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# Complementation between Nucleotide Binding Domains in an Anion-Translocating ATPase

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**The catalytic component of the oxyanion-translocating ATPase of the plasmid-encoded *ars* operon of *Escherichia coli* is a homodimer of the ArsA protein. This enzyme is an oxyanion-stimulated ATPase with two consensus nucleotide binding sequences in each subunit, one in the N-terminal (A1) half and one in the C-terminal (A2) half of the ArsA protein. The two halves of both the *arsA* gene and the ArsA protein exhibit similar nucleotide and amino acid sequences, respectively. The two halves of the *arsA* gene were subcloned into compatible plasmids. Neither alone was sufficient to confer resistance, but cells in which the *arsA1* and *arsA2* half genes were coexpressed were resistant to arsenicals. Genetic complementation was also observed in cells bearing plasmids with point mutations in the two halves of the *arsA* gene and between cells with plasmids carrying combinations of the *arsA1* or *arsA2* subclones and point mutations. In every case, complementation was observed only when one plasmid contained a wild-type *arsA1* sequence and the other contained a wild-type *arsA2* sequence. These results demonstrate that both sites are required for resistance but that the two nucleotide binding domains need not reside in a single polypeptide. We propose a model in which the ArsA dimer has two catalytic units, each composed of an A1 domain from one monomer and an A2 domain from the other monomer.**

Resistance to arsenite, arsenate, and antimonite in bacteria results from the action of a plasmid-encoded ATP-driven oxyanion extrusion pump, resulting in lowering of the intracellular concentration of the toxic compounds (4, 17). The genes for this novel anion pump were originally cloned from the IncF1 plasmid R773 of *Escherichia coli* (15). This oxyanion pump is unrelated to the other known families of ion pumps, such as the $F_0F_1$ and the E1E2 types of ion-translocating ATPases (9). Only two gene products, the 63-kDa catalytic ArsA protein and the 45.5-kDa inner membrane ArsB protein, are required for arsenite and antimonite resistance (16). Purified ArsA protein has been shown to exhibit oxyanion-stimulated ATPase activity (19) and to bind ATP by UV-catalyzed adduct formation (19) as well as by the use of the fluorescent analog 2′,3′-*O*-(2,4,6-trinitrophenylcyclohexadienylidene)adenosine-5′-triphosphate (8). The active form of the ArsA ATPase is a homodimer of two ArsA polypeptides (5).

The nucleotide sequence of the *arsA* gene shows two regions that are similar to one another. Both halves of the *arsA* gene product contain a similar but not identical consensus nucleotide binding sequence (2) that corresponds to a portion of a nucleotide binding domain (22). Hence, the *arsA* gene has been proposed to have arisen by a gene duplication and fusion event (2). By independent mutagenesis of the two nucleotide binding folds, it was previously shown that both domains are required for function (7, 10). Mutations in the N-terminal or A1 domain resulted in complete loss of ATPase activity and loss of photoadduct formation with [$\alpha$-$^{32}$P]ATP (7). Mutations in the C-terminal or A2 domain also resulted in loss of ATPase activity, but the mutant ArsA proteins retained the ability to form a photoadduct with ATP (10). These studies indicate that the site of UV-catalyzed reaction with ATP is in the A1 nucleotide binding fold.

In this study we created N- and C-terminal deletion mutants of the *arsA* gene to elucidate the role of the nucleotide binding domains of the ArsA protein. Each deletion mutant contained either the A1 or A2 nucleotide binding fold; there was a region of overlap between the sequences of the subclones. Photoadduct formation with [$\alpha$-$^{32}$P]ATP and studies of genetic complementation between deletion mutants as well as between deletion mutants and point mutants in the two consensus nucleotide binding sequences showed that the ArsA protein has two independent functional domains. The A1 domain formed a UV-catalyzed adduct with [$\alpha$-$^{32}$P]ATP in the absence of the A2 domain. However, either the A1 or A2 domain by itself was not sufficient for resistance. Complementation was observed when the two deletion subclones were coexpressed in the same cell. Similarly, the A1 subclone complemented A1 point mutants and the A2 subclone complemented A2 point mutants. A1 and A2 point mutants also complemented each other. These results indicate that both domains in the ArsA protein are required for function, although they need not be on the same polypeptide. Genetic complementation between the two domains may be facilitated by the dimeric nature of the ArsA protein, whereby the A1 nucleotide binding site on one subunit interacts with the A2 nucleotide binding site on the other.

