

**AN ARSENIC METALLOCHAPERONE FOR AN ARSENIC DETOXIFICATION
PUMP**

by

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DEDICATION

to

my wife, Hui-Wen Hsu

and

my mother, Nan Chan

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“I thank my God in all my remembrance of you.”— Phillipians 1:3

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CHAPTER 1

INTRODUCTION

1.1 Toxicity of Metalloids- Arsenic and Antimony

Arsenic (As) and antimony (Sb) are highly toxic metalloids that occur naturally in a number of minerals. They are also released into the environment by industrial and agricultural activities (Finkelman, 1999). Many incidents of arsenic and antimony contamination in the environment and cases of exposure have been reported in various countries.

1.1.1 Ubiquitous toxicants

Arsenic is a metalloid toxicant found in water, soil, and air from natural and anthropogenic sources. It is widely distributed throughout the earth's crust (Hindmarsh and McCurdy, 1986; Hughes, 2002). Anthropomorphic sources include arsenical-containing fungicides, pesticides and herbicides. Humans are exposed to arsenic mainly through either oral or inhalation routes. Oral exposure occurs via consumption of contaminated water, food, and drugs. Occupational exposure occurs mainly through inhalation via nonferrous ore smelting, semiconductor and glass manufacturing, or power generation by the burning of arsenic-contaminated coal (Yager and Wiencke, 1993).

Arsenic contamination of drinking water is a serious environmental problem worldwide because of the large number of contaminated sites that have been identified and the large number of people at risk (Chappell *et al.*, 1997). Acute and chronic arsenic exposure via drinking water has been reported in many

countries of the world including Taiwan, Mexico, Bangladesh, Inner Mongolia, Argentina, India, Thailand, as well as western South America. Arsenic occurs at high levels naturally, ranging from several hundreds to well over a thousand $\mu\text{g/L}$ (Brown and Ross, 2002; Tchounwou et al., 1999). Drinking water contamination by arsenic remains a major public health problem. In the United States, setting the maximum contaminant level (MCL) that regulates the concentration of arsenic in public water supplies has been an extraordinarily protracted process. Eventually in 2001 the US Environmental Protection Agency (EPA) lowered the MCL to 10 $\mu\text{g/L}$ from the standard of 50 $\mu\text{g/L}$ that had been established in 1942 (Smith *et al.*, 2002). The World Health Organization (WHO) standard is also 10 $\mu\text{g/L}$. If this is enforced, where in the world will water be drinkable?

Antimony, which is similar to arsenic, has a long history of usage for medical purposes. Arsenic- and antimony-containing drugs are today almost exclusively used in the treatment of acute promyelocytic leukemia (APL) and protozoan infections such as leishmaniasis, respectively (Barrett and Fairlamb, 1999; Borst and Ouellette, 1995; Soignet et al., 1998). Yet, both elements possess common toxicological properties.

1.1.2 Cause of diseases

Many different systems within the human body are affected by exposure to these metalloids. Arsenic may cause severe acute toxicity including gastrointestinal discomfort, vomiting, diarrhea, bloody urine, anuria, shock, convulsions, coma, and death (Hughes, 2002). It also causes chronic toxicity in humans. One of the hallmarks is from oral exposure to cause skin lesions, which have been characterized by hyperpigmentation, hyperkeratosis, and

hypopigmentation (Cebrian et al., 1983; Yeh et al., 1968). In Taiwan, blackfoot disease, a vasoocclusive disease that leads to gangrene of the extremities, is also observed in individuals exposed to arsenic in their drinking water (Tseng, 1977). Population-based epidemiological studies and clinical reports have shown the association of arsenic exposure with diseases of the peripheral vascular, cardiovascular and cerebrovascular systems (Chen et al., 1996; Chiou et al., 1997; Tseng et al., 1995), hypertension (Chen et al., 1995), and injury to the peripheral and central nervous systems (Bencko et al., 1977)). Arsenic ingestion is also associated with an increased incidence of other human diseases, such as atherosclerosis, diabetes, and cancers (Chen et al., 1995; Chiou et al., 1995).

Arsenic was one of the first chemicals recognized as a cause of cancer. Inorganic arsenic was classified by the International Agency for Research on Cancer (1987; Higginson and DeVita, 1980) and the US Environmental Protection Agency (EPA, 1988) as a known human carcinogen. As early as 1879, the high rates of lung cancer in miners were attributed in part to inhaled arsenic (Smith et al., 2002). A few years later, skin cancers were reported in patients treated with medicine containing arsenic. In 1930s and subsequent years, skin cancer has also been found in individuals exposed to arsenic through naturally contaminated drinking water (Cebrian et al., 1983; Tseng et al., 1968). Evidence that arsenic in drinking water could cause internal cancers came around 1960s to 1990s, showing tumor sites in lung, urinary tract, bladder, liver, and kidney (Biagini, 1966; Chen et al., 1985; Smith et al., 1992). Long-term occupational exposure has also been associated with increased prevalence of cancer of the

buccal cavity, pharynx, bone, large intestine, and rectum (Enterline *et al.*, 1995). The aggregate of worldwide data was sufficient to conclude that ingested inorganic arsenic was likely to cause several systemic diseases, as well as internal cancers (Bates *et al.*, 1992).

On the other hand, little work has been done on the toxicology of antimony. It also has been shown that trivalent antimony causes lung cancer in rats (Groth *et al.*, 1986). In the classification of International Agency for Research on Cancer (IARC), antimony trioxide (Sb_2O_3) is a possible human carcinogen (IARC 1989).

1.1.3 *Metalloids in medicine*

In spite of being toxicants, arsenic compounds have been used as medicinal agents for many centuries. In traditional Chinese medicine, arsenous acid ($\text{As}(\text{OH})_3$) or arsenic trioxide (As_2O_3) was used as a devitalizing agent prior to teeth fillings as well as treatment of other diseases such as psoriasis, syphilis, and rheumatosis (Shen *et al.*, 1997). In the 18th century, Dr. Thomas Fowler developed a therapeutic agent known as Fowlers solution by combining arsenic trioxide with potassium bicarbonate (Gallagher, 1998). Over the following hundred years, Fowlers solution was used to treat various diseases including malignant disease such as leukemia, Hodgkin's disease, and pernicious anemia as well as non-malignant diseases such as eczema, asthma, pemphigus, and psoriasis (Evens *et al.*, 2004). Arsenic was used as one of the standard treatments for chronic myeloid leukemia (CML) and other leukemias until the use of modern chemotherapy and radiation therapy introduced in the mid 1900's.

Recent scientific investigation and understanding of the various mechanisms of action of arsenic have led to renewed appreciation of arsenic as an effective anti-cancer agent. Contemporary studies have shown that arsenic trioxide (ATO) is an effective therapeutic agent for the treatment of acute promyelocytic leukemia (APL) (Niu *et al.*, 1999). The activity of ATO is also being investigated in other types of hemotologic malignancies. Preliminary results have been reported on various diseases such as multiple myeloma (MM), acute lymphoblastic leukemia (ALL) and myelodysplastic syndrome (MDS) (Verstovsek and Estrov, 2004). However systemic toxicity of ATO recorded in most patients under therapy is a problem that cannot be disregarded. To solve this problem, scientists are developing alternatives. For example, tetra-arsenic tetra-sulfide has shown to have an impressive response rate in patients (Lu *et al.*, 2002). There are also organic arsenic derivatives under development. Organic phenylarsenic acid (PAA) compounds with potent in vitro activity against human acute lymphoblastic leukemia cells showed 50% inhibition of cell growth (Liu *et al.*, 2003). S-dimethylarsino-glutathione has been identified as a lead compound among more than 100 derivatives and is currently being developed for clinical use. Arsenic compounds are being approved to be good therapeutic agents for at least leukemias.

The pentavalent antimonial drugs Pentostam and Glucantime are the first line treatment for leishmaniasis. High-dose long-term regimens of antimony have been shown to be highly effective for the treatment of cutaneous leishmaniasis (Berman, 1997).

1.1.4 Molecular mechanisms

Arsenic and antimony belong to group XV of the periodic table of elements. They are metalloids because they have both metallic and non-metallic properties. They exist in various forms, exhibiting different biological effects and degrees of toxicity (Abernathy et al., 1999; Snow, 1992).

Arsenic exists in inorganic and organic forms and in different oxidation states (-3, 0, +3, +5). In the case of environmental exposure, toxicologists are primarily concerned with arsenic in the trivalent and pentavalent oxidation states (Hughes, 2002). The more commonly known arsenic compounds, arsenite (As(III)) and arsenate (As(V)), are the anionic forms of arsenic acid and arsenous acid, respectively. Monomethylarsenic acids (MAs) and dimethylarsenic acids (DMAs) are methylated metabolites of inorganic arsenic.

The mechanism of pentavalent arsenic toxicity may be that it can replace phosphate in many biochemical reactions because they have similar structure and properties (Dixon, 1997). For example, arsenate reacts with glucose *in vitro* to form glucose-6-arsenate (Lagunas, 1980). Glucose-6-arsenate is a substrate for glucose-6-phosphate dehydrogenase and can inhibit hexokinase. In the human red blood cell arsenate can also replace phosphate in the sodium pump and the anion exchange transport system (Kenney and Kaplan, 1988). In *in vitro* arsenolysis studies arsenate uncouples formation of adenosine-5'-triphosphate (ATP) at both substrate level and mitochondrial level (Dixon, 1997; Gresser, 1981). Arsenolysis diminishes formation of ATP by the replacement of phosphate

with arsenate in the enzymatic reactions. Depletion of ATP by arsenate has also been observed in cellular systems in rabbit (Delnomdedieu et al., 1994) and in human erythrocytes (Winski and Carter, 1998) after *in vitro* exposure to arsenate.

