Dimerization Is Essential for DNA Binding and Repression by the ArsR Metalloregulatory Protein of Escherichia coli*

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Chun Xu and Barry P. Rosen‡

From the Department of Biochemistry and Molecular Biology, Wayne State University School of Medicine, Detroit, Michigan 48201

Arsenical resistance (ars) operons produce resistance to trivalent and pentavalent salts of the metalloids arsenic and antimony in cells of Escherichia coli. The first gene in the operon, arsR, was previously shown to encode a homodimeric trans-acting metalloregulatory repressor protein. Dimerization of ArsR was investigated using the yeast two-hybrid system in which the ArsR protein was fused to the Saccharomyces cerevisiae GAL4 DNA-binding domain and GAL4 activation domain to produce chimeric proteins. Transcriptional activation of lacZ reporter indicated that dimerization of the ArsR is stable in yeast. The results indicated that residues 1–8 of the DNA-binding domain, and a putative helix-turn-helix DNA-binding motif toes a homodimeric gene in the operon, using the yeast two-hybrid system in which the ArsR pressor protein. Dimerization of ArsR was investigated using the yeast two-hybrid system show that the amino-terminal 8 residues and carboxyl-terminal 28 residues are not required for dimerization of ArsR. These results were confirmed in vitro by size exclusion chromatography of each purified truncated ArsR. In addition, only arsR genes that produced proteins capable of dimerization exhibited metalloregulation in vivo and only ArsRs that could dimerize bound to DNA in vitro.

MATERIALS AND METHODS

E. coli Strains, Plasmids, and Media—The bacterial strains and plasmids used in this study are described in Table I. E. coli cells were grown in LB medium at 37 °C. Ampicillin (100 μg/ml), kanamycin (80 μg/ml), tetracycline (15 μg/ml), or chloramphenicol (20 μg/ml) were added as required. For protein expression 0.2 mM isopropyl-1-thio-β-D-galactopyranoside was used as inducer. Sodium arsenate or phenylarsine oxide were added at the indicated concentrations. All chemicals were obtained from commercial sources.

DNA Manipulation—Preparation of plasmid DNA was performed by using a Wizard DNA purification kit (Promega). Endonuclease digestions, DNA fragments separations and isolations, ligations, transformations, and Klenow fragment fill in were performed according to standard procedures (10) unless otherwise noted. The conditions for polymerase chain reaction were as described previously (2). Restriction endonucleases, T4 DNA ligase, Klenow fragment of DNA polymerase I and Taq polymerase were from Life Technologies, Inc. For DNA sequencing double-stranded DNA was isolated with a plasmid mini kit from QIAGEN and then sequenced by the method of Sanger et al. (11) using an ALFexpress system and a Cy5 labeled sequence kit (Pharmacia Biotech Inc.).

Yeast Two-hybrid Analysis—Cells of yeast strain SYP526 were grown in YPD or the appropriate selective minimal medium. Competent cells were prepared and transformed as described (12). Portions of the transformation mixture were spread on selective plates, after which the plates were incubated at 30 °C for 3–4 days. Yeast expression vectors pGBT9 containing the DNA-binding domain of the Saccharomyces cerevisiae GAL4 protein and pGAD424 with the GAL4 activation domain were from MATCHMAKER Two-hybrid System (CLONTECH Laboratories, Inc.). Full-length arsR was cloned in-frame in both pGBT9 and pGAD424 vectors by polymerase chain reaction (PCR)* mutation using primers P2 and P13 (Table II) to place an EcoRI site at the 5′ end and a BamHI site at the 3′ end of arsR, producing plasmids pGBT9-R and pGAD424-R. Before cloning into pGBT9 and pGAD424 vectors, the PCR product was cloned into pGEM-T vector (Promega), and the absence of random mutations was verified by DNA sequencing. The same methodology was applied for all subsequent PCR cloning. Deletion mutants producing carboxyl-terminal truncations were constructed by PCR cloning EcoRI/BamHI fragments using oligonucleotide P2 as a forward primer for all mutants and the following oligonucleotides as reverse primer: P5 for pGAD424-RJ62–117, P6 for pGAD424-RJ81–117, P7 for pGAD424-RJ84–117, P8 for pGAD424-RJ66–117, P9 for pGAD424-RJ88–117, P10 for pGAD424-RJ90–117, and P11 for pGAD424-RJ94–117. Plasmids pGAD424-RJ1–40 and pGAD424-
**ArsR Dimerization**

