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Purification and Characterization of an Arginine Regulatory Protein, ArgR, from *Pseudomonas aeruginosa* and Its Interactions with the Control Regions for the *car*, *argF*, and *aru* Operons

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Pseudomonas aeruginosa **ArgR, a regulatory protein that plays a major role in the control of certain biosynthetic and catabolic arginine genes, was purified to homogeneity. ArgR was shown to be a dimer of two equal subunits, each with a molecular mass of 37,000 Da. Determination of the amino-terminal amino acid sequence showed it to be identical to that predicted from the derived sequence for the *argR* gene. DNase I footprinting showed that ArgR protects a region of 45 to 47 bp that overlaps the promoters for the biosynthetic *car* and *argF* operons, indicating that ArgR exerts its negative control on the expression of these operons by steric hindrance. Studies were also carried out with the *aru* operon, which encodes enzymes of the catabolic arginine succinyltransferase pathway. Quantitative S1 nuclease experiments showed that expression of the first gene in this operon, *aruC*, is initiated from an arginine-inducible promoter. Studies with an *aruC*::*lacZ* fusion showed that this promoter is under the control of ArgR. DNase I experiments indicated that ArgR protects two 45-bp binding sites upstream of *aruC*; the 3′ terminus for the downstream binding site overlaps the −35 region for the identified promoter. Gel retardation experiments yielded apparent dissociation constants of 2.5×10^{-11}, 4.2×10^{-12}, and 7.2×10^{-11} M for *carA*, *argF*, and *aruC* operators, respectively. Premethylation interference and depurination experiments with the *car* and *argF* operators identified a common sequence, 5′-TGTCGC-3′, which may be important for ArgR binding. Alignment of ArgR binding sites reveals that the ArgR binding site consists of two half-sites, in a direct repeat arrangement, with the consensus sequence $TGTCGCN_8AAN_5$.**

We reported, in the adjoining paper, the cloning and characterization of a gene, *argR*, that plays a major role in control of biosynthesis and aerobic catabolism of arginine in *Pseudomonas aeruginosa* (19). The *argR* gene is the sixth and terminal gene of an operon for the transport of arginine and ornithine (12, 19, 20). The derived amino acid sequence for ArgR exhibits significant homology to the AraC/XylS family of regulatory proteins (19). Inactivation of *argR* by gene replacement resulted in abolition of the arginine repression of the biosynthetic operons, *car* and *argF*, encoding carbamoylphosphate synthetase (CPS) and anabolic ornithine carbamoyltransferase (aOTC), respectively (19). The *argR* inactivation also resulted in abolition of the arginine induction of enzymes of the arginine succinyltransferase pathway (19), which is considered the major route for catabolism of arginine by *P. aeruginosa* under aerobic conditions (9). Consistent with this role for ArgR, the inactivated derivative was unable to utilize arginine or ornithine as the sole carbon source (19).

This paper describes the purification and characterization of the regulatory protein encoded by *argR*. Interactions of the homogeneous protein with the control regions for the *car*, *argF*, and *aru* operons were investigated by various footprinting approaches.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. The bacterial strains and plasmids used are listed in Table 1. The Luria-Bertani (LB) enriched medium (16) was used with the following supplements as required: ampicillin at 50 μg/ml (*Escherichia coli*); carbenicillin at 200 μg/ml (*P. aeruginosa*); and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) at 0.03%. Minimal medium P described by Haas et al. (10) was used for the growth of *P. aeruginosa* with either L-glutamate or L-glutamate plus L-arginine (20 mM) as the sole source of carbon and nitrogen as indicated.

Transformation. For transformation of *E. coli* and *P. aeruginosa*, the method described by Chung et al. (6) was used for one-step preparation of competent cells.

Construction of the *aruC*::*lacZ* translational fusion. Plasmid pQF52, a broad-host-range *lacZ* translational fusion vector, was constructed from pQF50 (7) and pMC1871 (5). The *lacZ* gene on pQF50 was first removed by *Eco*RI digestion and self-ligation, and the resulting plasmid was designated pQF51. The *lacZ* translational fusion cassette on pMC1871 was isolated by *Sal*I digestion and cloned into pQF51 cut by the same restriction endonuclease. The gene arrangement in the resulting plasmid, pQF52, provides for transcriptional initiation of *lacZ* from the opposite direction to that of *bla*, thus eliminating a basal level of β-galactosidase expression that is otherwise observed with the parent vector, pQF50.

A 560-bp DNA fragment containing the *argR-aruC* intergenic region (Fig. 1c) was amplified from pSRS1 (19) by PCR (22) with two oligonucleotide primers designed to generate *Hin*dIII and *Ssp*I ends: oligo-1 (5′-TTAAGCTTCACTC CCGTCGAACGCG-3′) and oligo-2 (5′-GCAATATTTACCTGCGCATGGG GAGC-3′). The amplified fragment was digested with *Hin*dIII and *Ssp*I and ligated into *Hin*dIII-*Sma*I-cleaved pQF52 (Table 1). The resulting plasmid, pSC500, was used to transform *E. coli* DH5α, and transformants were selected on LB plates containing ampicillin and X-Gal. The orientation of the insert on the plasmid was confirmed by plasmid DNA sequencing.

β-Galactosidase assay. Logarithmically growing cultures were harvested at an optical density at 600 nm of 0.5. The cells were washed once and suspended in 20 mM potassium phosphate (pH 7.6) containing 1 mM EDTA. They were ruptured by passage through an Aminco French pressure cell, and the cell extract was centrifuged at 27,000 × *g* for 20 min at 4°C. β-Galactosidase activity was determined by the method of Miller (18). The relative levels of plasmid DNA under different growth conditions were determined by densitometric measurement of the stained and linearized DNA bands as described previously (14) and used to normalize β-galactosidase expression. The protein concentration was determined by the method of Bradford (2) with crystalline bovine serum albumin as a standard.