## MATERIALS AND METHODS

**Strains, plasmids, and phage.** The *E. coli* strains, plasmids, and phage used in this study are shown in Table 1.

**Media and growth conditions.** Cells were grown in LB medium (14) at 37°C. Antibiotics were added to 50 μg/ml unless otherwise noted. Sodium arsenite was added at the indicated concentrations to test for arsenical resistance.

**DNA manipulations.** The conditions used for plasmid isolation, DNA restriction endonuclease analysis, ligation, transformation, and sequencing were as described previously (20).

**Cloning of the A1 and A2 domains of the *arsA* gene.** The 5′

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TABLE 1. Strains, plasmids, and phage

| Strain, plasmid, or phage | Genotype or description | Reference or source |
|---|---|---|
| Strain JM109 | *recA1 thi hsdR17 supE44* (*lac-proAB*) (F' *traD36 proAB lacI*$^q$ Δ*lacZM15*) | Yanisch-Perron et al. (25) |
| Plasmids | | |
| pUM3 | *arsABC* genes of R773 cloned into the *Hin*dIII site of pBR322 (*arsABC*)[a] | Mobley et al. (15) |
| pUC18 | Cloning vector, Ap$^r$ | Yanisch-Perron et al. (25) |
| pSU2718 | Cloning vector, pACYC184 derivative, Kn$^r$ | Martinez et al. (13) |
| pPK56 | 3.2-kb *Eco*RI-*Hin*dIII fragment of mCMC49-1d1-56 cloned into the *Eco*RI-*Hin*dIII site of pUC18 (*arsAB*) | Kaur and Rosen (10) |
| pGR337 | Site-specific GR337 mutation in mCMC49-1d1-56 cloned into the *Eco*RI-*Hin*dIII site of pUC18 ($arsA_{GR337}B$) | Kaur and Rosen (10) |
| pKE340 | Site-specific KE340 mutation in mCMC49-1d1-56 cloned into the *Eco*RI-*Hin*dIII site of pUC18 ($arsA_{KE340}B$) | Kaur and Rosen (10) |
| pCMC1065 | GS20 mutation cloned into pACYC184 ($arsA_{GS20}BC$) | Karkaria et al. (7) |
| pCMC1079 | GD18 mutation cloned into pACYC184 ($arsA_{GD18}BC$) | Karkaria et al. (7) |
| pArsA1 | 1.1-kb *Eco*RI-*Hin*dIII fragment of mCMC49-3d1-22 cloned into the *Eco*RI-*Hin*dIII site of pUC18 (*arsA1*) | This study |
| pArsA2 | 3.66-kb fragment from pUM3 containing 1.25-kb C-terminal *arsA* and *arsBC* genes cloned into pUC18 (*arsA2BC*) | This study |
| pSU2718A1 | 1.1-kb *Eco*RI-*Hin*dIII fragment from pArsA1 cloned into *Eco*RI-*Hin*dIII site of pSU2718 (*arsA1*) | This study |
| pSU2718A2 | 3.66-kb fragment from pArsA2 cloned as a *Eco*RI-*Hin*dIII fragment into pSU2718 (*arsA2BC*) | This study |
| Phage | | |
| M13 phage mCMC49 | *arsABC* | Chen et al. (2) |
| mCMC49-3d1-22 | 1.1-kb 5' portion of *arsA* gene, *Bal*31 deletion of mCMC49 (*arsA1*) | This laboratory |

[a] The relevant *ars* genes present on the plasmids are given within parentheses.

1.1 kb of the *arsA* gene, encoding the first 323 residues of the N-terminal or A1 portion of the ArsA protein, was subcloned from M13 phage derivative mCMC49-3d1-22 as an *Eco*RI-*Hin*dIII fragment behind the *lac* promoter of pUC18, generating plasmid pArsA1. The portion of the gene for the A1 half of the ArsA protein is termed the *arsA1* gene.

To generate a plasmid encoding the C-terminal or A2 portion of the ArsA protein, plasmid pUM3 (15) was digested with *Ava*I. The ends were filled in with the Klenow fragment of DNA polymerase, and the DNA was further digested with *Hin*dIII. The 3.66-kb fragment was purified from a low-melting-point agarose gel and ligated into plasmid pUC18 that had been digested with *Sal*I, filled in with the Klenow fragment of DNA polymerase, and digested with *Hin*dIII. The resulting plasmid, encoding the last 417 residues of the ArsA protein, was designated pArsA2. The portion of the *arsA* gene encoding the A2 half of the ArsA protein (termed the *arsA2* gene) in plasmid pArsA2 was expressed under control of the *lac* promoter and utilized the translational start signals from the *lacZ* gene. This translational fusion carries 17 residues from β-galactosidase at its N terminus. The plasmid pArsA2 also contains the wild-type *arsB* and *arsC* genes.