The toxicity of trivalent arsenic is greater than pentavalent arsenic (Ellenhorn, 1997). Arsenite has been shown *in vitro* to react with thiol-containing molecules such as GSH and cysteine that have major roles in the activity of proteins (Scott *et al.*, 1993). As(III) binds to vicinal dithiols much more strongly than to monothiols. The binding of trivalent arsenic to critical vicinal thiol pairs may inhibit important biochemical reactions that could lead to toxicity. For example, pyruvate dehydrogenase (PDH) is a multisubunit complex that requires the cofactor lipoic acid, a dithiol, for enzymatic activity. Arsenite inhibits PDH perhaps by binding to the lipoic acid moiety (Hu et al., 1998; Szinicz and Forth, 1988). Methylated trivalent arsenical such as MAs(III) are potent inhibitors of GSH reductase and thioredoxin reductase (Lin et al., 1999; Styblo and Thomas, 1997). The inhibition may be also due to the interaction of trivalent arsenic with critical dithiol groups in these molecules. Inhibition of these enzymes may alter cellular redox status and eventually lead to cytotoxicity.

The metabolism of arsenic also has an important role in its toxic effects. Many mammalian species methylate inorganic arsenic (Vahter, 1994). Inorganic arsenic is metabolized by a sequential process involving a two-electron reduction of pentavalent arsenic to trivalent arsenic, followed by oxidative methylation to organic arsenicals, MAs and DMAs (Tamas and Wysocki, 2001). Because the acute toxicities of arsenite or arsenate are substantially greater than those of

MAs or DMAs, the metabolic production of these species following exposure to inorganic arsenic has generally been regarded as a mechanism of detoxification. However, recent studies suggest that the methylated arsenicals may even have higher carcinogenic potential than inorganic ones, arsenite and arsenate (Mass *et al.*, 2001; Thomas *et al.*, 2001). Evidence can be obtained from several toxicological studies. A recent study by Mass *et al.* (2001) has shown that the trivalent methylated arsenicals, MAs(III) and DMAs(III) are directly genotoxic. In rat hepatocytes, both methylarsine oxide ($\text{CH}_3\text{As(III)O}$) and dimethylarsinous iodide ($(\text{CH}_3)_2\text{As(III)I}$) are significantly more cytotoxic than arsenite (Styblo *et al.*, 2000). In Chang cells (a human liver cell line), monomethylarsonous acid (MAs(III)) was also found to be a more potent cytotoxin than arsenite (Petrick *et al.*, 2000).

In arsenic carcinogenicity, some mechanisms have been proposed; including genotoxicity, cell proliferation, altered DNA repair and DNA methylated oxidative stress, co-carcinogenesis, and tumor promotion (Hughes, 2002). Arsenite was shown to induce large deletion mutations in hamster-human hybrid cells (Hei *et al.*, 1998). Increased cell proliferation was observed in rat bladder after an exposure to DMAs (Wanibuchi *et al.*, 1996). A significant dose-dependent decrease in activity of a DNA repair enzyme, poly-(ADP-ribose)polymerase, was observed by Yager and Wiencke (Yager and Wiencke, 1997). The DNA of rat liver cells transformed by arsenite is globally hypomethylated and the effect is dependent on dose and length of exposure (Zhao *et al.*, 1997). Reactive oxygen species that can eventually alter the redox

status of the cell and present a stressful and toxic situation were detected in human-hamster hybrid cells within 5 min after exposure to arsenite. Arsenic trioxide can interact with benzo(a)pyrene (BP), a carcinogenic polycyclic aromatic hydrocarbon found in tobacco smoke, for co-carcinogenesis (Pershagen *et al.*, 1984). In tumor promotion, it has been observed in both rats and mice in multiple organs that DMA(V) promotes tumors (Wanibuchi *et al.*, 1996; Yamamoto *et al.*, 1995; Yamanaka *et al.*, 1996).

The variety of research indicates that not only the inorganic arsenicals, arsenite and arsenate, but also methylated species, MMAs and DMAs, exert a number of unique biological effects that are cytotoxic and genotoxic, and are potent inhibitors of the activities of some enzymes. Arsenic acts on cells through a variety of mechanisms that may influence numerous signal transduction pathways and result in vast cellular effects including apoptosis induction, growth inhibition, promotion or inhibition of differentiation, angiogenesis inhibition, and carcinogenesis induction. Responses may vary depending on cell type and the form of arsenic.

The biological properties and toxicological mechanisms of antimony have been only studied based on the antiprotozoan therapy in leishmaniasis. Pentavalent antimony inhibits glucose catabolism and ATP formation via glycolytic pathway and fatty acid β -oxidation in *Leishmania mexicana* (Berman *et al.*, 1987). Trivalent antimonite appears to interact with key sulfhydryl groups of leishmanial proteins, thereby causing enzyme inhibition (Roberts *et al.*, 1995).

1.2 Metalloid trafficking

All cells possess regulatory mechanisms to tightly control the cellular concentration of essential metals, such as zinc and copper and toxic metalloids, such as antimony and arsenic (Finney and O'Halloran, 2003; Gatti et al., 2000). This is necessary because even essential metals become toxic to the cell in excess due to their ability to catalyze cytotoxic reactions. Particularly because of the ubiquity in the environment, arsenic resistance mechanisms have evolved in every organism, both in prokaryotes and eukaryotes (Bhattacharjee *et al.*, 1999). Several transport proteins mediating the uptake or extrusion of metalloids have been identified and some of the metabolism systems have also been investigated.

1.2.1 Uptake systems

Arsenate uptake is catalyzed by two phosphate transporters, Pit and Pst in the prokaryote *E. coli* (Rosenberg *et al.*, 1977). The Pit system appears to be the predominant system (Willsky and Malamy, 1980). Similarly in the eukaryote *S. cerevisiae*, arsenate is taken up by several phosphate transporters (Bun-ya *et al.*, 1996; Yompakdee *et al.*, 1996).

Pathways for arsenite and antimonite uptake have recently been discovered. *E. coli* GlpF was identified as an arsenite transporter. GlpF is an aquaglyceroporin, a member of the aquaporin superfamily that transports neutral organic solutes such as glycerol and urea in *E. coli* (Borgnia et al., 1999; Sanders et al., 1997). In a screen of a random mutagenesis library turned up that GlpF is also an antimony transporter. Disruption of *glpF* greatly reduced the

level of uptake of both arsenite and antimonite. This clearly demonstrates that GlpF is the major uptake pathway for both metalloids in *E. coli* (Meng et al., 2004). Fps1p, a homolog of GlpF in yeast was recently shown to be the route of uptake of arsenite and antimonite (Wysocki et al., 2001).

An uptake system of trivalent metalloids in mammalian was also identified (Liu et al., 2002). In that study a mammalian aquaglyceroporin, rat AQP9, transported both arsenite and antimonite into transformed yeast cells and restored arsenite sensitivity of the arsenite-resistant yeast mutant. Another mammalian aquaglyceroporin, mouse AQP7, was microinjected into *Xenopus laevis* oocytes, and increased transport of arsenite was observed. These results demonstrate that these proteins can transport metalloids into cells. More recently the ability of hexose transporters to facilitate arsenic trioxide uptake in *Saccharomyces cerevisiae* was also examined and demonstrated that hexose permeases catalyze the majority of the transport of the trivalent metalloid arsenic trioxide (Liu et al., 2004).

Many mammals methylate trivalent inorganic arsenic in liver to species that are released into the bloodstream and excreted in urine and feces. One of the initial products of As(III) methylation is methylarsonous acid [MAs(III)], which is considerably more toxic than inorganic As(III). In a recent study, Liu and coworkers investigated the ability of GlpF, Fps1p, and AQP9 to facilitate movement of MAs(III) in rats and found that aquaglyceroporins differ both in selectivity for and in transport rates of trivalent arsenicals (Liu et al., 2006). In that study, the requirement of AQP9 conserved residue R219 for MAs(III)

movement was found to be similar to that found for As(III), suggesting that As(III) and MAs(III) use the same translocation pathway in AQP9. Considering the individual variability in sensitivity to arsenic in drinking water, we may assume that this variability is due in part to different levels of expression of aquaglyceroporins or hexose permeases in those individuals.

1.2.2 Metabolism systems

In general, the metabolism of arsenic which has been taken up into cells by uptake systems involves reduction, oxidative methylation and glutathione conjugation (Thomas et al., 2001; Thompson, 1993). Because trivalent arsenite (As(III)) is the preferred substrate for the methylation and extrusion reactions, there must be a source of reducing equivalents to convert pentavalent arsenate (As(V)) to arsenite. Subsequent methylation is an oxidative process, so there must be also a mechanism to convert the pentavalent arsenicals back to trivalent.

Reduction of As(V) to As(III) can occur nonenzymatically in the presence of a thiol such as glutathione (GSH) (Delnomdedieu et al., 1994a; Delnomdedieu et al., 1994b; Delnomdedieu et al., 1995; Scott et al., 1993). Reduction of arsenate to arsenite is linked to the formation of arsenotriglutathione (As(GS)₃). Arsenotriglutathione donates arsenite readily to dithiol-containing targets (Delnomdedieu *et al.*, 1993).

Enzymatic reduction systems have been investigated in both prokaryotes and eukaryotes. There are several independently evolved families of arsenate reductase enzymes. The first one was reported as the product of *arsC*, the last

gene of the *arsRDABC* operon of *Escherichia coli* plasmid R773 (Chen *et al.*, 1986). Homologues are found in many bacteria, both on plasmids and in chromosomes (Rosen, 1999). In the reaction cycle, arsenate first binds to ArsC reductase at an anion site and then forms a covalent arsenate thioester intermediate with a cysteine residue at active site. It is followed by a two-step reduction by glutaredoxin and glutathione to release arsenite. The second family of arsenate reductases is typified by the *arsC* gene product of *Staphylococcus aureus* plasmid pI258 (Ji and Silver, 1992a). This enzyme uses thioredoxin as the source of reducing potential (Ji *et al.*, 1994) and has two intramolecular cysteine residues that participate in the catalytic cycle (Messens *et al.*, 1999). The third family Acr2 is related to the superfamily of protein tyrosine phosphatases. LmACR2 from *Leishmania major* was shown to reduce both As(V) and Sb(V) (Zhou *et al.*, 2004) and was suggested to have phosphatase activity (Zhou *et al.*, 2006). It was proposed that LmACR2 is responsible for reduction of the pentavalent antimony in Pentostam to the active trivalent form of the drug in *Leishmania*. ScAcr2p from *S. cerevisiae*, on the other hand, does not exhibit phosphatase activity. Like the R773 ArsC, Acr2 has a single active site cysteine residue and uses glutaredoxin and glutathione as reductants (Mukhopadhyay and Rosen, 2001). But they are not related to each other. In mammalian cells, arsenate reductases also have been characterized *in vitro* to catalyze the reduction of arsenate to arsenite and of methylarsonic acid (MAs(V)) to methylarsonous acid (MAs(III)) (Radabaugh and Aposhian, 2000; Zakharyan and Aposhian, 1999b). These enzymes may serve a similar function as ArsC of the

bacterial *ars* operon. Recently the human activity has been attributed to the enzyme purine nucleotide phosphorylase (Radabaugh *et al.*, 2002). Whether it functions *in vivo* in arsenic detoxification is not yet known.