RNA preparation. *P. aeruginosa* PAO1 was grown in minimal medium to an optical density at 600 nm of 0.5 to 0.6. A 15-ml portion of the culture was transferred to a prechilled 125-ml flask, and the chilled culture was centrifuged at 12,000 × *g* at 0°C for 5 min. The cell pellet was suspended in 3 ml of a solution containing 0.02 M sodium acetate (pH 5.2), 1 mM EDTA, and 0.2% diethylpyrocarbonate, and then sodium dodecyl sulfate (SDS) was added to a final concentration of 0.5% (wt/vol). An equal volume of phenol equilibrated with 0.02 M sodium acetate (pH 5.2) was added, and the mixture was incubated at 65°C for

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TABLE 1. Strains and plasmids used in this study

Strains and vector	Genotype or description	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 relA1*	BRL[a]
P. aeruginosa		
PAO1	Wild type	D. Haas
PAO1214	*aotJ*	This study
PAO501	*argR*	This study
Plasmids		
pUC19	*bla lacZ′*	24
pMC1871	Vector for *lacZ* translational fusion	5
pQF50	*bla lacZ* transcriptional fusion vector	7
pQF51	*bla*; derived from pQF50	This study
pQF52	*bla lacZ* translational fusion vector; broad-host-range plasmid derived from pQF50	This study
pSRS1	*argR* clone in pUC19	19
pSM21	*argR* clone in pUC19	19
pKA58	Regulatory region of *car* in *Sma*I site of pUC19; nt 1 to 132 of Fig. 1a	19
pKF52	*argF*::*lacZ* transcriptional fusion; nt 1 to 380 of Fig. 1b	19
pSF54	Regulatory region of *argF* in *Sma*I site of pUC19; nt 121 to 380 of Fig. 1b	This study
pSC500	*aruC*::*lacZ* translational fusion	This study
pSC25	Regulatory region of *aruC* in *Sma*I site of pUC19; nt 258 to 559 of Fig. 1c	This study

[a] BRL, Bethesda Research Laboratories.

5 min with gentle shaking. The mixture was then centrifuged at 12,000 × *g* at room temperature for 5 min. The aqueous phase was extracted with an equal volume of phenol-chloroform (1:1, vol/vol), centrifuged as above, and transferred to a sterile 30-ml Corex centrifuge tube. Three volumes of 100% ethanol were added, and the mixture was incubated at −80°C for 30 min. The RNA precipitate was collected by centrifugation at 12,000 × *g* at 4°C for 30 min. The RNA pellet was dissolved in 300 μl of diethylpyrocarbonate-treated water. The concentration of RNA was determined by measurement of the absorbance at 260 nm.

S1 nuclease mapping. S1 nuclease mapping experiments with an end-labeled single-stranded probe were carried out basically as described by Greene and Struhl (8). A clone containing the *aruC* promoter (pSC500) was used to prepare radioactively labeled single-stranded DNA. A 27-mer which can hybridize with nucleotides (nt) 543 to 569 for *aruC* (Fig. 1c) was end labeled with [γ-^{32}P]ATP and annealed to double-stranded DNA of *Hin*dIII-cleaved pSC500. Extension of the labeled oligonucleotide for preparation of the single-stranded probe was carried out with Klenow fragment at 37°C, and the extended probe was eluted from a 6% polyacrylamide gel with a gel eluter (International Biotechnology, Inc.). Experiments were performed quantitatively to permit comparison of levels of transcripts under different growth conditions. The relative levels of transcripts were determined by obtaining scanning autoradiographs with a Molecular Dynamics personal laser densitometer.

Gel retardation analysis. The following DNA probes were used: the *Bam*HI-*Eco*RI fragment of pKA58 for the *car* promoter; the *Bam*HI-*Eco*RI fragment of pKF52 or the *Bam*HI-*Eco*RI fragment of pSF54 for the *argF* promoter; and the *Bam*HI-*Eco*RI fragment of pSC25 for the *aruC* promoter. In all cases, these DNA fragments were purified from a 1% low-melting-temperature agarose gel and labeled with either [α-^{32}P]dATP or [α-^{32}P]dGTP by using the Klenow fragment.

The binding reactions were carried out as described previously (11, 15). The radioactively labeled DNA probe (10^{-13} M) was allowed to interact with different concentrations of ArgR in 20 μl of 20 mM Tris-HCl (pH 7.6)–10 mM $MgCl_2$–100 mM dithiothreitol–10% (vol/vol) glycerol–50 mg of bovine serum albumin per ml. The reactions were allowed to equilibrate for 30 min at 25°C and terminated by the addition of an excess of cold DNA probe (10^{-11} M), and then the reaction mixtures were applied to a 5% polyacrylamide gel while the gel was running. After autoradiography of the gel, the intensity of the bound and unbound DNA bands was measured with a Molecular Dynamics personal laser densitometer. The apparent equilibrium binding constant, defined as the protein concentration required for half-maximal binding, was subsequently determined from a plot of the percentage of DNA bound against protein concentration.

Expression of *argR*. As described previously (19), plasmid pSM21 contains a 1.3-kb DNA fragment with the intact *argR* gene and its flanking regions cloned into the *Sma*I site of pUC19. Determination of the nucleotide sequence for pSM21 confirmed that expression of *argR* can be driven by the *lac* promoter of the parent vector, pUC19. Therefore, a recombinant strain of *E. coli* DH5α harboring pSM21 was used for overexpression of ArgR. Determination of ArgR by gel retardation and SDS-polyacrylamide gel electrophoresis showed that a significant amount of the ArgR protein was produced from this recombinant strain after overnight growth in LB medium.

Purification of the ArgR protein. A culture of *E. coli* DH5α harboring pSM21 was grown in LB medium (6 liter) overnight and harvested by filtration with the Pellicon Cassette System (Millipore Corp., Bedford, Mass.). Collected cells were washed in 20 mM potassium phosphate (pH 7.6) containing 1 mM EDTA and suspended at 0.5 g (wet weight) per ml in the same buffer. All solutions coming in contact with the enzyme in subsequent steps contained 1 mM EDTA. Phenylmethylsulfonyl fluoride was added to a final concentration of 1 mM immediately before the passage of the cell suspension through an Aminco French pressure cell at 8,000 lb/in^2. The crude extract was centrifuged at 27,000 × *g* for 30 min. Streptomycin sulfate (1 ml of 20% [wt/vol] solution per 10 ml of centrifuged extract) was added at 0°C with stirring and equilibrated for 10 min. After centrifugation at 27,000 × *g* for 30 min at 4°C, the supernatant was subjected to ammonium sulfate fractionation. The fraction precipitating at between 20 and 35% saturation was dissolved in 5 ml of 0.02 M potassium phosphate (pH 7.6). This solution was filtered through a Millipore membrane (0.45 μm pore size) and was subjected to gel filtration on a preparative Pharmacia Superdex200 (HR 16/60) column equilibrated with 0.02 M potassium phosphate (pH 7.6) and eluted with the same buffer. Fractions containing ArgR were detected by the gel retardation assay, combined, precipitated with ammonium sulfate (70% saturation), and centrifuged at 12,000 × *g* for 30 min. The precipitate was dissolved in 10 ml of 0.02 M potassium phosphate buffer (pH 7.6), and the solution was applied to a Pharmacia HiTrap Heparin (5-ml) column equilibrated with the same buffer. The column was washed with 55 ml of starting buffer, and the protein was eluted with a linear gradient of potassium chloride in 0.02 M potassium phosphate (pH 7.6). ArgR was eluted between 0.55 and 0.65 M KCl. Fractions containing ArgR were combined, concentrated with ammonium sulfate (70% saturation), and dissolved in 2 ml of 0.02 M potassium phosphate (pH 7.6) containing 0.8 M ammonium sulfate. The solution was applied to a Pharmacia phenyl-Superose column (HR 5/5) equilibrated with 0.02 M potassium phosphate (pH 7.6) containing 0.8 M ammonium sulfate. After the column was washed with 10 ml of this buffer, the protein was eluted with a linear gradient of 0.8 to 0 M ammonium sulfate in 0.02 M potassium phosphate (pH 7.6) buffer. ArgR was eluted at between 0.32 and 0.28 M ammonium sulfate.