For complementation studies, both the *arsA1* and *arsA2* genes were cloned as *Eco*RI-*Hin*dIII fragments under control of the *lac* promoter in the colE1-compatible pACYC184 derivative pSU2718 (12) and were designated pSU2718A1 and pSU2718A2, respectively. The ArsA protein was purified as described previously (6, 10).

**Photoadduct formation of the A1 or A2 half of the ArsA protein and [α-$^{32}$P]ATP.** Photolabeling was performed with either purified wild-type ArsA protein (6, 10) or cytosol from the cells expressing the *arsA1* or *arsA2* gene. Cells of *E. coli* carrying the indicated plasmids were grown in LB medium (14) to an optical density at 600 nm of 0.6 to 0.8 and induced with 0.25 mM isopropyl β-D-thiogalactopyranoside (IPTG). After 2 h at 37°C, the cells were harvested and lysed by a single passage through a French pressure cell at 20,000 lb/in$^2$. The lysate was centrifuged at 10,000 × *g* for 20 min to remove unbroken cells, and the supernatant solution was used directly for formation of photoadducts. Cytosol (25 to 50 μg of protein) was mixed with 5 μM ATP, 5 mM $MgCl_2$, and 10 μCi of [α-$^{32}$P]ATP in a total volume of 0.1 ml in a 96-well microtitration plate. The samples were exposed to UV light at 254 nm from a lamp placed directly on top of the microtitration plate for 30 min at 4°C (19). The protein was precipitated by adding 0.4 ml of 10% trichloroacetic acid. The precipitated protein was recovered by centrifugation and washed three times with 10% trichloroacetic acid. The pelleted material was suspended in sodium dodecyl sulfate (SDS) sample buffer and analyzed by polyacrylamide gel electrophoresis (PAGE) on a 12% polyacrylamide gel (11).

**Genetic complementation.** Cultures of *E. coli* JM109 bearing plasmids carrying the *arsA1* or *arsA2* gene or one of a variety of mutations in the *arsA* gene, alone or in combination, were grown in LB medium for 12 h at 37°C. Each culture was diluted 100-fold into 2 ml of LB medium containing 0, 3, 5, 7, or 9 mM sodium arsenite and 0.1 mM IPTG and incubated at 37°C with aeration. The turbidity of the cultures at 600 nm was measured after 12 h. A portion of each culture (0.5 ml) was centrifuged, and the pellets were suspended in 0.1 ml of SDS sample buffer and boiled for 10 min. Portions (10 μl) were analyzed by SDS-PAGE on 12% polyacrylamide gels and immunoblotted with anti-ArsA antiserum.

Complementation was also examined on solid LB agar medium containing 0.1 mM IPTG and various concentrations of sodium arsenite. Each strain was streaked to give

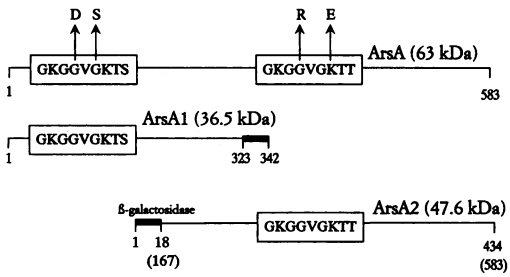


FIG. 1. Diagrammatic representation of the ArsA, ArsA1, and ArsA2 proteins. The ArsA protein has 583 residues and two consensus sequences for nucleotide binding sites (boxed sequences). Within those two sequences are shown mutations GD18, GS20, GR337, and KE340 (arrows). The nucleotide sequence for the first 323 residues was cloned to produce plasmid pArsA1, which encodes the 342-residue ArsA1 protein containing 323 residues of the ArsA protein. Because the message does not terminate for an additional nine codons, the ArsA1 polypeptide contains nine additional residues. The nucleotide sequence for ArsA residues 167 through 583 was ligated to the sequence for the first 17 residues of the *lacZ* gene, generating plasmid pArsA2, which encodes the 434-residue ArsA2 protein.

isolated single colonies. The plates were incubated at 37°C for 18 to 24 h and visually scored for growth.

**Materials.** All restriction enzymes and nucleic acid-modifying enzymes were obtained from Bethesda Research Laboratories. All other chemicals were obtained from commercial sources.

## RESULTS

**Creation of deletion derivatives of ArsA protein.** To elucidate the roles of the two nucleotide binding domains of the ArsA protein and to study the interactions between them, deletion clones of the *arsA* gene were created. In addition a number of previously isolated mutants with point mutations in either the A1 or A2 nucleotide binding folds (7, 10) were utilized (Table 1).