Methylation of arsenic was found not only in mammalian cells, but also in prokaryotic cells. It requires a methyltransferase and a methyl group donor S-adenosylmethionine (SAM) (Hughes, 2002; Thomas *et al.*, 2001). In *in vitro* assay from rat liver extracts, GSH was shown to promote methylation (Buchet and Lauwerys, 1988). An enzyme that catalyzes synthesis of MAs from arsenite has been purified from rabbit liver and shown to require both SAM and a thiol for activity (Zakharyan *et al.*, 1995). Notably, arsenite can also be chemically methylated in a thiol-dependent reaction (Buchet and Lauwerys, 1985; Zakharyan and Aposhian, 1999a). However, the contribution of chemical methylation to the overall conversion of inorganic arsenic to methylated products has not been quantified. Pentavalent methylarsinic acids (MAs(V)) are stable methylated metabolites of inorganic arsenic and are primarily excreted in the urine (Hughes, 2002). The trivalent methylarsinous acids (MAs(III)) are intermediates in the metabolic pathway of arsenicals and can exert distinct biological effects (Thomas *et al.*, 2001). It seems likely that methylation of arsenic may increase cellular toxicity rather than contributing toward detoxification. However a prokaryotic ArsM from *Rhodopseudomonas palustris* was identified recently and shown to confer As(III) resistance to an arsenic-sensitive strain of *Escherichia coli* (Qin *et al.*, 2006). ArsM catalyzes the formation of a number of methylated intermediates from As(III), with trimethylarsine as the end product.

The net result is loss of arsenic, from both the medium and the cells. Because ArsM homologues are widespread in nature, this microbial-mediated transformation is proposed to have an important impact on the global arsenic cycle.

1.2.3 Extrusion systems

In addition to metabolizing these toxic metalloids, both prokaryotes and eukaryotes can develop resistance systems when exposed to these metalloids. As devastating as are those ecological environmental catastrophes, it is the chronic exposure to the low levels of arsenic that exist naturally in both water and soil (Smedley and Kinniburgh, 2002) that provides evolutionary pressure to maintain arsenic resistance or detoxification systems in most, if not all living organisms, including humans (Rosen, 2002b). In eukaryotes, the resistance can be conferred by MRPs (multidrug resistance-associated proteins), members of the ABC transport ATPases superfamily (Cole *et al.*, 1994), which has been known to catalyze export of GS-conjugates (Leier *et al.*, 1994). MRP1-catalyzed export of glutathione from cells was increased by arsenite, suggesting that MRP1 may function as a $\text{As}(\text{GS})_3$ carrier (Zaman *et al.*, 1995). In human liver MRP2 extrudes arsenic-glutathione complexes into bile and may be a major route of arsenic detoxification in humans (Kala *et al.*, 2000).

In the yeast *S. cerevisiae* an MRP homolog, Ycf1p, has been shown to be a transporter that pumps $\text{Cd}(\text{GS})_2$ (Li *et al.*, 1996; Szczyпка *et al.*, 1994) as well as $\text{As}(\text{GS})_3$ and $\text{Sb}(\text{GS})_3$ (Ghosh *et al.*, 1999) into the vacuole. In addition to Ycf1p,

S. cerevisiae has a plasma membrane transporter Acr3p that is homologous to an arsenite carrier protein in *B. subtilis* and mediates the efflux of arsenite (Ghosh et al., 1999; Wysocki et al., 1997). Recent data indicates that Acr3p is also involved in antimonite resistance (Tamas and Wysocki, 2001) (Wysocki *et al.*, 2001). Ycf1p and Acr3p are two independent systems that may provide major pathways for arsenic and antimonite detoxification in yeast.

In bacteria there are better-studied mechanisms of arsenite and antimonite extrusion that use pumps where energy is supplied either by the membrane potential of the cell or by arsenite-translocating ATPase (Dey and Rosen, 1995; Rosen, 2002a). To date, all bacteria with sequenced genomes have *ars* (arsenic resistance) operons, either intrinsic (chromosomal) or acquired (plasmid-encoded). Some bacteria have three-gene *arsRBC* operons that use ArsB alone to extrude metalloids, while some have two additional genes *arsD* and *arsA* to form five-gene *arsRDABC* operons and to extrude metalloids by ArsAB complex coupled utilization of ATP (Rosen, 1999).

In both three-gene and five-gene *ars* operons, *arsB* encodes an integral membrane protein with 12 membrane-spanning segments (Wu *et al.*, 1992). ArsB appears to be an antiporter that catalyzes metalloid-proton exchange (Meng et al., 2004). ArsR is a *trans*-acting repressor protein (Ji and Silver, 1992b; Rosenstein et al., 1992; Wu and Rosen, 1991) that belongs to a novel family of small metalloregulatory proteins (Shi et al., 1994; Wu and Rosen, 1991; Wu and Rosen, 1993b). It negatively regulates the transcription of each *ars* operon and can be derepressed by binding arsenite or antimonite. ArsC, as mentioned above,

is an arsenate reductase. Pentavalent arsenate is reduced to trivalent arsenite prior to extrusion or sequestration (Rosen, 2002a).

Other pathways for arsenic detoxification in bacteria were also proposed. For example, the *Sinorhizobium meliloti* *ars* operon includes an aquaglyceroporin (*aqpS*) in place of *arsB* (Yang *et al.*, 2005). The presence of AqpS in an arsenic resistance operon is interesting, since aquaglyceroporin channels have previously been shown to adventitiously facilitate uptake of arsenite into cells, rendering them sensitive to arsenite (Liu *et al.*, 2002). Yang and coworkers proposed that when *S. meliloti* is exposed to environmental arsenate, arsenate enters the cell through phosphate transport systems and is reduced to arsenite by ArsC. Internally generated arsenite flows out of the cell by downhill movement through AqpS. Thus, AqpS confers arsenate resistance together with ArsC-catalyzed reduction.

1.2.4 The *arsD* and *arsA* genes

E. coli has a chromosomal three-gene *arsRBC* operon that confers moderate resistance to arsenite and antimonite. However, when *ArsDA* is present in *E. coli* as the five-gene *arsRDABC* operon from plasmid R773, cells are more resistant to these metalloids. It has been shown that the ArsAB ATPase is a much more efficient extrusion pump than ArsB alone (Dey and Rosen, 1995). It has been proposed that the five-gene *arsRDABC* operons may arise by insertion of the *arsDA* genes into a three-gene operon because *arsD* and *arsA* genes are almost always next to each other in the operons (Rosen, 2002a)

(Appendix A). To date, this phenomena can be found in nearly 50 *ars* operons either in chromosomes or plasmids with only two exceptions.

ArsA is a member of a family of ATPases that probably arose from GTPases (Leipe *et al.*, 2002). It is normally bound to ArsB (Dey *et al.*, 1994), but in the absence of ArsB, ArsA is found in the cytosol and can be purified as a soluble protein. ArsA has two halves, A1 and A2, that are homologous to each other (Walker *et al.*, 1982). The study of crystal structure of ArsA from *E. coli* R773 revealed that it has three domains (Zhou *et al.*, 2000). First, there are two nucleotide-binding domains (NBDs) which are folded structures that both contain residues from both A1 and A2. Both NBDs are required for activity. Second, there is a single metallo-binding domain (MBD) composed of the two halves of ArsA at the opposite end from the NBDs. The MBD consists of a number of residues including Cys113, Cys172, Cys422, His148, His453 and Ser420 (Bhattacharjee *et al.*, 1995; Bhattacharjee and Rosen, 1996; Bhattacharjee and Rosen, 2000). ArsA ATPase activity is activated by the binding of metalloids to the MBD. A recent study showed that ArsA binds a single Sb(III) with high affinity only in the presence of Mg²⁺-nucleotide (Ruan *et al.*, 2006). Mutation of the codons for Cys113 and Cys422 eliminated Sb(III) binding to purified ArsA. Metalloid stimulation of ArsA activity enhances the ability of the pump to reduce the intracellular concentration of metalloid, and confers an evolutionary advantage. Third, there are signal transduction domains (STDs) in each half of the protein and they connect the single MBD to the two NBDs. They can be recognized by a 12-residue signature sequence (D₁₄₂TAPTGHTRILL and D₄₄₇TAPTGHTRILL).

R773 ArsD is a homodimer of two 120-residue subunits. It has been shown to function as a second *trans*-acting *ars* repressor (Wu and Rosen, 1993a) that binds to the same operator site as ArsR. Although ArsD has a metalloid affinity as high as ArsR, but has a low DNA affinity that is two-orders of magnitude lower than ArsR (Chen and Rosen, 1997). ArsR controls the basal level of expression of the operon, while ArsD may control the maximal expression to prevent the toxicity resulting from high-level production of the membrane protein ArsB. The vicinal pairs of cysteine residues of ArsD have been shown to be involved in coordinating the metalloids (Li *et al.*, 2001). It appears to have two or even three metalloid binding sites per monomer (Cys12-Cys13, Cys112-Cys113 and Cys119-Cys120), but only two of them, the Cys12-Cys13 and Cys112-Cys113 pairs, were shown to be required for *in vivo* activity and to coordinate metalloids. Recently, a kinetic study showed that the metalloid binding of ArsD is cooperative between the four binding sites of the dimer (Li *et al.*, 2002). In addition to its role as a regulatory protein, ArsD might have additional functions.