The purified ArgR protein was dialyzed against 0.02 M potassium phosphate (pH 7.6) and stored at 4°C. Although the ArgR solution is stable for several months under these conditions, all gel retardation assays and footprinting experiments were performed within a few days of preparation of the protein to avoid possible changes during storage.

Polyacrylamide gel electrophoresis. Protein samples were applied to 8 to 25% (wt/vol) polyacrylamide gels in the presence of SDS by using the Pharmacia Phast System. The gels were electrophoresed at 250 V and 10 mA for a total of 65 V · h and stained with Coomassie blue as specified by the manufacturer.

Determination of the amino-terminal amino acid sequence. The purified ArgR protein was directly spotted on a polyvinylidene difluoride membrane, and the amino-terminal amino acid sequence was determined with a Beckman LF3200 gas-phase protein sequenator at the Molecular Genetics Facility of Georgia State University.

Molecular mass determination. The molecular mass of the native ArgR was determined by molecular sieving on a Pharmacia Superose 6 HR column (10/30) with proteins with known molecular masses: trypsin inhibitor (21,000 Da), 3-phosphoglycerate kinase (47,000 Da), and phosphorylase (97,400 Da). The proteins were eluted with 0.1 M potassium phosphate (pH 7.6), and elution of ArgR was monitored by the gel retardation assay and SDS-polyacrylamide gel electrophoresis.

DNase I footprinting analyses. DNase I footprinting was carried out as previously described (15). The reaction mixture (200 μl) contained 10^{-11} M uniquely end-labeled restriction fragment carrying the operator region, 0.7 to 90 × 10^{-11} M homogenous ArgR protein, 50 mM Tris-HCl (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 μg of sheared salmon sperm DNA, and 50 mg of bovine serum albumin. After incubation for 30 min at 25°C, pancreatic DNase I (0.2 μg; Boehringer) was added. The digestion was allowed to proceed for 2 min and then terminated by the addition of 20 μl of 3 M sodium acetate, 10 μg of tRNA, and 600 μl of ethanol. After precipitation with ethanol at −70°C, the pellet was dissolved in 20 μl of formamide-dye mixture and the reaction products were analyzed on an 8% denaturing polyacrylamide sequencing gel against a G+A sequencing ladder (17).

Premethylation interference experiments. DNA methylation was carried out according to the protocol described by Baldwin (1). The concentration of the repressor used in the binding reaction was determined empirically such that the amounts of bound and unbound DNA fractions of methylated DNA were approximately equal. The bound and free DNA fractions were separated by elec-

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(a)

                                                                                    -35                             -10       *+1
GTACGACATGCAGGATGTGCTCGGCCTACGCTGAGGCGCTGCCGCACGTCGTCTTATTGGTGGACCGGAATGTCGCGATTCTGTAAACTACAGCTTTAGT  100

                                     ---------------->                <-----------------
GTGTCCACTAAAAGCAGCGCAGCATGAATCGAAAAAAAGCGGGATGACTCTTCACGGTGTCATCCCGCTTTTTACACCTGCGCGACCAGTCAGGCTTGA  200

TTTACGGGAGGTCTTCTTGACTAAGCCAGC  230
       S.D.
          carA: M  T  K  P  A

(b)

GCCGTGCCGGCGGCGCGGCGGCGAGTGTTTCTCCCGTGTGCATGTTCGACCTCCCTGGTCCGAAACCCGCCTCCTCCATGAAGGTATTCGGGCGCGCACA  100
GCATAAAGCGTCGCCCCGCCCAACCGATAGATCGACGAGGCGGGACTTTGGCGGCGGGACAACGGCGAAGACCCGGGAAAAACCCCGTAACCCGCGCCTT  200

          -35             -10        *+1
CCAAGCCCTCCTTGTGTTTCCGCGACATTTCCTTATAAGATCGCGCCTTCCCCTATTTTCCCGCACGTTGCGGTCCCGCCGCAGGTCCAGTCCCCTTTTC  300

TCTCGCGAAAGCGGGCCAGTGCCTCGGCCAGCAGTAAAAATAAAAGGTAGTCAGCGATGAGCGTACGGCACTTTCTCTCG  380
                                           S.D.
                                                  argF: M  S  V  R  H  F  L  S

(c)

CTTCACTCCCGTCGAACGCGGCTGATCGCCAACGGCGCGGCGGTTTCAGCCGGGCTTTGCCACGCCCCGGGAGGCGGATCGCCTCGGGAAGTCGCCGAGT  100
 F  T  P  V  E  R  G  -
TCGCGACGGGCCGTCGGAATGATCGCCTCCAGCAGTTCCAGCGGAACGTCGCTGGCGAACAGGTTGACCACGAAACGCCGGCCGACGTCGTCGCGAAAGA  200
CCTCGAACAGCGGCTGTTCGCCGCGCCAGCACTCCAGGCCGAGGCCGTCGCGCGGATTGACGTCGGAGGCGAAGACGAGACGGTAGTCGATTCCCTCGAT  300
GGGCATGGCTTGTTTCCCTGCTGTTCCCGCCCGCGGGGCGGCGTAGAAGAAGTATGCACCCGAGTGCATCGCGCGTTTACACTGCGCCTTCCCGATGCTT  400

