream of biosynthetic genes that are negatively controlled by arginine (23, 27). Finally, the RocR protein of *B. subtilis*, which functions as an activator of the *roc* operons for arginine and ornithine catabolism in this organism, has been identified as a member of the NtrC family of regulatory proteins (7). Interestingly, AhrC was also reported (26) to activate an arginine catabolism operon (*rocABC*) in this organism.

While this work identifies ArgR as an important element in the regulation of arginine biosynthesis and catabolism, it is certainly not the only regulatory element for the network of arginine pathways in *P. aeruginosa*. The ability of the *argR* derivatives of *P. aeruginosa* (PAO1214 and PAO501) to utilize arginine as the sole nitrogen source at wild-type rates under aerobic conditions clearly indicates that other arginine catabolic pathways are not regulated by ArgR. Furthermore, Winteler and Haas (49) have identified another protein, Anr, that induces the arginine deiminase pathway, which functions in arginine utilization under anaerobic conditions.

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