It is striking that the *arsD* and *arsA* genes are always found together in the bacterial and archaeal arsenic resistance operons or gene clusters identified to date. The linkage of these two genes further suggests that ArsD and ArsA might have associated functions in arsenic detoxification. Since ArsA is the catalytic subunit of the ArsAB As(III)-translocating ATPase, it is reasonable to consider that the 26 kDa homodimeric ArsD, a cytosolic protein with three vicinal cysteine pairs per monomer (Figure 1) that potentially form multiple As(III) binding sites,

serves as a metallochaperone for intracellular arsenite, transferring metalloid to the pump.

1.3 Metallochaperones

Protein-protein interactions are intrinsic to virtually every cellular process – for example, DNA replication, transcription, translation, splicing, secretion, cell cycle control, signal transduction, and intracellular trafficking of metal ions. They are all currently the subject of great interest.

There are a large number of transient protein-protein interactions, which in turn control a large number of cellular processes. These include modifications of proteins such as protein kinases, phosphatases and proteases, the recruitment and assembly of the transcription complex to specific promoters, the transport of proteins across membranes, the folding of native proteins catalyzed by chaperonins, and the delivery of metal ions by metallochaperones.

One-third of all proteins require metal cofactors for function. Metalloproteins play key roles in many biological processes, including respiration, photosynthesis, nerve transmission, and defense against toxic agents such as arsenic and antimony. These proteins are housed in a wide variety of intracellular locations or are exported to the extracellular milieu. The complexity of metalloenzyme active sites ranges from one metal ion with several protein ligands to polynuclear clusters. Assembly of these metal centers as well as delivery of specific metal cofactors to diverse locations involves many accessory or helper proteins. On the other hand, all cells possess metallosensors that switch on or off the expression

of systems that control the level of the metals. Membrane transporters, predominantly transport ATPases, frequently participate in metal ion homeostasis. A further level of regulation is afforded by metallochaperones that can sequester metals in the cytoplasm, buffering their concentration, and deliver them to protein targets, such as transporters for extrusion (Finney and O'Halloran, 2003; Rae et al., 1999; Rosenzweig, 2002). This recently identified class of accessory proteins, called metallochaperones, binds metal ions and delivers them directly to target enzymes via protein-protein interactions.

1.3.1 Copper Chaperones

The current picture of metallochaperone-mediated cofactor assembly derives primarily from studies of copper chaperones (Rosenzweig, 2002). Prior to 1997, there were no established molecules that served this function. *In vitro*, most copper enzymes easily acquire their metal without any auxiliary proteins. For example, the copper- and zinc-dependent enzyme superoxide dismutase (SOD1) binds copper ions *in vitro* with the K_d around 10^{-15} M. Yet in a living cell, where cytoplasmic free copper concentration is estimated to be less than 10^{-18} M, SOD1 relies heavily upon an auxiliary factor for acquiring copper (Rae et al., 1999). The first identified copper metallochaperone for SOD1 is a protein involved in the lysine biosynthetic pathway, namely LYS7 in yeast and CCS (copper chaperone for SOD) in humans (Culotta et al., 1997; Horecka et al., 1995).

More copper chaperones were then discovered consequently. Cox17, which localizes to both the cytosol and inner membrane space of the mitochondria, has been proposed to deliver copper to the inner mitochondrial membrane protein Sco1. Atx1 is also approved as a cytosolic yeast copper chaperone that delivers copper to the transport ATPase Ccc2 in the trans-Golgi network. The human homolog of Atx1, Hah1 (Atox1), interacts with the copper-transporting ATP7A and ATP7B pumps, and mutations in these pumps lead to genetic disorders such as Menkes and Wilson Diseases (Walker *et al.*, 2002). The *cop* operon of *Enterococcus hirae* encodes a metallochaperone, CopZ, a metalloregulated repressor, CopY, and two copper pumps, CopA and CopB (Solioz and Stoyanov, 2003). Under conditions of limiting copper, CopA catalyzes copper uptake, while CopB catalyzes export when copper reaches toxic levels. CopA-Cu(I) interacts with CopZ to load it with Cu(I) for delivery to the repressor CopY.

All the types of copper chaperones bind Cu(I) with multiple cysteine ligands. Atx1-like chaperones are characterized by a conserved CXXC motif in N-terminus. However the X-ray structures of Atx1 indicate the presence of two or even three sulfur ligands (Pufahl *et al.*, 1997; Rosenzweig, 2001). Two of these ligands probably derive from the CXXC motif, and the third could either be an exogenous thiol or a cysteine from a second Atx1 molecule. The CCS chaperones has been shown that four cysteine residues from both the N-terminal CXXC motif and the C-terminal CXC motif form the metal binding sites (Eisses *et al.*, 2000). Yet only the C-terminal CXC motif appears poised to deliver metal ions to the SOD1 site. Likewise Cox17, the proposed chaperone for cytochrome

c oxidase, binds copper ions with cysteine ligands. It binds three ions using a conserved CCXC motif, in which all three cysteines are required to produce active cytochrome c oxidase. CopZ, characterized by a MxCxxC metal binding motif, transfers copper to CopY. The copper binding stoichiometries of CopZ were determined and found to be one copper(I) per CopZ (Cobine et al., 2002). X-ray absorption studies suggested a mixture of two- and three-coordinate copper in Cu(I)CopZ. In this case, CopY has a higher affinity for copper than CopZ. The copper transfer between CopZ and CopY was dependent on electrostatic interactions.

1.3.2 Other Metallochaperones

Metallochaperones are also believed to deliver nickel ions to enzymes such as urease, hydrogenase, and CO dehydrogenase. For assembly of the urease dinuclear nickel site, apo urease forms a complex with proteins, UreD, UreF, and UreG, and is then activated by the addition of nickel, bicarbonate, GTP, and a putative metallochaperone called UreE (Soriano *et al.*, 2000). UreE binds six Ni(II) ions, but only two sites are involved in delivery to urease. These two sites are five or six coordinate with 2-4 histidine ligands. The mechanism of metal transfer is expected to differ from those for Atx1 and CCS because two Nickel ions must be transferred between histidine ligands rather than one metal ions being transferred either by thiol exchange or from cysteine to histidine coordination environments.

While there is clearly a need for cells to control the levels of redox-active metals such as copper, recent studies have shown that cells exert tight control over cytosolic concentrations of relatively low toxic metals such as zinc (Outten and O'Halloran, 2001), suggesting that metallochaperones might exist to control the cellular levels of other soft metals. The advances in understanding copper and nickel chaperones underscore the possibility that additional metallochaperones exist for biologically relevant metal ions. Although genetic and biochemical data indicate that accessory proteins are required for assembling many other metal cofactors, a metallochaperone function has not been assigned definitively to any of these proteins. Numerous gene products involved in assembly of metal cofactors are being identified and may function as metallochaperones.

With eight cysteines per monomer, high affinity for metalloids, ArsD has a high potential for being the one which is required for assembling arsenic- or antimony-bound ArsA.

CHAPTER 2

MATERIALS AND METHODS

2.1 Strains, plasmids and media.

Cell strains and plasmids used were given in Table I and II. *E. coli* strain JM109 and JM110 were used for molecular cloning. *E. coli* strain BL21(DE3) was used for protein expression and purification, and AW3110 was used for resistance and transport assays. *S. cerevisiae* strain AH109 was used for two-hybrid analysis (Clontech). Plasmids pET28a (Km^r) (Novagen) and pSE380 (Ap^r) (Invitrogen) were used as cloning vectors, and plasmid pGBT9 and pACT2 (Ap^r) were used as *S. cerevisiae* - *E. coli* shuttle vectors (Clontech). *E. coli* cells were grown in Luria-Bertani (LB) medium (Sambrook et al., 1989) at 37°C. Ampicillin (100 µg/ml), tetracycline (10 µg/ml), chloramphenicol (50 µg/ml), kanamycin (40 µg/ml) and isopropyl-β-D-thiogalactopyranoside (IPTG; 0.1-0.3 mM) were added as required. Yeast cells were grown in complete YPD medium or minimal SD medium (Adams et al., 1998) with the appropriate supplements at 30°C. Growth in liquid culture was estimated from the absorbance at 600 nm.

2.2 DNA manipulations.

Plasmid purification, restriction digestion, gel electrophoresis, polymerase chain reaction (PCR), ligation, dephosphorylation, sequencing and *E. coli* transformations were carried out as described (Sambrook et al., 1989). Site-directed mutagenesis was performed using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Primers used for PCR and

mutagenesis were listed in Table III. Transformation of yeast cells was carried out using a Geno FAST-Yeast transformation kit (Geno Technologies, St. Louis, MO).

2.3 Construction of *ars* plasmids.

Plasmids with the *arsDAB*, *arsAB*, *arsB* and *arsD* genes were constructed as follows. Plasmid pET28a was changed to pET28a1 by replacing the *HindIII* site with a *StuI* site and introduction of a *HindIII* site behind the *XbaI* site in the multiple cloning region by PCR (Table III). The *arsAB* genes were excised from plasmid pAlterAB1 as a *HindIII-EcoRI* fragment and ligated with *HindIII-EcoRI* digested pET28a1, generating plasmid pET-AB. Similarly, the *arsB* gene was excised from pAlterAB2 into the *HindIII* and *EcoRI* sites of pET28a1, generating plasmid pET-B. An *XbaI-HindIII*-truncated *arsD* gene was made by PCR, digested with both restriction enzymes and ligated with similarly digested pET-AB, generating plasmid pET-DAB. For construction of a plasmid with a full-length *arsD* gene, the truncated *arsD* Δ 119-120 gene on pArsD6H Δ 119-120 (Li et al., 2001) was modified by introduction of an additional *XbaI* site immediately following the *EcoRI* site; the sequence for Cys-119 and Cys-120 was inserted by PCR; the gene was then isolated as a *EcoRI-BamHI* fragment and ligated with *EcoRI-BamHI* digested pSE380, producing plasmid pSE-D. This plasmid was transformed into *E. coli* BL21(DE3) for purification of non-tagged full-length ArsD. The *arsDAB*, *arsAB* and *arsB* genes were then cloned into plasmid pSE-D using the *XbaI* and *XhoI* sites from pET-DAB, pET-AB and pET-B, generating plasmids

pSE-DAB, pSE-AB and pSE-B, respectively. The pSE-DAB and pSE-AB plasmids were used for molecular competition assays. The *arsD* gene was also cloned as follows. A 380-bp PCR product containing the P_{BAD} promoter region from pBAD/Myc-HisA was cloned into pACYC184 using the *BclI* and *EcoRI* sites, generating pACBAD. The full-length *arsD* gene was PCR cloned into this plasmid using the *NcoI* and *EcoRI* sites, generating pACBAD-D, which was co-transformed with pSE-AB or pSE-B into *E. coli* strain AW3110 and used for transport assays.