                                                                       -35(?)              -10
TCTGTCGCATTTCCGAAAGCCGCGGAAATTCCCGGGTTCCGCGTATAACAAGTTGTCGCATGGCGGCAATGCCGGCCCGGTCCCAATCCCTACAATCCTT  500

  *+1
TCATCGATAGCCGCCTTCAGGCAGGAGAAACACCGATGTCCGCTCCCCATGCGCAGGTAGAACGTGCCGA  570
                       S.D.
                              aruC: M  S  A  P  H  A  Q  V  E  R  A  D
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FIG. 1. Nucleotide sequences of the *P. aeruginosa* promoters and flanking regions for *car* (a), *argF* (b), and *aruC* (c). The transcriptional initiation sites are indicated by asterisks above the nucleotides and labeled +1, and the −10 and −35 regions of each promoter are indicated accordingly. Also shown are the relevant Shine-Dalgarno (S.D.) sequences for each gene. The hypersymmetric sequence required for pyrimidine regulation of the *car* operon (14) is defined by converging arrows. The ArgR-binding sites as determined in this study are underlined, and their nucleotide sequences are italicized.

trophoresis through a 5% polyacrylamide gel. The gel was autoradiographed for 2 h. The DNA bands of interest were excised from the gel, placed into 400 μl of elution buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, 0.1% [wt/vol] SDS), and incubated overnight at 37°C with gentle shaking. Following centrifugation, the eluted DNA was coprecipitated with 10 μg of tRNA by using ethanol. The pellet was dissolved in 45 μl of water, and 5 μl of piperidine was added to start the cleavage reaction. After incubation at 90°C for 15 min, 50 μl of 0.6 M sodium acetate and 400 μl of ethanol were added to

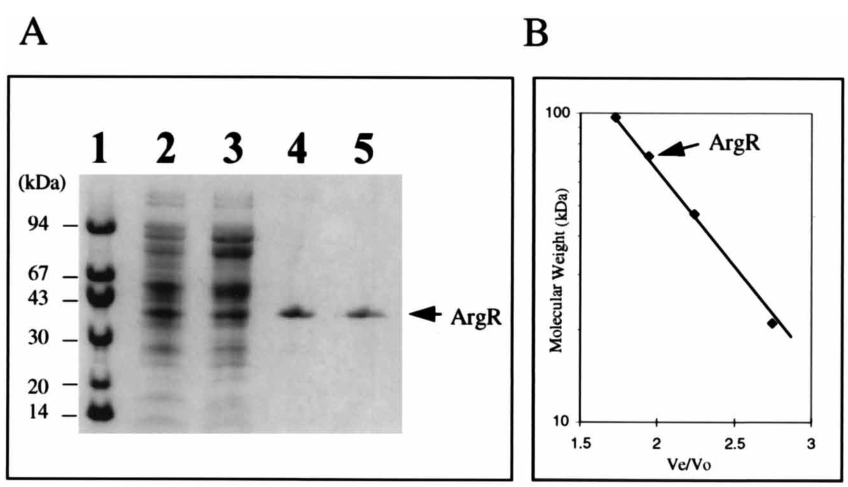

FIG. 2. (A) Purification of the ArgR protein. A cell extract was prepared from *E. coli* DH5α harboring pSM21 and subjected to fractionation as described in Materials and Methods. Samples were run on an SDS–8 to 25% polyacrylamide Phast gradient gel (Pharmacia) and stained with Coomassie brilliant blue. Lanes: 1, polypeptide molecular weight standards; 2, ammonium sulfate fraction; 3, gel filtration fraction; 4, HiTrap heparin affinity fraction; 5, purified ArgR after hydrophobic phenyl-Superose column chromatography. (B) Determination of the molecular mass of ArgR by gel permeation. Gel permeation experiments were carried out with a Pharmacia Superose-6 HR column in the presence of 0.1 M potassium phosphate (pH 7.6). The standards used were phosphorylase (97,400 Da), 3-phosphoglycerate kinase (47,000 Da), and trypsin inhibitor (20,100 Da). Ve, elution volume; V0, void volume.

TABLE 2. Effect of exogenous arginine on the expression of β-galactosidase from an *aruC*::*lacZ* fusion in *P. aeruginosa* and an *argR*::Gm derivative

Strain[a] (genotype)	Growth conditions[b]	Sp act[c] (nmol/mg/min)
PAO1 (wild type)	Glutamate	324 ± 15
	Glutamate + arginine	4,611 ± 40
PAO501 (*argR*::Gm)	Glutamate	140 ± 4
	Glutamate + arginine	210 ± 6

[a] An *aruC*::*lacZ* translational fusion was constructed and transformed into PAO1 and PAO501 as described in Materials and Methods.
[b] Cells were grown in minimal medium P supplemented with 20 mM glutamate or 20 mM glutamate and arginine as indicated.
[c] Specific activities are the mean and standard error for cultures at each growth condition.

precipitate the DNA. The pellet was air dried and redissolved in 15 μl of formamide-dye solution for analysis on an 8% denaturing polyacrylamide sequencing gel.

Missing contact probing experiments. The depurination reaction (G+A) described by Brunelle and Schleif (3) was used to modify the DNA. After depurination with formic acid, the free and bound DNA were separated, eluted, and cleaved with piperidine as described above.

RESULTS

Purification of ArgR and determination of its molecular weight. The ArgR protein was purified from an *E. coli* strain harboring pSM21, in which *argR* is expressed from the *lac* promoter as described in Materials and Methods. The final product was judged homogeneous as indicated by SDS-polyacrylamide gel electrophoresis (Fig. 2A). The molecular mass of the protomer was determined from a plot of electrophoretic mobility against the logarithms of the molecular mass of known polypeptides (Fig. 2A). A value of 37,000 Da was obtained.

The molecular mass of the native protein was estimated by molecular sieving as described in Materials and Methods. A value of 74,000 Da was obtained (Fig. 2B), indicating that ArgR is a dimer of equal subunits.

Determination of the amino-terminal amino acid sequence. The purified ArgR protein was subjected to amino-terminal sequence determination as described in Materials and Methods. The first 10 residues were Thr-Ala-Gln-Pro-Gln-Arg-Ile-Gly-Phe-Leu. This sequence is identical to that derived from the nucleotide sequence of cloned *argR* (19), with the first methionine residue being removed posttranslationally.