The subclones of the *arsA* gene encoded either the N-terminal (A1) or the C-terminal (A2) nucleotide binding fold, with an overlap between the two (Fig. 1). Plasmid pArsA1, carrying the *arsA1* gene, encoded a polypeptide with a predicted mass of 36,544 Da containing residues 1 through 323 of the ArsA protein, including the A1 consensus sequence G-15KGGVGKTS-23, and 19 residues at the C terminus from the vector sequence. Plasmid pArsA2, carrying the *arsA2* gene, encoded a polypeptide with a predicted mass of 47,571 Da containing residues 1 through 17 of β-galactosidase fused to residues 167 through 583 of the ArsA protein, including the A2 consensus sequence G-334KGGVGKTT-342. In these constructs expression of both the A1 and A2 polypeptides is under the control of *lac* promoter.

Neither the *arsA1* nor the *arsA2* portion of the *arsA* gene alone conferred high-level arsenite resistance on cells, whereas cells expressing a wild-type *arsA* gene with the *arsB* gene were resistant to at least 10 mM sodium arsenite (Fig. 2A and B). In this assay medium, resistant cells were still capable of growth at 15 mM sodium arsenite (data not shown). These results indicate that neither half of the ArsA protein alone is sufficient for function. Both the A1 and A2 peptides were produced, as determined by immunoblotting with anti-ArsA antiserum (Fig. 3). When subjected to SDS-PAGE, each exhibited approximately the size predicted from the nucleotide sequence; 36.5 kDa for the A1 polypeptide and 47.6 kDa for the A2 polypeptide.

**Direct photolabeling of ArsA and deletion peptides in cytosol.** Binding of ATP to the isolated A1 and A2 polypeptides was examined by photoadduct formation with [α-$^{32}$P]ATP. The cytosol fractions from cells expressing the two polypeptides individually were irradiated with UV in the presence of [α-$^{32}$P]ATP with or without $Mg^{2+}$. The samples were separated by SDS-PAGE, transferred to nitrocellulose, and subjected to immunodetection with anti-ArsA serum. The nitrocellulose was autoradiographed. Comparison of the autoradiograph and immunoblot allowed unambiguous assignment of the positions of the 36.5-kDa ArsA1 and 47.6-kDa ArsA2 polypeptides. Note that photolysis of the ArsA protein occurs during UV irradiation, producing smaller immunoreactive species (Fig. 4 and 5). The A1 polypeptide

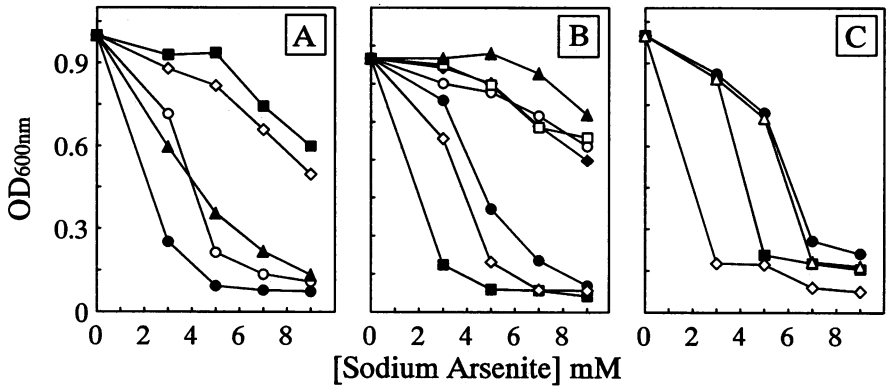


FIG. 2. Arsenite resistance in cells bearing combinations of *arsA* mutants and partial *arsA* clones. After 12 h of growth in LB medium at 37°C, cells of *E. coli* JM109 bearing the indicated plasmids were diluted 100-fold into fresh medium containing 0, 3, 5, 7, or 9 mM sodium arsenite. Expression of the *ars* genes was induced by adding 0.1 mM IPTG. The optical density at 600 nm ($OD_{600}$) was measured after 12 h of growth at 37°C with aeration. The cells had the following plasmids. (A) ●, pUC18 (vector); ○, pCMC1065 ($arsA_{GS20}BC$); ▲, pKE340 ($arsA_{KE340}B$); ■, pPK56 (wild type, *arsAB*); ◇, pCMC1065 ($arsA_{GS20}BC$) and pKE340 ($arsA_{KE340}B$). (B) ■, pArsA1 (*arsA1*); ◇, pCMC1079 ($arsA_{GD18}BC$); ●, pSU2718A2 (*arsA2BC*); ◆, pArsA1 (*arsA1*) and pCMC1065 ($arsA_{GS20}BC$); ○, pSU2718A2 (*arsA2BC*) and pKE340 ($arsA_{KE340}B$); □, pArsA1 (*arsA1*) and pSU2718A2 (*arsA2BC*); ▲, pArsA1 (*arsA1*) and pCMC1079 ($arsA_{GD18}BC$). (C) ◇, pSU2718A1 (*arsA1*); ■, pSU2718A1 (*arsA1*) and pKE340 ($arsA_{KE340}B$); △, pArsA2 (*arsA2BC*) and pCM1065 ($arsA_{GS20}BC$); ●, pArsA2 (*arsA2BC*).