For used in yeast two-hybrid assays, plasmids were constructed as follows. The *arsR* gene was engineered with an *EcoRI* site at the 5' end, followed immediately by a *NcoI* site and a *BamHI* site after the stop codon at the 3' end by PCR and then cloned into the GAL4 DNA-binding domain (BD) fusion plasmid pGBT9 through *EcoRI* and *BamHI* sites and the activation domain (AD) fusion plasmid pACT2 through *NcoI* and *BamHI* sites, generating pGBT-R and pACT-R, respectively. The *arsA*, *arsC* and *arsD* genes were cloned similarly using the *NcoI* and *BamHI* sites on pGBT-R and pACT2, generating pGBT-X (BD-X) series and pACT-X (AD-X) series plasmids. The truncated *ArsD* genes were PCR-amplified and cloned into plasmid pGBT9 through *EcoRI* and *BamHI* sites, generating pGBT-Dx (BD-ArsDx) series and. For DMA cross-linking, the C-terminally truncated *ArsD* (*ArsD*₁₋₁₀₉) with an N-terminal histidine tag was cloned from pGBT-D₁₋₁₀₉ into pET28a through *EcoRI* and *Sall* sites, generating pET-hD₁₋₁₀₉. For construction of the N-terminal maltose binding protein (MBP)-*ArsD* chimera, an *EcoRI-Sall* fragment containing the entire or modified *arsD* gene was

cloned from pGBT-D or pGBT-Dx into pMAL-c2X plasmid, generating plasmid pMAL-D or pMAL-Dx.

2.4 Resistance assays.

E. coli strain AW3110 cells harboring the two plasmids were grown in LB medium overnight and diluted 50-fold into LB medium containing 0.05% arabinose and various concentrations of sodium arsenite, and the absorbance at 600 nm was monitored by microplate reader SPECTRA max 340PC (Molecular Devices) with a path length of 0.24 cm at 37°C.

2.5 Molecular competition assays.

Molecular competition growth assays were performed as follows. Cells of *E. coli* strain AW3110 bearing either pSE-AB or pSE-DAB were grown overnight and mixed at a 1:1 ratio. The mixture was then diluted 1:1000 in LB medium containing 10 μ M sodium arsenite at 37°C daily for 9 days. The plasmids were extracted from the mixed culture and analyzed by digestion with *Xba*I and *Bam*HI. The digested DNA fragments were run on a 1% agarose gel containing 0.5 μ g/ml ethidium bromide. The bands were visualized at 302 nm and digitized using UN-SCAN-IT software (Silk Scientific, Inc.).

2.6 Transport assays.

For transport assays, *E. coli* strain AW3110 was co-transformed with pSE380 series and pACBAD series plasmids. Cultures were grown overnight in LB

medium and diluted 50-fold into LB medium at 37°C. After 1 hr 0.05% arabinose was added to induce ArsD expression, and the cells were harvested at an A_{600} of 1. The cells were washed and suspended in 1/10 of the original volume in a buffer consisting of 75 mM HEPES-KOH, 0.15 M KCl and 1 mM $MgSO_4$, pH 7.5, 22°C. Transport assays were performed with 10 μ M sodium arsenite, as described (Dey and Rosen, 1995). Arsenic was determined by inductively coupled mass spectrometry with a PerkinElmer ELAN 9000. Protein expression levels were determined by immunoblotting using anti-ArsA and anti-ArsD antibodies.

2.7 Yeast two-hybrid analysis.

A GAL4-based yeast two-hybrid system (Fields and Song, 1989) (Clontech Laboratories, Inc.) was used to determine protein-protein interactions. AH109, a GAL4-activating *HIS3* reporter yeast strain, was co-transformed with *ars* gene-fused BD-X series and AD-X series plasmids. The transformed cells were cultured overnight in SD medium at 30°C and then washed, suspended and adjusted to A_{600} of 1 in 20 mM Tris-HCl pH 7.5. Portions of the cell suspensions (1 μ l) were inoculated on SD agar plates lacking histidine without or with 0.1 mM sodium arsenite in serial 10-fold dilutions and incubated at 30°C for 2-3 days. As a positive control, pVA3 (BD-p53) was expressed with pTD1 (AD-T antigen); as a negative control, vector plasmid pGBT9 was expressed with pACT2.

2.8 Protein purification.

Cells bearing the indicated plasmids were grown in LB medium overnight at 37°C and then diluted 50-fold into 1 L of the same medium containing 100 µg/ml ampicillin or 40 µg/ml kanamycin. Proteins were expressed by induction with 0.3 mM IPTG at A_{600} of 0.6-0.8 for 3 hr. Wild type ArsD was purified from cultures of *E. coli* strain BL21(DE3) bearing plasmid pSE-D. Induced cells were harvested by centrifugation and washed once with a buffer containing 20 mM Tris-HCl, pH 7.5, 0.2 M NaCl, 1 mM EDTA and 5 mM DTT (buffer A). The cells were suspended in 5 ml of buffer A per g of wet cells and lysed by a single passage through a French press at 20,000 psi. Diisopropyl fluorophosphate was added at 2.5 µl/g wet cells immediately following lysis. Unbroken cells and membranes were removed by centrifugation at 10,000-150,000 x g for 1 hr at 4°C. ArsD was purified as described (Li et al., 2001) and stored at -80°C until used.

The MBP-ArsD chimera was purified from BL21(DE3)/pMAL-D. Cytosol was applied to a 1 x 10 cm amylose column (New England Biolabs) pre-equilibrated with buffer A. The column was washed with 120 ml of buffer A, and the chimeric protein was eluted with buffer A containing 10 mM maltose. MBP-ArsD-containing fractions were identified by SDS PAGE, pooled, concentrated, and stored in small aliquots at -80°C until use. ArsA with a six histidine tag at the C-terminus and the C-terminally truncated ArsD with an N-terminal histidine tag were expressed in BL21(DE3) harboring plasmids pSE-AB and pET-hD₁₋₁₀₉, respectively. The proteins were purified as described (Bhattacharjee and Rosen, 2000) and were stored at -80°C until use. Protein concentrations were

determined according to the method of Bradford (Bradford, 1976) or from the absorption at 280 nm (Gill and von Hippel, 1989).

2.9 Crosslinking assays.

Crosslinking studies with bBBr were described previously (Bhattacharjee and Rosen, 1996). Purified ArsD and ArsA were buffer exchanged into 50 mM MOPS, 0.2 M NaCl, pH 7.5 by using micro-spin gel filtration column (Bio-Rad). The proteins were quantified and expressed as molar concentration of ArsA or ArsD monomer. Proteins (16 μ M each) were incubated with 0.5 mM bBBr and/or 1 mM each of potassium antimonyl tartrate, $MgCl_2$ and ATP for 30 min at room temperature. Samples were analyzed by SDS PAGE using a step gradient gel with 8% (to resolve ArsA) and 16% (to resolve ArsD) acrylamide. Formation of fluorescent crosslinked products was visualized at 365 nm, and crosslinked proteins by immunoblotting with antiserum directed against ArsA, ArsD or CadC. The membranes were stripped for reaction with the next antibody by incubation in a buffer containing 62.5 mM Tris-HCl (pH 6.8), 100 mM β -mercaptoethanol and 2% SDS at 50°C for 30 min.

Crosslinking with DMA was performed by incubation of proteins (30 μ M each) with 10 mM DMA in a buffer containing 0.1 M $NaHCO_3$, pH 9.4, for 2 hr at room temperature. The reactions were terminated by addition of SDS sample buffer and incubation in boiling water for 3 min. 2.5 mM $MgCl_2$, 2 mM ATP, 1 mM potassium antimonyl tartrate and 1 mM sodium arsenite were added, as indicated. Samples were analyzed by SDS PAGE using a step gradient gel of

10% and 16% acrylamide followed by Coomassie Blue staining or immunoblotting with anti-ArsA, anti-ArsD or anti-ArsC antibodies.

2.10 Measurement of Metalloid Binding.

The buffer used for purification of ArsD was exchanged with a buffer containing 50 mM MOPS-KOH, pH 7.5 (Buffer A), using a Bio-Gel P-6 Micro Bio-Spin column (Bio-Rad). Purified protein was incubated at 4 °C with indicated concentrations of potassium antimonyl tartrate. After 1 hour, each sample was passed through a Bio-Gel P-6 column pre-exchanged with the Buffer A. Portions (30 µl) were diluted with 2% HNO₃, and the quantity of metalloid measured by inductively coupled mass spectrometry with a PerkinElmer ELAN 9000. Antimony standard solutions in the range of 0.5–10 ppb in 2% HNO₃ were obtained from Ultra Scientific, Inc. (North Kingstown, RI). The concentration of ArsD derivatives was calculated from the Bradford assay (BioRad).