### Table I

| Strains and plasmids | Genotype/description | Ref. |
|----------------------|----------------------|------|
| **Yeast** SFY526 | MATa ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3 112 can1 gal4-542 gal80-538 | 19 |
| **E. coli** JM109 | recA1 supE44 endA1 hsdR17 tyrA69 relA1 thi-1 (lac-proAB) F' [traD36 proAB' lacIq'] lacZΔM15 | 10 |
| **BL21 (DE3)** | hsdS gal (λcI857 ind1 Sam7 nin5 lacUV5-T7 gene1) | 20 |

### Plasmids

- **pGBT9**
  - GAL4(1–147) DNA-binding domain, TRP1, Ap+

- **pGAD424**
  - GAL4(768–881) activation domain, LEU2, Ap+

- **pVA3**
  - murine p53(12–309) in pGBT9, TRP1, Ap+

- **pTD1**
  - SV40 large T-antigen (84–708) in pGAD3F, LEU2, Ap+

- **pGEM-T**
  - PCR cloning vector, Ap+

- **pT7-7**
  - Expression vector, Ap+

- **pACYC184**
  - Cloning vector, Cm+, Tc+

- **pET28a**
  - Cloning and expression vector, Km+

- **pET29b**
  - Cloning vector, expression vector, Km+

- **pGDBAR2**
  - lacZ under control of ars promoter/operator, Tc+

- **pGBT9R**
  - Full-length of arsR gene fused to GAL4(1–147), DNA-binding domain in pGBT9, TRP1, Ap+

- **pGAD424R series plasmids**
  - Full-length or various deletions of arsR gene fused to GAL4(768–881) activation domain in pGAD424, LEU2, Ap+

- **pETR series plasmids**
  - Full-length or various deletions of arsR gene cloned in pET28a or pET28b, Km+

### Table II

| Oligonucleotide primers | Name | Sequence (5'–3') | Location in arsR gene | Restriction site |
|-------------------------|------|------------------|-----------------------|-----------------|
| P1                      | ATCAGGAGCCGCCATTGTC | −14 to +5          | NdeI                  |
| P2                      | GATTCGAGCCGAAAATGCT  | −15 to +7           | EcoRI                 |
| P3                      | GAATTCGAGCGCAATATGTC  | +32 to +51         | EcoRI                 |
| P4                      | GAATTCGACCAGCGAAGCCAA | +115 to +138      | EcoRI                 |
| P5                      | CGGGATCCACAGCAGCGG   | Complementary to +170 to +190 | BamHI |
| P6                      | GATATCCATGTTGAATGCG   | Complementary to +224 to +244 | BamHI |
| P7                      | GATATCCTTCGCCCGTCTG    | Complementary to +236 to +280 | BamHI |
| P8                      | GATATCCTATTTTGCGCGAGC  | Complementary to +244 to +265 | BamHI |
| P9                      | GATATCCTATCAATTGCGG    | Complementary to +253 to +275 | HindIII |
| P10                     | GAATTCGTTACCAGCGTCTGAG | Complementary to +264 to +286 | BamHI |
| P11                     | CGGGATCTCTTTCTACCGGAG  | Complementary to +274 to +286 | BamHI |
| P12                     | GAATTCGATGCGCAGAATGC  | Complementary to +274 to +286 | BamHI |
| P13                     | GAATTCGAGGTATTAACACTTC  | End of arsR gene | BamHI |

*U*nderlined nucleotides are changes from the wild type sequence to introduce the indicated restriction sites.