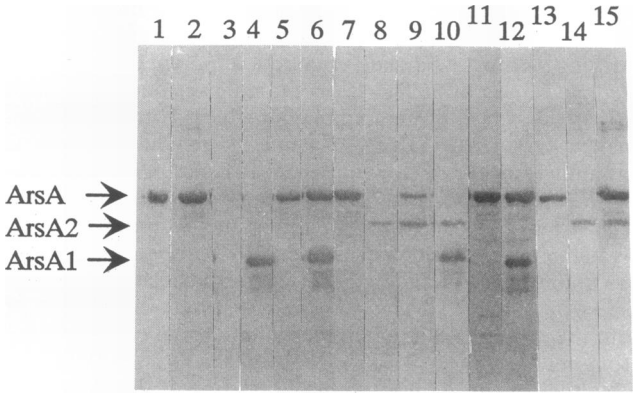


FIG. 3. Analysis of coexpressed gene products. Cultures of *E. coli* JM109 bearing the indicated plasmids were induced with 0.1 mM IPTG. Cell suspensions (10 μl) were boiled in SDS sample buffer, and cellular protein was separated by SDS-PAGE on 12% polyacrylamide gels. Immunoblotting was performed with antiserum prepared against the ArsA protein. Lanes: 1, purified wild-type ArsA protein (5 μg); 2, pPK56 (wild-type arsA); 3, pUC18 (vector); 4, pArsA1 (ArsA1); 5, pCMC1065 ($ArsA_{GS20}$); 6, pCMC1065 ($ArsA_{GS20}$) and pArsA1 (ArsA1); 7, pKE340 ($ArsA_{KE340}$); 8, pSU2718A2 (ArsA2); 9, pKE340 ($ArsA_{KE340}$) and pSU2718A2 (ArsA2); 10, pArsA1 (ArsA1) and pSU2718A2 (ArsA2); 11, pCMC1079 ($ArsA_{GD18}$); 12, pCMC1079 ($ArsA_{GD18}$) and pArsA1 (ArsA1); 13, pGR337 ($ArsA_{GR337}$); 14, pSU2718A2 (ArsA2); 15, pGR337 ($ArsA_{GR337}$) and pSU2718A2 (ArsA2). Arrows indicate the migration positions of the 63-kDa ArsA, 36.5-kDa ArsA1, and 47.6-kDa ArsA2 polypeptides, as determined from known standards.

formed a photoadduct in a $Mg^{2+}$-dependent manner to the same extent as the wild-type protein did (Fig. 4). In contrast, no photoadduct formation was observed with cytosol containing the A2 polypeptide (Fig. 5). A protein slightly larger than the ArsA2 present in the cytosol preparations photolabeled in the absence of $Mg^{2+}$ (Fig. 4, lane 5; Fig. 5, lane 8) but not in its presence (Fig. 4, lane 6; Fig. 5, lane 7). This band is also present in cytosol from cells without any *ars* genes and is unrelated to the ArsA2 polypeptide (data not shown).