2.11 Metalloid transfer assays.

An assay was developed to demonstrate that ArsA releases Sb(III) from ArsD. Cells of *E. coli* strain BL21(DE3) expressing MBP-ArsD lysed in the presence of 1 mM potassium antimonyl tartrate in buffer A lacking DTT. The cytosol containing the MBP-ArsD-Sb(III) complex was applied to a 2-ml amylose column, which was washed with 20 ml of the same buffer. BSA or purified ArsA (1 ml of 20 µM) was then applied to the columns with 1 mM ATP, ADP, ATPγS and/or MgCl₂, as indicated. The column was then washed with 8 ml of buffer,

and MBP-ArsD was eluted with 3 ml of 10 mM maltose. From SDS PAGE, fraction 2 contained nearly all of the BSA or ArsA with little MBP-ArsD, and fraction 11 contained nearly all of the MBP-ArsD with little or no BSA or ArsA. The concentration of BSA, ArsA or ArsD was calculated from the absorption at 280 nm. Antimony was quantified by inductively coupled mass spectrometry (ICP-MS) with a PerkinElmer ELAN 9000. Metalloid transfer efficiency is calculated as $([\text{Sb(III)}]_{\text{ArsA}}/[\text{ArsA}])/([\text{Sb(III)}]_{\text{ArsD}}/[\text{ArsD}])$.

To further demonstrate that ArsA accepts As(III) from ArsD, the amount of arsenic on each protein was determined following interaction. To determine the amount of As(III) bound to ArsA, a mixture of 3 μM his-tagged ArsA, 25 μM sodium arsenite, 2.5 mM MgCl_2 , 2 mM ATP γS with or without 9 μM MBP-ArsD was incubated at 37°C for 10 min. To isolate the As(III)-ArsA complex, MBP-ArsD and free As(III) were removed by centrifugation through a Bio-spin gel filtration column (Bio-Rad) with a 0.3 ml layer of amylose resin applied at the top of the spin column. ArsA and bound arsenic concentrations were measured by protein and ICP-MS determinations. The amount of As(III) bound to ArsD was determined similarly by adding excess ArsA (9 μM) to ArsD (3 μM) mixture. His-tagged ArsA and free As(III) were removed using a gel filtration spin column with a layer of 0.3 ml of Ni-NTA resin applied at the top of the spin column, and the amount of ArsD and As(III) determined.

2.12 ATPase assays.

The ATPase activity was estimated using a coupled assay (Vogel and Steinhart, 1976), as described (Hsu and Rosen, 1989). MBP-ArsD was buffer exchanged into 50 mM MOPS-KOH, pH 7.5, 0.25 mM EDTA using a Micro Bio-Spin Chromatography Column (Bio-Rad) and then added at a final concentration of 3 μ M into an assay mixture containing the same buffer plus 5 mM ATP, 1.25 mM phosphoenolpyruvate, 0.25 mM NADH, 10 units of pyruvate kinase and lactate dehydrogenase with or without various concentrations of potassium antimonyl tartrate or sodium arsenite. ArsA was added to a final concentration of 0.3 μ M. The mixture was prewarmed to 37°C, and the reaction was initiated by the addition of 2.5 mM MgCl₂, which was measured at 340 nm. The linear steady state rate of ATP hydrolysis was used to calculate specific activity. The reaction volume was 1 ml for assays in 2 ml cuvettes or 0.2 ml for microplate reader assays.

CHAPTER 3

RESULTS

3.1 ArsD confers elevated resistance to arsenic upon cells expressing the arsenical pump.

To examine whether ArsD and ArsA have linked functions in arsenic detoxification, the *arsD* gene was co-expressed with the *arsAB* genes from compatible plasmids under control of heterologous promoters. The plasmids were expressed in *E. coli* strain AW3110, in which the chromosomal *arsRBC* operon had been deleted (Carlin *et al.*, 1995). By itself, *arsB* confers low-level resistance, while *arsAB* expression confers resistance at considerably higher levels (Figure 2A and B) (Dey and Rosen, 1995). Cells co-expressing *arsD* with *arsB* were no more resistant to arsenite than cells express only *arsB*, while cells co-expressing *arsDAB* were significantly more resistant to higher concentrations of arsenite compared to cells expressing only *arsAB*. Since an immunoblot established that *arsD* did not affect the levels of ArsA produced (Figure 5), the data are consistent with interaction of ArsD with ArsA to increase the efficiency of the ArsAB pump.

3.2 ArsD confers an competitive advantage to cells growing in subtoxic concentrations of arsenite.

Arsenic is a ubiquitous toxic metal contaminant and health hazard in drinking water worldwide (Smedley and Kinniburgh, 2002). When arsenite

resistance was compared between cells expressing the *arsAB* genes or the *arsDAB* genes, the latter showed a modest increase in resistance, with the greatest differences observed at millimolar concentrations of arsenite, amounts of arsenite that are toxic under laboratory settings (Figure 2A and B). Does the presence of the *arsD* gene confer an evolutionary advantage on the host organism for growth in concentrations of arsenite frequently found in the environment? A molecular competition experiment was devised to examine this question. Two sets of cells of *E. coli* strain AW3110 were allowed to compete with each other in a mixed culture for growth in the presence of a sub-toxic concentration (10 μ M) of arsenite, which is in the range of what is found in the environment (Smedley and Kinniburgh, 2002). One set of cells had a plasmid with *arsAB* under control of the *tac* promoter, while the other set had *arsDAB* in the same vector. Each day the culture was diluted 1000-fold, and the relative amounts of the *arsDAB* and *arsAB* plasmids were analyzed by restriction digestion (Figure 3A). After nine days of growth, cells with *arsDAB* had largely replaced those with only *ArsAB* (Figure 3B), indicating that the *arsD* gene provides a competitive advantage for growth in the low concentrations of arsenite that are ubiquitous in soil or surface waters.

3.3 *ArsD* enhances the ability of the pump to lower the intracellular concentration of arsenite

To demonstrate that *ArsD* enhances the ability of the pump to lower the intracellular concentration of arsenite, the effect of the *ars* genes on arsenite

accumulation was examined in intact cells. Higher rates of extrusion result in lower accumulation of arsenite (Dey and Rosen, 1995). Cells of the arsenite-hypersensitive strain AW3110 with no *ars* genes (vector plasmids pSE380 and pACBAD) accumulated approximately 150 pmol As(III)/10⁹ cells/10 min (Figure 4). Cells expressing only *arsB* accumulated arsenite to approximately 22 pmol As(III)/10⁹ cells/10 min, reflecting the ability of ArsB to catalyze arsenite/proton exchange (Meng et al., 2004). Expression of *arsAB* resulted in decreased accumulation to approximately 7 pmol As(III)/10⁹ cells/10 min, reflecting more efficient arsenite extrusion by the ArsAB pump than by ArsB alone (Dey and Rosen, 1995). Cells co-expressing *arsD* and *arsAB* exhibited substantially less accumulation of arsenite (approximately 1 pmol As(III)/10⁹ cells/10 min) than those with *arsAB*. Cells expressing *arsD* with only *arsB* accumulated arsenite to approximately the same level as cells expressing only *arsB*, indicating that ArsD does not affect activity of ArsB. The results of immunoblotting showed that *arsD* does not affect the levels of ArsA produced (Figure 5). These results clearly show that ArsD increases the efficiency of the ArsAB pump.

3.4 Interaction of ArsD with ArsA *in vivo*

Yeast two-hybrid analysis was applied to demonstrate that ArsD and ArsA physically interact (Figure 6A). ArsA interacted with ArsD but not with the ArsR repressor or the ArsC arsenate reductase. ArsD interacted with ArsA and with itself, which would be expected since ArsD is a homodimer (Chen and Rosen, 1997), but not with ArsR or ArsC. ArsR, which is a homodimer, also interacts with

itself, but not with ArsD or ArsA. These results indicate specific interaction of ArsD and ArsA. When 0.1 mM potassium antimonyl tartrate was added to the medium, the cells grew more slowly, but there was no effect on the ability of BD-ArsD to interact with AD-ArsA (Figure 6B). Thus, ArsD and ArsA interact in the absence of added metalloid. However, the presence of some metal or metalloid in the yeast cytosol that promotes interaction cannot be ruled out.

3.5 Interaction of ArsD with ArsA *in vitro*

Direct physical interaction between ArsD and ArsA was observed by chemical crosslinking with two different crosslinkers. Since both proteins have metalloid binding sites composed of cysteine residues (Bhattacharjee and Rosen, 1996; Li *et al.*, 2001), it was reasonable to consider that they might interact at those sites. Crosslinking was performed using (4,6-bis(bromomethyl)-3,7-di-methyl-1,5-diazabicyclo[3.3.0]octa-3,6-diene-2,8-dione (dibromobimane or bBBr) (Invitrogen Corporation, Carlsbad, California), a fluorogenic, homobifunctional thiol-specific crosslinking reagent that becomes highly fluorescent when its two alkylating groups react with cysteine residues that are within 3 to 6 Å of each other (Kosower *et al.*, 1980). ArsA forms intramolecular crosslinks with bBBr at its metalloid binding site (Bhattacharjee and Rosen, 1996). When ArsD was treated with bBBr, subjected to sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE), and immunoblotted with anti-ArsD, it formed a number of species that correspond to dimers and higher order species (Figure 7). All ArsD bands, including the monomer, became fluorescent

following reaction, showing that both intra- and intermolecular crosslinking had occurred (Figure 7, top panel). Since ArsD is a functional dimer, intermolecular crosslinking is not unexpected. When an equimolar mixture of ArsD and ArsA was reacted with bBBr, a crosslinked species was detected that reacted with both anti-ArsA and anti-ArsD antibodies (Figure 7, lane 2, second and third panels). This species migrated as a band with an apparent mass of approximately 90 kDa, the predicted mass of an ArsD dimer crosslinked to a monomer of ArsA. It should be pointed out that the intensity of the bands from one blot to the next cannot be directly compared because different polyclonal antibodies react with their antigens differently and because the stripping process removes variable amounts of the antigenic species. As a control, ArsA was reacted with CadC, a Cd(II)-responsive regulatory protein of similar size to ArsD and with a metal binding site formed of cysteines that react intramolecularly with bBBr (Wong *et al.*, 2002). No ArsA-CadC adducts were observed using anti-ArsA (Figure 7, lanes 6-8, second panel) or anti-CadC (lanes 6-8, bottom panel) antibodies. The amount of the ArsD-ArsA crosslinked product was increased by addition of MgATP (Figure 7, lane 4, second and third panels). As expected, addition of Sb(III) did not increase crosslinking since the thiol groups that coordinate the metalloid would have reacted with bBBr (Figure 7, lane 3, second and third panels). In these and other *in vitro* experiments Sb(III) was used rather than As(III) since both ArsD and ArsA have higher affinity for trivalent antimony, a softer and more thiophilic metal than arsenic.