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R11–11 containing amino-terminal deletion mutants were generated by cloning EcoRI BamHI fragments from PCR using either oligonucleotide P3 (for deletion of 1–11 amino acid residues) or P4 (for deletion of 1–40 amino acid residues) as forward primer and oligonucleotide P13 as reverse primer. In the case of deletion of residues 1–8, an Muni/BamHI DNA fragment from plasmid pGAD424-R was cloned into vector plasmid pGAD424 that had been digested with EcoRI and BamHI, generating plasmid pGAD424-R1–8.

Protein-protein interaction was analyzed by in vivo measurement of β-galactosidase activity using a filter assay (12). The yeast reporter strain SFY526 was grown on YPD medium or SD synthetic medium containing adenine hemisulfite and all required amino acids. To select for transformants containing pGBT9-derived plasmids, tryptophan was omitted from the reading medium at 30 °C. Positive colonies appeared within 10 h. Plasmids pVA3, encoding a murine p53/GAL4 DNA-binding domain hybrid, and pT1D, expressing an SV40 large T-antigen/GAL4 activation domain hybrid, were used as positive controls. The vectors alone (pGBT9 and pGAD424) were used as negative controls.

**Expression and Purification of ArsR Proteins**—Vector plasmids pET28a and pET28b (Novagen) were used to construct recombinant plasmids for expression of arsR deletions and genes with six histidine codon tags (His6-tag). Plasmid pETR, encoding His6-tagged wild type ArsR, was generated by ligation of an Ndel fragment from the plasmid pCR0 (2) into the Ndel site of vector plasmid pET28a. The orientation of arsR was confirmed by restriction endonuclease digestion. Except for construction of plasmid pETRN9, all arsR deletion mutants were produced by PCR mutagenesis, with several steps of subcloning through vectors pGEM-T and pT7-7 to achieve in-frame fusions. The correct reading frames were verified by DNA sequencing. For deletion genes encoding carboxyl-terminal truncations, the forward primer was the oligonucleotide P1, in which an EcoRI site of vector plasmid pET28a. The orientation of arsR was confirmed by restriction endonuclease digestion. Except for construction of plasmid pETRN9, all arsR deletion mutants were produced by PCR mutagenesis, with several steps of subcloning through vectors pGEM-T and pT7-7 to achieve in-frame fusions. The correct reading frames were verified by DNA sequencing. For deletion genes encoding carboxyl-terminal truncations, the forward primer was the oligonucleotide P1 (for deletion of residues 1–40), was constructed by cloning a PCR fragment produced using oligonucleotide P4 as the forward primer and P13 as the reverse primer into vector plasmid pET28a. For the deletion of arsR codons 1–8, an Muni/HindIII fragment from plasmid pGAD424-R was cloned into vector pET28b that was digested with EcoRI and HindIII, generating plasmid pETRN9.

Cells of E. coli strain BL21 (DE3) bearing pETR series plasmids were grown overnight in LB medium containing kanamycin at 37 °C. The culture was diluted to 100-fold with fresh, prewarmed medium and grown at 37 °C. When the culture reached an A600 of 0.8, expression of arsR genes was induced by addition of 0.2 mM isopropyl-1-thio-β-D-galactopyranoside for an additional 3 h. Induced cells were harvested by centrifugation and washed once with buffer A (20 mM Tris-HCl, pH 7.9, 5 mM imidazole, 0.5 mM NiCl2, and 5 mM β-mercaptoethanol). The pelleted cells were suspended in 5 ml of buffer A per g of wet cells and disrupted by a single passage through a French pressure cell at 20,000 p.s.i. Unbroken cells and membranes were removed by centrifugation at 150,000 × g for 1 h. The soluble fraction was loaded onto a 1.2-cm diameter column filled with 8 cm of ProBond resin (Invitrogen) pre-equilibrated with buffer A. The column was washed with 10 volumes of...
ArsR Dimerization

Fig. 1. Yeast two-hybrid analysis of ArsR homodimerization. A gene fusion of the entire arsR gene to the sequence for the GAL4 DNA-binding domain (left) was co-expressed with the indicated sequences from arsR fused to the GAL4 activation domain (right) by co-transformation into yeast SFY536 cell. Expression of the lacZ reporter gene was scored by generation of blue color in filter assays of β-galactosidase activity. Each assay was performed in duplicate and repeated two or three times.