Evidence for an arginine-inducible *aruC* promoter. We recently reported that *aruC*, encoding the third enzyme of the catabolic arginine succinyltransferase pathway, is under the control of *argR* (19). We also reported in this work that *aruC*, the first gene of the *aru* operon, is found in *P. aeruginosa* 500 bp downstream of *argR*. To study the existence of promoter activity in the *arg-aruC* intergenic region, an *aruC*::*lacZ* translational fusion, pSC500, was constructed as described in Materials and Methods. The chromosomal insert of pSC500 contains the last seven codons of *argR*, the entire *arg-aruC* intergenic region, and the first nine codons of *aruC* (Fig. 1c), with the ninth codon of *aruC* fused in frame to *lacZ* on the vector plasmid pQF52. Expression of the fusion was much weaker in *E. coli* relative than in *P. aeruginosa* on X-Gal indicator plates. Measurements of β-galactosidase activity expressed from the fusion in PAO1 (Table 2) show that exogenous arginine induces *aruC* expression 14-fold. Similar experiments with PAO501, in which *argR* was inactivated by gene replacement (19), show that *aruC* expression in the fusion is no longer induced by arginine (Table 2).

S1 nuclease mapping was performed quantitatively with RNA extracted from cells grown in the absence or presence of arginine. The results (Fig. 3) show four consecutive bands corresponding to nt 503 to 506 of the sequence in Fig. 1c, whose intensity is induced 12-fold by growth in the presence of arginine. Multiple bands are usually considered the result of nibbling by S1 nuclease. Accordingly, the most distal site, corresponding to nt 503 (Fig. 1c), is the most likely transcriptional initiation site for *aruC*. This site is preceded by a good −10 sequence (5 of 6 bases) and a weak −35 sequence (1 of 6 bases)

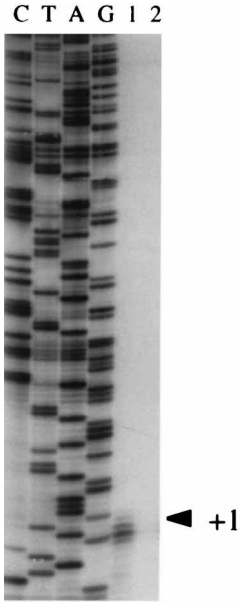

FIG. 3. S1 nuclease mapping of the 5′ end of the *aruC* transcript from *P. aeruginosa*. A single-stranded DNA fragment (nt 1 to 569 [Fig. 1c]) which has sequence complementary to the mRNA was labeled at the 5′ end as described in Materials and Methods. After hybridization to cellular RNA, the hybridization mixture was treated with S1 nuclease and analyzed on a 6% sequencing gel. The dideoxy ladder (lanes G, A, T, and C) was derived with the same primer that was used to generate the probe for S1 nuclease mapping. The cells were grown with 20 mM arginine (lane 1) or glutamate (lane 2) as the sole source of carbon and nitrogen. Also shown is the 5′ terminus of the arginine-inducible mRNA, defined as +1, corresponding to the residue at nt 503 in Fig. 1c.

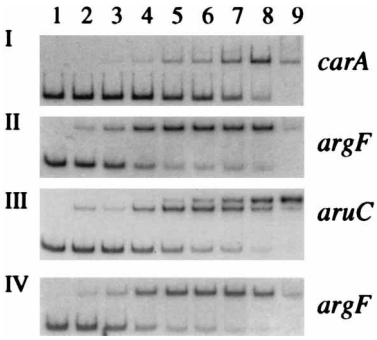

FIG. 4. Gel retardation experiments. The radioactive ^{32}P-labeled *carA* (panel I), *argF* (panel II), and *aruC* (panel III) operator DNA fragments were incubated with various ArgR concentrations in the absence of L-arginine. The reactions were terminated by the addition of excess unlabeled operator DNA and applied to a 5% polyacrylamide gel while the gel was running. Experiments were also conducted with the *argF* operator DNA fragment in the presence of 1 mM arginine (panel IV). Lanes 1 to 9 contained ArgR concentrations of 0, 1×10^{-12}, 3×10^{-12}, 6×10^{-12}, 12×10^{-12}, 25×10^{-12}, 50×10^{-12}, 100×10^{-12}, and 200×10^{-12} M, respectively.