Because the interaction of the two proteins appears to involve cysteine

residues, crosslinking was examined with dimethyl adipimidate (DMA) (Pierce Biotechnology, Inc. Rockford, IL), a homobifunctional imidoester that crosslinks free amines within 8.6 Å of each other, including N-termini and ε-amino groups of lysine residues, and does not modify cysteine thiolates (Figure 8). ArsA has 75 amino groups (74 lysines and the amino terminus), and ArsD has 15, so there are a large number of potential sites of crosslinking with DMA. Not surprisingly, a number of crosslinked species were observed that reacted with anti-ArsA or anti-ArsD sera, and several that appeared to react with both. The most prominent was a band observed after staining with Coomassie Blue or immunoblotting with either anti-ArsA or anti-ArsD that migrated with an apparent MW of approximately 130 kDa (indicated by the asterisk in Figure 8, top, second and third panels). The position of this band is higher than predicted for the ArsA-ArsD complex. However, bifunctional crosslinking reagents such as DMA are known to retard electrophoretic mobility as a result of intramolecular crosslinks that prevent unfolding by SDS (Sieber *et al.*, 2002). To demonstrate specificity of crosslinking between ArsA and ArsD, no crosslinking of ArsA and ArsC was observed (Figure 8, bottom panel). Again, crosslinking of ArsD and ArsA was enhanced by the presence of nucleotide. There was also an additional enhancement by either As(III) or Sb(III), but this was difficult to quantify by DMA crosslinking. In agreement with the yeast two-hybrid results, these *in vitro* data suggest direct interaction of ArsD and ArsA through the As(III) binding sites of the two proteins. The requirement for nucleotide suggests that ArsD interacts preferentially with a nucleotide-bound conformation of ArsA.

3.6 Transfer of metalloids from ArsD to ArsA

To explore whether ArsD-ArsA interactions give rise to transfer of metalloid, the ability of ArsA to abstract Sb(III) from ArsD was determined. For these assays, cytosol from cells expressing a maltose binding protein (MBP)-ArsD fusion were incubated with Sb(III), following which the MBP-ArsD-Sb(III) complex was bound to an amylose column, which was then washed with 10 column volumes of buffer to remove other proteins. When the column was eluted with BSA and MgATP, little Sb(III) came off with in the BSA-containing fractions (Figure 9A). Subsequent application of buffer with maltose then eluted nearly homogeneous MBP-ArsD protein in fraction 11 with Sb(III). In contrast, when the column was eluted with ArsA and MgATP, more Sb(III) came off with ArsA in fraction 2 and less with ArsD in fraction 11, consistent with transfer of metalloid from ArsD to ArsA (Figure 9B). The elution fractions were analyzed with SDS-PAGE (Figure 10). The effect of nucleotides on Sb(III) transfer was examined using similar assays (Figure 11). Mg^{2+} enhanced transfer with ATP but was not effective alone. Little Sb(III) eluted with ArsA and Mg^{2+} without ATP in fraction 2, and most of the metalloid eluted with ArsD in fraction 11 (Figure 11D). Among the various conditions, MgATP was the most effective, followed by $MgATP_{\gamma}S$, MgADP and ATP alone (Figure 11A, B, C and 12), indicating that the nucleotide enhances transfer but this process is not dependent upon its hydrolysis. Furthermore, when ArsA was incubated with $MgATP_{\gamma}S$ using a similar metalloid transfer assay, ArsA bound more As(III) in the presence of excess ArsD than in

its absence (Figure 13). In contrast, under the same conditions, ArsD bound less As(III) with excess ArsA than in its absence, consistent with transfer of metalloid from ArsD to ArsA. ArsD, with a K_d of 1.7 μM (Li *et al.*, 2002), has higher affinity for Sb(III) than does ArsA, with a K_d of 540 μM (Walmsley *et al.*, 2001). However, the affinity of ArsA for Sb(III) is substantially increased by binding of nucleotides ($K_d = 8 \mu\text{M}$) (Ruan *et al.*, 2006). Considering that ArsA has a greater affinity for metalloids in the presence of nucleotides, it is not surprising that metalloid transfer from ArsD to ArsA is similarly enhanced by nucleotides.

3.7 ArsD enhances the catalytic activity of ArsA

The effect of ArsD on the catalytic activity of ArsA was investigated. The ATPase activity of ArsA is stimulated by As(III) (Hsu and Rosen, 1989). When ArsD was added to the ATPase assay, ArsD increased the apparent affinity for arsenite 60-fold, from approximately 1.2 mM to 20 μM (Figure 15A). A similar increase in affinity for Sb(III) was observed (Figure 15B). This was not the result of increased thiol buffering of arsenite, since dithiothreitol could not replace ArsD (Figure 14A and B). ArsD did not greatly affect the K_m of ArsA for ATP at a concentration of arsenite (0.5 mM) or antimonite (10 μM) which is below saturation in the absence of ArsD but is sufficient to saturate the enzyme in the presence of ArsD (Figure 16A and B). Significantly, at a sub-saturating concentration of arsenite, ArsD increased the V_{max} with ATP by approximately 3-fold. Thus, the functional consequence of the ArsD-ArsA interaction appears to be an increase in the efficiency of the catalytic subunit of the ArsAB pump at low

concentrations of the substrate of the pump, arsenite.

3.8 Cysteine residues in ArsD contribute to metalloid binding sites (MBSs)

Alignment of the primary sequence of homologues of the R773 ArsD indicates that they possess an absolutely conserved vicinal cysteine pair, Cys12-Cys13 and a single conserved cysteine, Cys18 (Fig. 2). Two other vicinal cysteine pairs, Cys112-Cys113 and Cys119-Cys120 are found in some homologues but not others. We previously showed that none of the three vicinal cysteine pairs are required for repression (Chen and Rosen, 1997; Li *et al.*, 2001). Although all the vicinal pairs are capable of binding As(III), Cys12-Cys13 and Cys112-Cys113, but not Cys119-Cys120, are required for derepression by As(III).

Binding of Sb(III) to purified MBP-ArsD derivatives were measured by rapid gel filtration. The binding was measured as a function of Sb(III) concentration. A background binding was observed with only the maltose binding protein (MBP). The MBP fused wild type ArsD binds metalloid with a stoichiometry of six Sb(III) per dimer and an apparent K_d of 10^{-6} M (Figure 17A), suggesting that there are three metalloid binding sites (MBSs) on an ArsD monomer. The actual K_d could be lower since this assay is not very sensitive. An ArsD derivative ArsD₁₋₁₁₈, in which Cys119 and Cys120 were replaced with a six-histidine tag, was able to bind four Sb(III) per dimer, suggesting the third vicinal cysteine pair Cys119-Cys120 (MBS3) on ArsD has a metalloid binding capacity of two per dimer. The truncation ArsD₁₋₁₀₉, in which a stop codon was added after the codon for residue 109 and only the first cysteine pair Cys12-Cys13 (MBS1) was present, was able

to bind two Sb(III) per dimer. Similarly the mutant ArsD_{1-118,C12/13A}, in which the first cysteine pair had been changed to an alanine pair, the third pair had been replaced with six-histidine tag and only the second pair Cys-112-Cys113 (MBS2) was present, was able to bind two Sb(III) per dimer. These results are consistent with previous observations that each cysteine pair is competent to bind metalloid (Li *et al.*, 2001).

Because the cysteine pair Cys12-Cys13 and the Cys18 are absolutely conserved, further Sb(III) binding assays were performed based on these cysteine mutants. The mutants ArsD_{1-118,C12A}, ArsD_{1-118,C13A} and ArsD_{1-118,C18A}, in which the pair Cys119-Cys120 was replaced with a six-histidine tag and one of the three N-terminal cysteines was changed to alanines, were able to bind only two Sb(III), indicating that either MBS1 or MBS2 was eliminated (Figure 17B). Similar mutants based on ArsD₁₋₁₀₉, in which the Cys112-Cys113 and Cys119-Cys120 were deleted and only two of the three N-terminal cysteines were present, were unable to bind any Sb(III), suggesting that Cys18, in addition to Cys12 and Cys13, is required to form the MBS1 (Figure 18). However, the possibility that Cys18-to-Ala mutant does not fold properly could not be eliminated.

3.9 MBS1 in ArsD is the active site for metalloid transfer and ArsA activation.

When ArsA was incubated with As(III) in the presence of excess ArsD_{1-118,C18A} in the As(III) transfer assay, ArsA bound much less As(III) than that in the

presence of wild-type ArsD or that in the absence of ArsD (Figure 19). This suggests that this ArsD mutant, which binds As(III) in MBS2 but not in MBS1, does not transfer As(III) to ArsA, but instead competes with ArsA for binding As(III). And of that the cysteines in MBS1 are responsible for delivering metalloids to ArsA.

The ability of ArsD derivatives to activate ArsA ATPase activity was also examined (Figure 20). In the presence of ArsD₁₋₁₁₈, a truncated ArsD lacking Cys119-Cys120, the half maximal concentration of As(III) was 10 μ M (Figure 20A), similar to that of wild-type ArsD. In the presence of ArsD₁₋₁₀₉, a truncated ArsD lacking the Cys112-Cys113 and Cys119-Cys120 pairs, the half maximal concentration of As(III) was 25 μ M, also similar to that of wild-type ArsD. These data suggest that MBS2 and MBS3 are not required for ArsD to activate ArsA. In contrast, in the presence of a C12A/C13A derivative (ArsD_{1-118,C12/13A}), the concentration of As(III) required for half-maximal stimulation of ATPase activity was 1.5 mM, similar to that of ArsA in the absence of ArsD. This mutant ArsD, which lacks MBS1, was not able to activate ArsA. These data are consistent with participation of MBS1, but not MBS2 or MBS3, in increasing the affinity of ArsA for metalloid.