buffer A, followed by 6 volumes of buffer A containing 60 mM imidazole and eluted with a linear gradient of 0.1–0.5 M imidazole in buffer A. Fractions of 1.5 ml were collected and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (14). Fractions containing the His6-tagged ArsRs were pooled, concentrated, and stored at −70 °C until use. The mass of purified wild type and truncated His6-tagged ArsR was determined using a Waters high pressure liquid chromatography equipped with Synchropak size exclusion column (Synchron, Inc.) and a precolumn. Proteins were eluted at 1 ml/min with degassed buffer B (50 mM Tris acetate, pH 7.5, 0.5 mM KCl, and 14.4 mM β-mercaptoethanol). Bovine serum albumin (64 kDa), carbonic anhydrase (29 kDa), and trypsin inhibitor (17 kDa) were used as standards.

Gel Mobility Shift Assay—Gel mobility shift assay was performed as described previously (15). A DNA fragment containing the ars operator/promoter was produced by PCR. The purified PCR product was digested with EcoRI to produce a 153-base pair fragment. The DNA was labeled with [α-32P]dATP and the Klenow fragment of DNA polymerase 1 and purified using a Wizard DNA clean-up kit (Promega).

Expression of lacZ Reporter Genes—The lacZ reporter plasmid pGBDAR2 was constructed to monitor the regulatory properties of arsR genes. An EcoRI-HindIII fragment from plasmid pGBDAR1 (16) was filled in with the Klenow fragment of DNA polymerase and ligated into plasmid pACYC184 that had been digested with EcoRI and PvuII, generating plasmid pGBDAR2.

Overnight cultures of E. coli strain BL21 (DE3) harboring both pGBDAR2 and pET series plasmids were diluted 50-fold into 3 ml of fresh LB medium containing 80 μg/ml kanamycin and 15 μg/ml tetracycline. After 2 h of shaking at 37 °C, cells were induced with 50 μM sodium arsenite and grown for another 2 h. After centrifugation of 1 ml of cell culture, the pellet cells were suspended in 0.5 ml of Z buffer, followed by which the cells were permeabilized by adding 30 μl of 1% SDS and 50 μl of chloroform, with vortexing for 30 s. The reaction mixture contained 50 μl of cell extract, 0.1 ml of 8 mg/ml o-nitrophenyl-β-D-galactopyranoside, and 0.85 ml of Z buffer. β-Galactosidase activity was estimated from the release of nitrophenol at 420 nm at 37 °C and was expressed in Miller units (13).

RESULTS

Assay of ArsR Dimerization Using the Yeast Two-Hybrid System—Intersubunit interactions were investigated using the yeast two-hybrid protein-protein interaction assay. When yeast reporter strain SFY536 was cotransformed with pGBT9-R (arsR cloned in-frame and carboxyl-terminal to the S. cerevisiae GAL4 DNA-binding domain) and vector plasmid pGAD424 or with vector plasmid pGBT9 and pGAD424-R (arsR cloned in-frame and carboxyl-terminal to the GAL4 activation domain), the colonies were white. However, when the plasmids pGBT9-R and pGAD424-R were coexpressed, blue colonies expressing lacZ were observed (Fig. 1). These results demonstrate that dimerization of ArsR is detectable with the yeast two-hybrid system.

The products of deletions of arsR from the 5’ or 3’ ends were examined for their ability to interact with a wild type arsR gene product (Fig. 1). All of the deletions were fused to the 3’ end of the sequence for the GAL4 activation domain in the vector plasmid pGAD424. Plasmid pGAD424-RΔ90–117, with deletion of codons 90–117, and plasmid pGAD424-RΔ94–117, with deletion of codons 94–117, each produced blue colonies. However, deletion of an additional two codons (pGAD424-RΔ88–117) abolished β-galactosidase activity, as did further deletions to codons 85, 83, 80, or 61 (Fig. 1). Deletion from the 5’ end of the gene were analyzed. Deletion of codons 1–11 (pGAD424-RΔ1–11) or 1–40 (pGAD424-RΔ1–40) also prevented transcriptional activation of lacZ. In contrast, deletion of only the first eight codons (pGAD424-RΔ1–8) resulted in lacZ expression when cotransformed with pGBT9-R (Fig. 1). These results suggest that amino-terminal residues 1–8 and carboxyl-terminal residues 90–117 of ArsR are not required for dimerization.