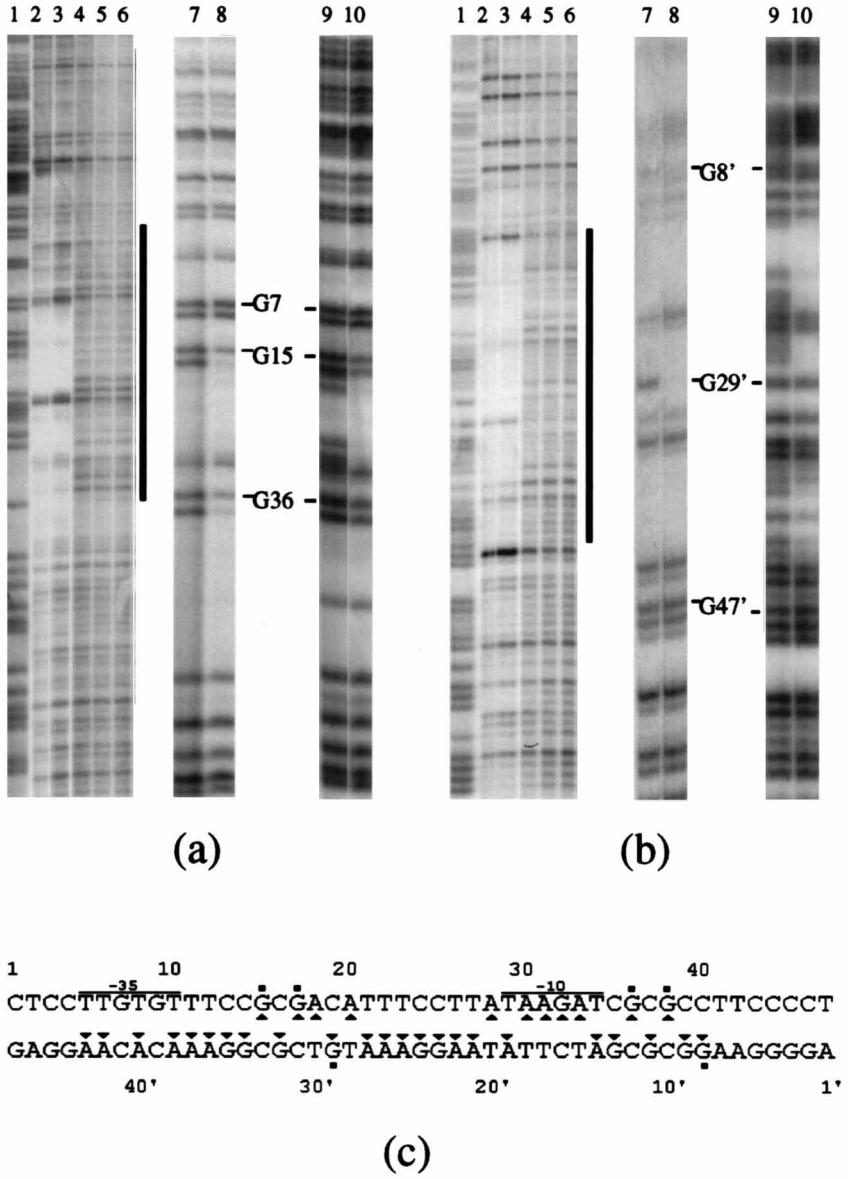

FIG. 5. Footprinting analyses of ArgR with the *argF* control region. The DNA fragments used were labeled at the 3′ end of the sense strand (a) and antisense strand (b) of the *argF* operator. Lanes: 1, the corresponding G+A Maxam-Gilbert sequencing ladder; 2 to 6, DNase I footprinting with decreasing concentrations of ArgR: 90×10^{-11}, 18×10^{-11}, 3.6×10^{-11}, 0.7×10^{-11}, and 0 M, respectively (the protection region is indicated by a bar); 7 and 8, premethylation footprint sequences of unbound and bound DNA, respectively; 9 and 10, are the depurination footprint sequences of unbound and bound DNA, respectively. These two forms of DNA have been separated and modified as described in Materials and Methods. Also shown are several guanine residues, which are numbered according to the description in Fig. 5c. (c) Summary of the results of the three footprinting analyses. The nucleotide sequence of a region defined by DNase I footprinting is shown and numbered accordingly. Nucleotides detected by premethylation interference footprinting are indicated by solid squares, and those detected by depurination footprinting are indicated by solid triangles. Also shown are the −35 and −10 regions of the *argF* promoter.

on the basis of homology to the consensus sequence of sigma-70 promoters of *E. coli.*

Binding of ArgR to the *car*, *argF*, and *aruC* regulatory regions. Gel retardation experiments were carried out with the purified ArgR protein of *P. aeruginosa* and DNA fragments carrying the regulatory regions of the *car*, *argF*, and *aruC* loci as described in Materials and Methods. The results (Fig. 4) show that ArgR binds with different affinities to the *car*, *argF*, and *aruC* operators. The relative amounts of free and bound DNA bands in Fig. 4 were estimated densitometrically. Plots of percent bound DNA against the concentration of ArgR yield apparent dissociation constants of 2.5×10^{-11}, 4.2×10^{-12}, and 7.2×10^{-11} M for the *carA*, *argF*, and *aruC* operators, respectively. The results in Fig. 4 also show a single retarded band for the *car* and *argF* probes and two retarded bands for the *aruC* probe (panel III). The variation in the intensities of the two bands observed in response to the increase in the concentration of the *aruC* probe suggests the presence of two ArgR-binding sites on this DNA fragment.

The gel retardation experiments shown in panels I to III of Fig. 4 were performed in the absence of arginine from all reagents. Inclusion of L-arginine (1 mM) in the reaction mixture did not change the dissociation constant for the *argF* operator (panel IV). Similarly, arginine had no effect on the

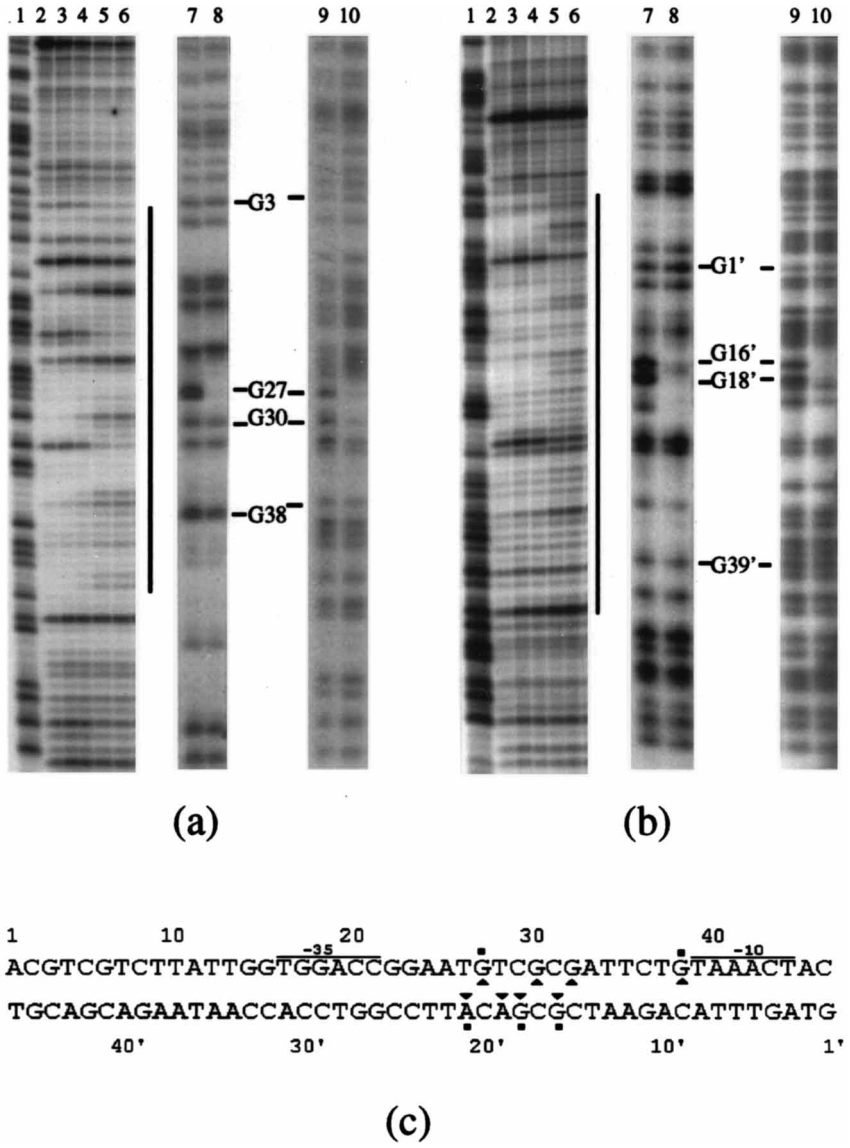

FIG. 6. Footprinting analyses of ArgR with the *car* control region. The DNA fragments used were labeled at the 3′ end of the sense strand (a) and antisense strand (b) of the *car* operator. (c) Summary of results of the three footprinting analyses. Descriptions of the panels are identical to those in the legend to Fig. 5.

dissociation constants for the *car* and *aruC* operators (data not shown). Several intermediates of arginine metabolism that could serve as effectors for ArgR (L-ornithine, agmatine, and glutamate) were also examined in gel retardation experiments. None of these compounds affected the dissociation constant for ArgR binding to the *argF* operator (data not shown).