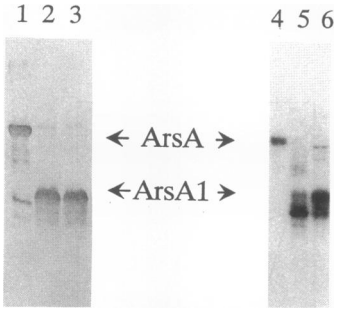


FIG. 4. Photoadduct formation of the ArsA and ArsA1 polypeptides with [α-$^{32}$P]ATP. UV-catalyzed adduct formation between purified wild-type ArsA (5 μM, final concentration) or N-terminal 36.5-kDa deletion peptide (25 μg of cytosolic protein) and [α-$^{32}$P]ATP was performed as described in Materials and Methods. Proteins were separated on SDS–12% PAGE gels. The gels were transferred electrophoretically to nitrocellulose and immunoblotted with antiserum against the ArsA protein (lanes 1 through 3). The immunoblot was autoradiographed (lanes 4 through 6). Lanes: 1 and 4, purified wild-type ArsA protein (3 μg); 2, 3, 5, and 6, 25 μg of cytosol containing the ArsA1 polypeptide. The reactions were performed with (lanes 1, 3, 4, and 6) or without (lanes 2 and 5) 5 mM $MgCl_2$. Arrows indicate the migration positions of the 63-kDa ArsA and the 36.5-kDa ArsA1 polypeptides, as determined from known standards.

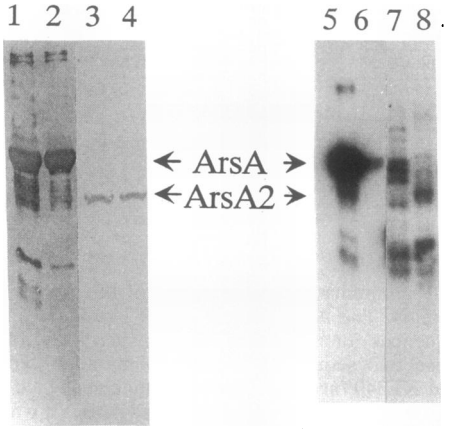


FIG. 5. Photoadduct formation of the ArsA and ArsA2 polypeptides with [α-$^{32}$P]ATP. UV-catalyzed adduct formation was carried out as described in Materials and Methods. Samples were electrophoresed on SDS–12% PAGE gels and then immunoblotted with antiserum against the ArsA protein (lanes 1 through 4) and autoradiographed (lanes 5 through 8). Lanes: 1, 2, 5, and 6, purified wild-type ArsA (10 μg); 3, 4, 7, and 8, 50 μg of cytosol containing the ArsA2 polypeptide. The reactions were performed with (lanes 1, 3, 5, and 7) and without (lanes 2, 4, 6, and 8) 5 mM $MgCl_2$. Arrows indicate the migration positions of the 63-kDa ArsA and the 47.6-kDa ArsA2 polypeptides, as determined from known standards.

These data are in agreement with the results with intact ArsA proteins that had mutations in either the A1 or A2 nucleotide binding consensus sequences, where mutations in the A1 sequence eliminated photoadduct formation but mutations in the A2 did not (7, 10). When cytosols containing the A1 and A2 polypeptides were mixed before labeling with [α-$^{32}$P]ATP, only the A1 polypeptide formed a photoadduct (data not shown); these results are identical to those with the A1 polypeptide alone (Fig. 4, lanes 5 and 6).

**Genetic complementation.** Arsenical resistance requires two intact nucleotide binding consensus sequences in the ArsA protein (7, 10). Since purified ArsA protein has been shown to form a functional homodimer in vitro (5), the possibility that the presence of only one of each type of nucleotide binding domain in each monomer would allow for resistance was explored.

Various combinations of point mutants and partial clones were coexpressed in the same cells on compatible plasmids (Fig. 2). The *recA* mutant strain *E. coli* JM109 was used to prevent recombination. Complementation between subunits or subclones was assayed by resistance to various concentrations of sodium arsenite in liquid LB medium. Qualitatively similar results were obtained when complementation assays were done on solid LB agar medium. In each case a wild-type *arsB* gene was present on one of the plasmids.

Complementation was observed when cells expressing plasmids with point mutations in the A1 consensus sequence (G-15KGGVGKTS-23) coexpressed a plasmid with a point mutation in A2 consensus sequence (G-334KGGVGKTT-343). Cells expressing either plasmid pCMC1065, which has the mutation GS20 in the A1 sequence, or plasmid pKE340, which has the KE340 mutation in the A2 sequence, were

only slightly more resistant than cells with vector alone to low concentrations of arsenite (Fig. 2A). At higher arsenite concentrations, cells with either mutant plasmid were fully inhibited and indistinguishable from those with vector alone. When the two plasmids were expressed in the same cell, the cells were as resistant to arsenite as were cells with plasmid pPK56, which has a wild-type *ars* operon (Fig. 2A). Complementation between a plasmid with the mutation TI22 (7) in the A1 sequence and the mutation KE340 in the A2 sequence was also observed (data not shown).