Properties of Purified ArsR Proteins—The wild type arsR gene and six deletion mutants were cloned into plasmids pET28a or pET28b (Novagen). The resulting gene products, ArsRH6, RN9H6, RN41H6, and RN89H6, contained the His6 tag at their amino terminus, whereas RC80H6, RC85H6, and RC93H6 contained the His6 tag at their carboxyl terminus. The proteins were produced by expression of the genes in E. coli BL21 (DE3), and each protein was purified to greater than 90% homogeneity by chromatography on Ni-NTA columns. The yield of each protein was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

In Vivo Regulatory Properties of arsR Deletions—To investigate the relationship between dimerization and function of ArsR, the repressive and metalloregulatory activities of arsR and deletion mutants were assayed in vivo using lacZ gene expression as a reporter. Eight plasmids, pETRN41, pETRN9, pETRN12, pETRN41, pETRC80, pETRC85, pETRN89, and pETRC93, were individually cotransformed with plasmid pGBDAR2, in which transcription of the lacZ gene was under control of the up-stream arsR promoter, into E. coli strain BL21 (DE3). β-Galactosidase activity was measured in cells harboring both plasmids (Fig. 2). Expression of the gene for His6-tagged ArsR repressed lacZ expression in the absence of inducer and induced in the presence of sodium arsenite. The metalloregulatory activities of the genes encoding RN9H6, RN89H6, and RC98H6 were similar to that of the wild type, although basal lacZ expression was somewhat higher. In contrast, lacZ expression in cells with the deletion mutants encoding RN41H6, RC80H6, and RC85H6 was constitutive. Because RN41H6, RC80H6, and RC85H6 are monomers and RN9H6, RN89H6, and RC98H6 are dimers, these results suggest that dimerization is essential for the repressive and metalloregulatory activities of the arsR.

In Vitro DNA Binding Activity of ArsR Repressors—Gel mo-
ArsR Dimerization

**FIG. 2. In vivo regulation by arsR genes.** Cells of *E. coli* strain BL21 (DE3) bearing both plasmid pGBDΔR2, in which lacZ was under control of the *ars* promoter, and individual members of the pETR series were assayed for β-galactosidase activity as described under "Materials and Methods." Cells were uninduced (open bars) or induced with 50 μM sodium arsenite (solid bars).

**FIG. 3. ArsR protein-DNA interactions.** Mobility shift assays were performed as described under "Materials and Methods." The PCR product containing the *ars* promoter region digested with EcoRI was radiolabeled with [α-32P]dATP and incubated with 3 μg of purified His6-tagged ArsR or truncated proteins. The binding mixtures were analyzed on 6% polyacrylamide gels. All lanes contained probe DNA. Lane 1, no protein; lane 2, wild type ArsRH6; lane 3, RN9H6; lane 4, RN41H6; lane 5, RC80H6; lane 6, RC85H6; lane 7, RN89H6; lane 8, RC93H6. The positions of free DNA probe and the ArsR-DNA complex are indicated.

**FIG. 4. Affinity of wild type and truncated ArsRs for promoter DNA.** ArsRs at the indicated protein concentrations were incubated with 0.25 μM purified DNA probe, and the binding mixtures were analyzed on 6% polyacrylamide gels. The gels were dried, and the amount of free probe and protein-probe complex was quantified with an AMBIS radioactive analysis system. Proteins: □, wild type; ○, RN9H6; △, RN89H6; and ▲, RC93H6.