DNA footprinting analyses. Three footprinting approaches, DNase I, premethylation, and depurination, were used to characterize the interactions of ArgR with the control region of the *argF* operon. DNase I footprinting analysis was used to define the target site for ArgR binding. The results (Fig. 5) shows that binding of ArgR protects a 47-bp region from nuclease digestion. Specific base interactions that are crucial for ArgR binding were analyzed by premethylation interference and missing-contact probing experiments. As shown in Fig. 5a and b, methylation of the G-15, G-17, G-36, and G-38 residues on the sense strand and the G-8, and G-29, residues on the antisense strand of the *argF* operator sequence strongly interfere with the binding of ArgR. These figures also show that depurination of these guanine residues as well as most of the adenine residues in the region defined by DNase I footprinting inhibit the formation of the ArgR-*argF* operator complex. A schematic presentation of the combined data (Fig. 5c) clearly indicates that the ArgR binding site in the *argF* regulatory region completely overlaps the previously determined promoter sequence for this gene (13).

Similar experiments (Fig. 6a and b) were carried out with the *car* operator. The combined data (Fig. 6c) showed that ArgR protects a stretch of 46 bp on both strands from nuclease digestion. This binding site completely overlaps the −35 and −10 regions of the *car* promoter as previously defined by Kwon et al. (14). However, in contrast to the *argF* operator (Fig. 5), the results of premethylation interference and depurination experiments indicate that ArgR-nucleobase contacts are specifically focused within a TGTCGC sequence between the −35 and −10 regions.

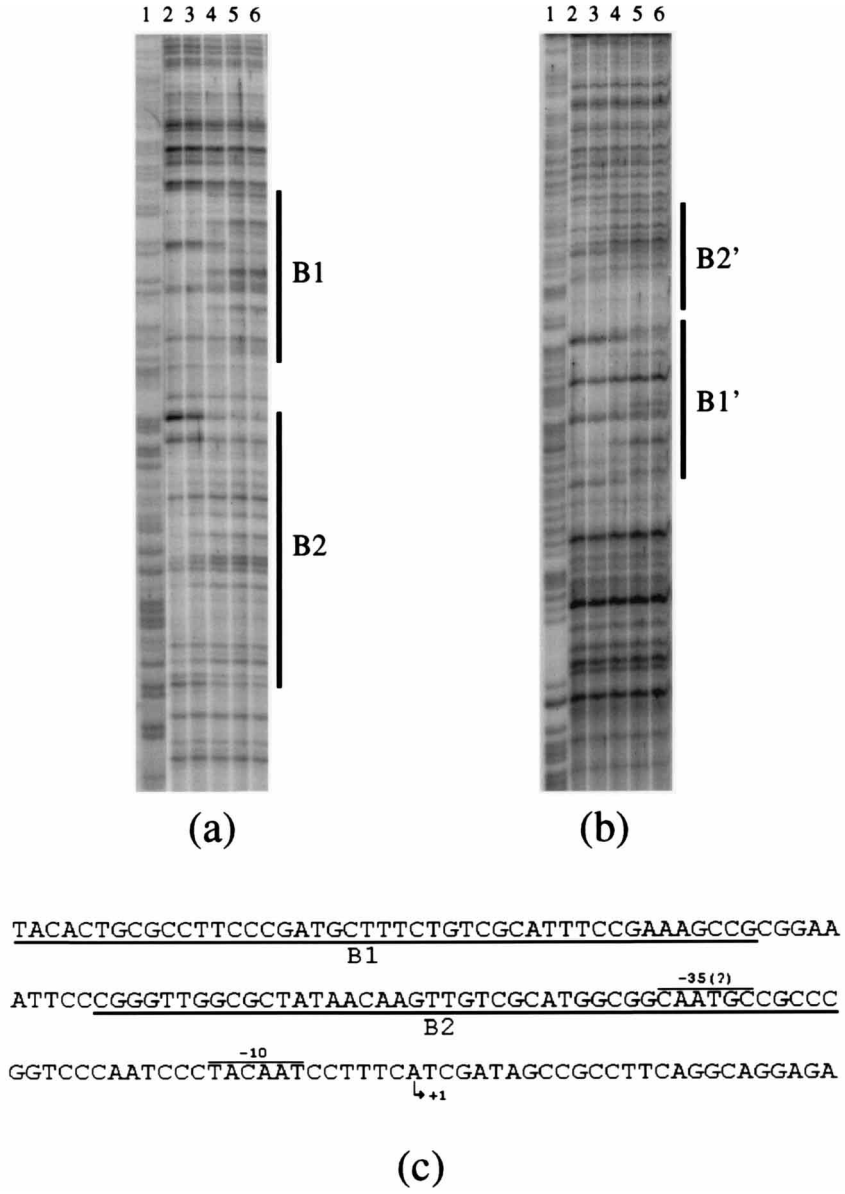

FIG. 7. DNase I footprinting analysis of the *aruC* operator. (a and b) Results obtained with the sense and antisense strands, respectively. Lanes: 1, G+A Maxam-Gilbert sequencing reaction; 2 to 6, DNase I footprinting with decreasing concentrations of ArgR, respectively, as described in the legend to Fig. 5. (c) Nucleotide sequence of the regulatory region of *aruC*. The putative −35 and −10 promoter elements are labeled, and the transcriptional initiation site is also indicated by an arrow and labeled +1. B1, first binding site; B2, second binding site.

DNase I footprinting was similarly used to characterize the interactions of ArgR with the control region of *aruC*. In contrast to the *car* and *argF* operators, the results (Fig. 7a and b) showed the presence of two ArgR-binding sites, each composed of 45 bp, on the *aruC* control region. These results also showed that ArgR binds to the upstream binding site at lower concentrations of the protein. As shown in Fig. 7c, the two consecutive ArgR-binding regions are separated from each other by 10 bp, and the 3′ terminus of the downstream binding site overlaps with the −35 region of the arginine-inducible promoter of *aruC*.