Similarly, plasmid pArsA1 (expressing the 36.5-kDa A1 polypeptide) complemented plasmids pCMC1065 and pCMC1079 (with GS20 and GD18 mutations, respectively) in the A1 site (Fig. 2B). For unexplained reasons, plasmid pCMC1079 (GD18) did not complement plasmid pKE340 (KE340) (data not shown). Plasmid pSU2718A2, expressing the 47.6-kDa A2 polypeptide, complemented plasmid pKE340, which has the KE340 point mutation in the A2 nucleotide binding domain (Fig. 2B). Coexpression of both the *arsA1* gene in plasmid pArsA1 and the *arsA2* gene in plasmid pSU2718A2 also restored high-level arsenite resistance (Fig. 2B).

As expected, subclones did not complement point mutations in the other nucleotide binding domain. Cells coexpressing plasmid pSU2718A1, which expresses the *arsA1* gene, and plasmid pKE340, which has a wild-type A1 domain and a mutation in the A2 domain, were no more resistant to arsenite than were cells with pKE340 alone (Fig. 2C). Similarly, cells coexpressing plasmid pArsA2, which encodes an A2 domain, and plasmid pCMC1056, which encodes a wild-type A2 domain and a mutated A1 domain, were as sensitive to arsenite as were cells with pArsA2 alone (Fig. 2C). Note that constructs with the *arsA2* gene appear to confer some resistance (Fig. 2B and C). This is actually the result of expression of the *arsB* gene. In the absence of a bound ArsA protein, the ArsB protein alone confers partial resistance to and transport of arsenite (3a). We hypothesize that the ArsB protein alone functions as a secondary oxyanion porter (18). Plasmid pGR337, expressing the ArsA2 point mutation GR337, was not complemented by any other plasmid. This lack of complementation may result from improper interactions between the mutant GR337 protein and other ArsA proteins.

**Immunoblot analysis of coexpression of the proteins.** To demonstrate that the compatible plasmids were expressing the expected Ars1, ArsA2, and/or mutant ArsA polypeptides, immunoblot analysis was performed with anti-ArsA serum. Discrete bands corresponding to the full-length mutant ArsA proteins and to the two truncated ArsA1 and ArsA2 peptides were observed (Fig. 3).

## DISCUSSION

The nucleotide sequence of the *arsA* gene shows that the N-terminal and C-terminal halves of the ArsA protein are similar to one another, indicating that the *arsA* gene arose through a gene duplication and fusion event (2). Both halves of the ArsA protein contain similar but not identical consensus nucleotide binding sequences (23). This consensus sequence, GXXGXGKT(S), found in many nucleotide binding proteins, forms part of a nucleotide binding domain, as shown by the X-ray crystal structure determination of adenylate kinase (22). Mutagenesis of each nucleotide binding sequence in the ArsA protein separately has shown that both the A1 and A2 nucleotide binding consensus sequences contribute independently to the catalytic activity of this protein (7, 10). Mutations in either fold resulted in loss of ATPase activity. An important difference between the two sets of mutants was in their abilities to form a photoadduct with [$\alpha$-$^{32}$P]ATP. Mutants with point mutations in the A1 site completely lost the ability to form the photoadduct, whereas all mutants with mutations in the A2 site retained this ability, indicating that the A1 site is the site of photoadduct formation. The results of the present study show that the A1 portion of the ArsA protein contains an independent nucleotide binding domain. The N-terminal 36.5-kDa polypeptide encoded by the *arsA1* subclone of the *arsA* gene, like the wild-type protein, retained the ability to form a $Mg^{2+}$-dependent photoadduct with [$\alpha$-$^{32}$P]ATP. Expression of the ArsA1 polypeptide by itself was not sufficient to confer arsenite resistance in cells containing a wild-type *arsB* gene. Hence it appears that the A2 nucleotide binding domain, although required for the activity of the ArsA protein, is not required for $Mg^{2+}$-ATP binding at the A1 site. The 47.6-kDa A2 polypeptide did not exhibit photoadduct formation with $Mg^{2+}$-ATP, consistent with the results obtained with point mutants in A1 site (7). The ArsA protein has been shown to bind 2 mol of nucleotide per mol of protein (8). Hence, either the A2 nucleotide binding domain is a lower-affinity ATP binding site or the amino acid residues in the vicinity of that site are not capable of adduct formation in the UV-catalyzed reaction.

Domain analysis has been used to demonstrate interactions of multiple sites within single polypeptides. Results similar to those described in this report have been obtained with the SecA protein, the catalytic subunit of the protein translocation ATPase of *E. coli* (13). When polypeptides containing two fragments of the SecA protein with overlapping N- and C-terminal halves were mixed in vitro, the N-terminal polypeptide, which contains a nucleotide binding consensus sequence, formed a photoadduct with [$\alpha$-$^{32}$P]ATP. Since the SecA protein has been proposed to function as a dimer, dimerization may stabilize the association of the interacting polypeptides.