**FIG. 5. Effect of phenylarsine oxide on protein-DNA complex formation.** Mobility shift assays of the [α-32P]dATP-labeled DNA probe containing the *ars* promoter and 3 μg of purified wild type His6-tagged ArsR or truncated proteins RN9H6, RN89H6, and RC93H6. To induce 50 μM phenylarsine oxide (PAO) was added in the indicated lanes. The positions of free DNA probe and the ArsR-DNA complex are indicated.

for regulation? We have shown previously that when gene fusions were constructed between *arsR* and *blaM*, fusions encoding ArsR-β-lactamase chimerae retained metalloregulation when the fusion site was at *arsR* codons 92 or greater (2). In contrast, gene fusions to *arsR* codons 79 or less lost metalloregulation. However, the oligomeric state of the products of the gene fusions was not determined. In this study truncated ArsR proteins were produced with the objective of investigating the relationship between dimerization and function of the ArsR protein.

Three methods were used to analyze the results of truncations as follows: the yeast two-hybrid system, *in vivo* regulatory properties in *E. coli*, and biochemical analyses *in vitro*. The yeast two-hybrid system has been used to characterize protein-protein interactions (17). This assay was used to determine regions of ArsR that could be deleted without preventing homodimerization (Fig. 1). A full-length *arsR* gene was fused to the sequence for the GAL4 DNA-binding domain, and full-length and partial *arsR* sequences were fused to the GAL4 activation domain. Full-length ArsR and those with carboxy-terminal truncations retaining residues 1–93 or 1–89 were able to interact with a full-length ArsR, whereas the products of deletions that retained the sequences for residues 1–87, 1–85, 1–83, 1–81, and 1–61 did not interact with the full-length ArsR.

For amino-terminal truncation mutants, the product of deletion of codons for the first 8 amino acids of ArsR retained the ability to interact with a full-length ArsR. In contrast, removal of the first 11 or 40 residues of ArsR abolished interaction. Thus the dimerization domain of ArsR must be con-
and residues indicated. The boxed metalloregulatory proteins (3, 4). Members of this family have moter (Fig. 3). These data are all consistent with dimerization and RC85H6 proteins lost their ability to bind to the inducer (Fig. 5). In contrast, the monomeric RN41H6, RC80H6, promoter (Fig. 3) and dissociated from the DNA upon binding of dimerize (Fig. 2). Similarly the trans filtration as a dimer, retained the ability to bind to the inducer and derepress in the presence of inducer.

If dimerization is required for the metalloregulatory activity of ArsR, then there should be a correlation between the aggregation state of the protein and its ability to repress in the absence of inducer and derepress in the presence of inducer. The in vivo regulatory properties of the wild type araR and deletion mutants were examined using a lacZ reporter gene under control of the ara promoter. There was a clear correlation between the ability of an araR gene to regulate transcription of the reporter gene in trans and the ability of its gene product to dimerize (Fig. 2). Similarly the in vitro DNA binding properties of the truncated ArsRs correlated with their ability to dimerize. RN9H6, RN89H6, and RC93H6, each of which eluted from gel filtration as a dimer, retained the ability to bind to the ara promoter (Fig. 3) and dissociated from the DNA upon binding of inducer (Fig. 5). In contrast, the monomeric RN41H6, RC80H6, and RC85H6 proteins lost their ability to bind to the ara promoter (Fig. 3). These data are all consistent with dimerization being essential for ArsR function.

The chromosomal ArsR is a member of the ArsR family of metalloregulatory proteins (3, 4). Members of this family have variable lengths, with a core of similar residues and variable lengths on the amino and carboxyl termini. The smallest is ArsR of the archebacterium Methanococcus jannaschii, with only 89 residues (18). That homolog aligns with residues 2–90 of the E. coli ArsR, with all of the identities between ArsR residues 9 and 89 (Fig. 6). Similarly, the degree of similarity between the E. coli and plasmid R773 ArsRs, both of which consist of 117 residues, is 75% along its entire sequence but 86% within residues 9–89 (not shown). This is consistent with the results of the present study that demonstrate that a core sequence of approximately 80 residues is sufficient for all of the regulatory properties of the ArsR repressor: dimerization, DNA binding, and metal recognition.

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Fig. 6. Alignment of ArsR repressors from E. coli and M. jannaschii. Shown are the amino acid sequences of ArsR from E. coli (top) and M. jannaschii (bottom) (18), with identical (:) and conserved (. ) residues indicated. The boxed residues contain the core sequence of ArsR.