DISCUSSION

Recent work from our laboratory (19) described the cloning of *argR*, which controls the expression of a number of anabolic and catabolic *arg* genes. Plasmid pSM21, carrying the cloned *argR* downstream from a *lac* promoter, was used for expression and purification of the encoded protein. Determination of the amino-terminal amino acid sequence for the purified protein showed that the first 10 residues are identical to those of the derived sequence for the cloned *argR* (19). Furthermore, the molecular mass of the protomer determined by SDS-polyacrylamide gel electrophoresis (37,000 Da [Fig. 2A]) is identical to that predicted from the derived sequence (19). These results confirm that the purified protein is indeed that encoded by the *argR* gene.

Amino acid sequence comparison indicated that the ArgR protein of *P. aeruginosa* is a member of the AraC/XylS family of transcriptional regulators (19). Inactivation of *argR* by gene replacement abolished arginine repression of the biosynthetic

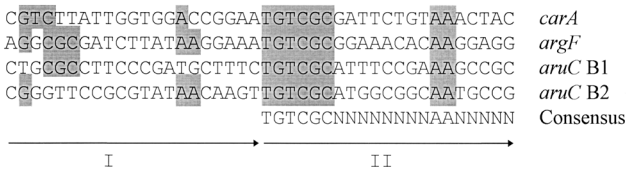

FIG. 8. Sequence alignment of ArgR-binding sites. The sequences were obtained from the results of DNase I footprintings and aligned by placing the TGTCGC of each sequence at an identical position. The first and second halves of the binding sites are depicted by arrows and labeled I and II, respectively. The consensus sequence was deduced from the nucleotides of the second half-sites that are essential for ArgR binding. Nucleotides identical to the corresponding positions in the $TGTCGCN_8AAN_5$ consensus sequence are shaded.

enzymes, carbamoylphosphate synthetase (*car*) and anabolic ornithine carbamoylphosphate transferase (*argF*). Inactivation of *argR* also abolished the induction of the *aru* operon, encoding enzymes of the arginine succinyltransferase pathway (19). This pathway is considered the major pathway for aerobic utilization of arginine (9). The results reported here on the expression of *aruC*, the first gene in the *aru* cluster, show the presence of an arginine-inducible transcript initiated 33 bp upstream of *aruC* (Fig. 3). Furthermore, experiments with an *aruC*::*lacZ* fusion show that arginine induction of this promoter requires the presence of a functional ArgR (Table 2).

DNase I footprinting experiments (Fig. 5 to 7) showed that ArgR protects a region of 45 to 47 bp in the regulatory regions for *argF*, *car*, and *aruC*. For the two biosynthetic operons, *argF* and *car*, the ArgR-binding site completely overlaps the promoters of these operons. These results indicate that ArgR exerts its negative effect on the expression of *argF* and *car* through steric hindrance. In contrast to these results, DNase I footprinting analysis for the *aruC* control region showed that the protected region covers two ArgR-binding sites (B1 and B2, Fig. 7). The presence of two binding sites is consistent with the presence of two retarded bands in gel retardation experiments (Fig. 4, panel III). The 3′ terminus for the downstream site overlaps the putative −35 region for the arginine-inducible promoter. Such an overlap is consistent with reports for other transcriptional activators; for AraC, the overlap with the −35 region of the promoter was found to be essential for activation (4, 21). The role of the two ArgR-binding sites in the control of *aruC* expression must await additional studies.

The results of premethylation interference and depurination footprinting experiments identify a common sequence, 5′-TGT CGC-3′, which is important for the binding of ArgR to the *argF* and *car* operators (Fig. 5 and 6). The identified sequence is located between the −10 and −35 regions of the two promoters. However, the TGTCGC sequence is present in opposite orientations in the two operons relative to the transcriptional start sites. This sequence is present on the sense strand of the *car* operator (Fig. 6c) whereas it is present on the antisense strand of the *argF* operator (Fig. 5c). When the protected regions of the two genes are aligned such that the common sequence, TGTCGC, is in the same orientation (Fig. 8), the TGTCGC sequence position relative to the 45-bp binding site is identical for the two operons.

As shown in Fig. 8, alignment of all the ArgR-binding sites reveals the presence of a conserved $TGTCGCN_8AA$ sequence in the second half of the binding sites. Moreover, analysis of the alignment of the first and second halves of the binding sites reveals a consensus sequence of $TGTCGCN_8AAN_5$ in a direct-repeat structure. While the ArgR-binding site is somewhat larger than the AraC-binding site, the direct repeat structure is similar to that of the AraC-binding sites and is in contrast to many prokaryotic DNA-binding sites consisting of two inverted-repeat sequences (23). While the arrangement of the half-sites in the binding site might not be important for the function of ArgR in negative control of the biosynthetic operons, it could prove important in its role as an activator of *aruC* expression. Studies with AraC showed that the two half-sites recognized by the AraC dimeric protein must be in the same direct-repeat orientation for full activation of transcription of the *araBAD* promoter (4, 21).

Interestingly, the first half-site of the ArgR-binding site appears more degenerate than the second half-site, and the extent of this degeneration is dependent on the target gene (Fig. 8). Such variation might account for the different affinities that ArgR exhibits with different control regions (a range of 3- to 17-fold [Fig. 4]). Thus, the lower level of degeneration of the first half-site for *argF* could account for the higher affinity observed with this operator relative to the *car* and *aruC* operators (Fig. 4).

The presence of L-arginine does not affect the in vitro binding of *P. aeruginosa* ArgR to the operators examined in this work (Fig. 4). Significantly, studies with AraC (4, 21) have shown that the coeffector arabinose is not required for the DNA-binding activity of AraC. Instead, AraC binding to the polymerase-proximal half-site is sufficient for strong transcriptional activation, and the role of arabinose is to induce the correct occupancy of the protein. Thus, it is possible that arginine plays a similar role here and that the effect of exogenous arginine is mediated through modulation of expression of *argR* itself. This proposal is consistent with the observed induction of ArgR by exogenous arginine (19) and with the finding that the transport operon that encompasses *argR* is also under the control of ArgR (18a). Additional information about the regulation of the transport operon might provide insights into the mechanism by which the effect of exogenous arginine is mediated.

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