Similarly, in vivo formation of heterodimeric mercuric reductase proteins between point mutants has been reported (3). In that study a two-plasmid system in which two mutant genes were placed on separate compatible plasmids was utilized. Coexpression led to mercury-resistant phenotypes that were distinguishable from the phenotype of either mutant alone. Reconstitution of an active lactose carrier in vivo by simultaneous expression of two *lacY* DNA segments has also been shown (1, 24).

Previous studies have demonstrated that the ArsA protein functions as a dimer in solution and presumably also in the membrane-bound complex (5). This conclusion is supported by the complementation experiments. Since the A1 and A2 halves of the ArsA protein appear to contain independent nucleotide binding domains, it was of interest to determine whether coexpression of point mutations in the A1 or A2 consensus sequences would lead to restoration of function and hence arsenite resistance. Various combinations of plasmids were coexpressed such that, out of four potential nucleotide binding domains in a dimer (two A1 sites and two A2 sites), the cells contained at least one wild-type A1 sequence and one-wild type A2 sequence. This was achieved by using plasmids encoding (i) two full-length mutated *arsA* genes, (ii) one full-length *arsA* gene and one half-length *arsA1* or *arsA2* gene, or (iii) two half-length *arsA* genes. These combinations are represented diagramatically in Fig. 6. Importantly, point mutants in two halves of the ArsA protein complement each other, and their coexpression

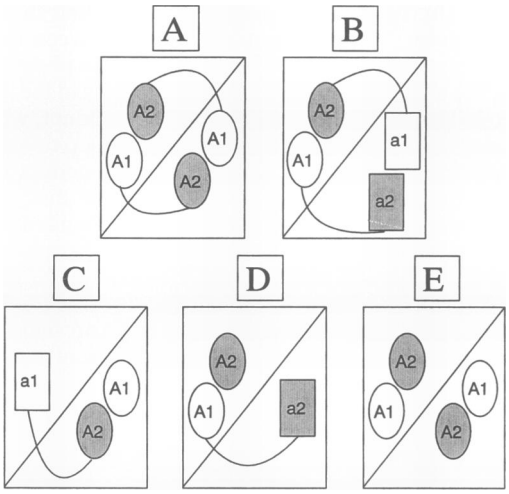


FIG. 6. Model of the interaction of the A1 and A2 nucleotide binding domains in an ArsA dimer. The ArsA protein is represented with two domains connected by a linker peptide. The wild-type domains are indicated as A1 and A2 in ellipses, and the mutant domains are indicated as a1 and a2 in rectangles. The combinations of domains that would be postulated from the complementation data to form active dimers are shown. In each dimer there are two potential catalytic units, each composed of an A1 domain and an A2 domain contributed by different subunits. The enzyme has ATPase activity with only one complete catalytic unit. Dimers: A, wild-type ArsA dimer; B, an ArsA1 point mutant (such as GS20) and an ArsA2 point mutant (such as KE340); C, an ArsA1 polypeptide and an ArsA1 point mutant; D, an ArsA2 polypeptide and an ArsA2 point mutant; E, ArsA1 polypeptides and ArsA2 polypeptides.

results in a high level of arsenite resistance. Coexpression of two truncated proteins was also sufficient for conferring arsenite resistance. Further, the results of photoadduct formation with [$\alpha$-$^{32}$P]ATP demonstrate that the 36.5-kDa A1 peptide by itself retains nucleotide binding and, in combination with the A2 peptide, forms a functional complex in the cell. Hence the A1 and A2 domains contribute independently to the function of the ArsA protein.

We propose a model in which only two of the four potential nucleotide binding domains in a dimer, one from the A1 half and one from the A2 half, are sufficient to form a catalytic unit (Fig. 6D through G). The results indicate that dimerization of ArsA protein facilitates complementation between the A1 and A2 domains. This is reminiscent of the interaction of the $\alpha$ and $\beta$ subunits of the $F_1$ ATPase, where ATPase activity requires positive interactions of the two (21). ATP binding to the $\alpha$ subunit produces a conformational change that is transmitted to the $\beta$ subunit as a positively cooperative effect on ATP hydrolysis or synthesis.

The isolation and purification of the ArsA1 and ArsA2 polypeptides are in progress. Biochemical reconstitution of a functional oxyanion-stimulated ATPase by mixing of the two polypeptides would demonstrate their independent contributions to a common function.

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