Figure 20B shows the effects of single residue substitutions in MBS1 of ArsD on activating ArsA. Mutants ArsD_{1-118,C12A}, ArsD_{1-118,C13A} and ArsD_{1-118,C18A}, in which the MBS1 was eliminated and only MBS2 was active, were unable to activate ArsA. Cysteine-to-serine substitutions were also investigated in the same assay and found to have similar results as those with alanine substitutions

(Figure 20B). Since metalloids alone augment the ATPase activity of ArsA, it seems plausible that Cys12, Cys13 and Cys18 are directly responsible for delivering arsenite to ArsA to enhance its affinity. In other words, the affinity for arsenite in the ArsD-ArsA complex would be largely determined by ArsD rather than by ArsA.

3.10 Effects of elimination of MBSs in ArsD on protein-protein recognition

To verify if the ArsD mutants are stable and able to dimerize in yeast, the ability of the cysteine-to-alanine mutants to interact with wild-type ArsD were examined. All of the mutants are able to interact with wild-type ArsD, indicating that they are produced and stable in yeast (Figure 21A). Although the interaction with the mutant C12A/C13A ArsD seemed to be weaker than that with others, yeast two-hybrid results are not quantitative. To determine if the cysteine residues are involved in ArsD-ArsA interactions, the ability of six cysteine-to-alanine and three cysteine-to-serine ArsD mutants to interact with ArsA was examined. The effect of 100 μ M Sb(III) or 50 μ M As(III) in the medium were also tested. Two mutants were still able to interact with ArsA. These were ArsD_{1-118,C112A} and ArsD_{1-118,C112A/C113A}, in which Cys119 and Cys120 were replaced with a six-histidine tag, and Cys112 or both Cys112 and Cys113 were replaced with alanines (Figure 21B). Both mutants retain Cys12, Cys13 and Cys18. In contrast, the mutants lacking Cys12, Cys13 or Cys18 were unable to interact. These include ArsD_{1-118,C13A}, ArsD_{1-118,C18A}, ArsD_{1-118,C12A/C13A}, ArsD_{1-118,C12S}, ArsD_{1-118,C13S}, ArsD_{1-118,C18S} (Figure 21B and C). This is consistent with a role for

the MBS1 in interaction with ArsA. However the mutant ArsD_{1-118,C12A}, in which the Cys12 was changed to alanine, seemed to be able to interact with ArsA when incubated longer (Figure 21C) or in the presence of Sb(III) (Figure 22A). However, the same mutant was unable to interact in the presence of As(III) (Figure 22B). This result is unexplained. Also the similar mutant ArsD_{1-118,C12S}, in which the Cys12 was changed to serine, was unable to interact under all conditions. The requirement of Cys12 for ArsD to interact with ArsA in yeast two-hybrid is not clear at this point.

3.11 Protein-protein interaction domain on ArsD

Furthermore truncations of ArsD which lack the first 7, 11 or 13 residues or the last 11, 27 or 43 residues were assayed with the yeast two-hybrid system in order to determine what regions of ArsD are responsible for dimerization and interaction with ArsA. These truncations were examined against wild-type ArsD. The truncated ArsD derivatives, which lack less than 43 residues in the C-terminus (1-77), were able to interact with both wild type ArsD and ArsA (Figure 23A and B). In contrast, the truncated ArsD lacking more than 7 residues in the N-terminus (8-120) was unable to interact with both proteins, suggesting the involvement of the N-terminus of ArsD in protein-protein recognition. Further truncations of ArsD which lack the first 2 or 4 residues or the last 55 or 77 residues were also tested. All of them were able to interact (Figure 24). However those lack more than 4 residues in the N-terminus (5-120) or more than 27 residues in the C-terminus (1-93) of ArsD seemed to have weaker interactions,

although, again this assay is not quantitative. These data suggest that the N-terminus of ArsD is the core region which is responsible for protein-protein interactions (Figure 24).

3.12 Effects of elimination of MBSs in *arsD* gene on arsenic accumulation and resistance

We examined whether removal of individual vicinal cysteine pairs or the conserved Cys18 affected the ability of ArsD to enhance the activity of the ArsAB pump *in vivo*. While cells expressing the *arsD*_{1-118, C112A/C113A} mutant, which encodes an ArsD derivative in which Cys119 and Cys120 were deleted, and Cys112 and Cys113 changed to alanines, *in trans* with *arsAB* accumulated As(III) to nearly the same extent as those expressing *arsDAB*, cells expressing the mutant *arsD*_{C12A/C13A} or *arsD*_{C18A} *in trans* with *arsAB* accumulated only slightly more As(III) than cells with only *arsAB* (Figure 25). These data are consistent with our idea that Cys12, Cys13 and Cys18 are involved in the functional interaction of ArsD with the ArsAB efflux pump.

We also examined whether the same ArsD mutants affected the resistance conferred by the ArsAB pump. In comparison to cells expressing wild type *arsDAB*, cell expressing an *arsD*_{1-118, C112A/113A} mutant *in trans* with *arsAB* had only a moderate reduction in arsenite resistance. In contrast, cells expressing a mutant *arsD*_{C12A/C13A} or *arsD*_{C18A} were no more resistant than cells with only *arsAB* (Figure 26A and B). These results suggest that the Cys12-Cys13 pair and Cys18, but not Cys112-Cys113 or Cys119-Cys120, are required

for activation of the ArsAB pump.

3.13 Effects of mutations in ArsA on ArsD-ArsA interaction.

In the ArsA crystal structure, there are three Sb(III) in its metalloid binding domain. One Sb(III) is connected to Cys113 from A1 and Cys422 from A2 (Site 1), a second to Cys172 from A1 and His453 from A2 (Site 2), and the third to His148 from A1 and Ser420 from A2 (Site 3). Thus, the three metalloid atoms act as molecular glue to bring the A1 and A2 halves of ArsA together, an event that is linked to activation of ATP hydrolysis (Zhou *et al.*, 2000). A recent mutagenesis study suggested that site 1 is a high affinity site (Ruan *et al.*, 2006).

C113A/C422A ArsA had a basal ATPase activity similar to that of the wild type but lacked metalloid-stimulated activity. In this study, we investigate the effects of the elimination on each of these sites in ArsA on ArsD-ArsA interaction. The paired residues in each site were changed to alanines, two at a time. When either one of these sites was eliminated, the mutated ArsA no longer interacts with ArsD by yeast two-hybrid analysis (Figure 27A). Although each of the three mutants had basal ATPase activity, only the site 2 mutant, H148A/S420A ArsA, had a 2-fold stimulation of ATPase activity by Sb(III). However none of them was further enhanced by ArsD (Figure 27B). These results suggest that all of the three sites are required for interaction with ArsD, and at least ArsA sites 1 and 3, if not all, are required for metalloid-stimulated ATPase activity.

CHAPTER 4

DISCUSSION

Metallochaperones for copper have been shown to protect both prokaryotic and eukaryotic cells from excess of copper by sequestering the metal for delivery to transport ATPases that act as extrusion pumps to confer resistance on the cell (Rosenzweig, 2002). In addition, copper chaperones have been identified that are necessary for sequestering copper in the cytosol to protect cells from intracellular copper-scavenging systems such as metallothioneins and for delivery and incorporation into metalloenzymes such as superoxide dismutase (Finney and O'Halloran, 2003; Rae et al., 1999). Resistance to many toxic metals is conferred by efflux pumps, including transport ATPases and resistance-nodulation-cell division (RND) transporters (Legatzki et al., 2003; Silver and Phung le, 2005). However, to date, the only other metallochaperones identified appear to be involved in delivery to metal-containing proteins. For example, a nickel chaperone delivers Ni^{2+} to the enzyme urease (Mulrooney and Hausinger, 1990), and the chaperone frataxin delivers iron to partners for assembly of iron-sulfur clusters and heme (Mansy and Cowan, 2004). Here we report the existence of a chaperone for the toxic metalloid arsenic that enhances resistance by delivering the metalloid to the ArsAB efflux pump. Our data indicate that ArsD is a bifunctional protein. We had originally identified it as a weak transcriptional repressor of the *arsRDABC* operon of *Escherichia coli* plasmid R733 (Wu and Rosen, 1993a). We now demonstrate that it has a second function as a

metallochaperone to sequester and deliver metalloids to the ArsAB transport ATPase.

ArsD binds As(III) or Sb(III), and interacts with and transfers metalloids to ArsA. The yeast two-hybrid results indicate that ArsD interacts with ArsA in the absence of added metalloid. However when the active metalloid binding site (MBS1) on ArsD was eliminated by changing the cysteine residues to alanines, little interaction was detected. It implies that either these cysteines provide bonding networks between the two proteins or there is metalloid bound to this site. Since all of the three cysteines in this site are required for binding metalloid, there is little possibility that disulfide bonds could be formed between cysteines in the two proteins. Cysteine-to-serine substitutions which might retain hydrogen bonding networks, however, eliminated the interaction too. Furthermore these serine-substituted ArsD mutants neither activate ArsA ATPase activity nor bind Sb(III) in this mutated site (data not shown), consistent with the idea that it is the metalloid-bound form of ArsD which mainly interacts with ArsA. Furthermore DMA crosslinking results showed an enhanced interaction by either As(III) or Sb(III). We propose that ArsD interacts with ArsA with low affinity in the absence of metalloid and high affinity when metalloid is bound, and in the yeast cytosol, the low affinity form interacts.

ArsD has been shown to bind Sb(III) with higher affinity than ArsA. For transfer of metalloid from a high affinity binding site on one protein to a relatively lower affinity site on another protein, interaction of the two proteins must produce conformational changes in ArsD and/or ArsA. Indeed, the binding of Sb(III) to

ArsD is so tight that its dissociation takes several hours in the absence of ArsA, but the release rate is stimulated by more than four orders of magnitude by ArsA (data not shown). The fact that the MBSs in ArsA and MBS1 in ArsD appear to be involved in the interaction suggests that metalloid binding sites in both proteins are brought into close proximity, allowing transfer of metalloid directly from the binding site on ArsD to the binding site on ArsA. It is plausible that this interaction destabilizes the metalloid binding sites on ArsD, reducing its affinity, while stabilizing the metalloid binding sites on ArsA by occluding the metalloid within the complex, thus enabling transfer of metalloid to ArsA. In this manner, the thermodynamically unfavorable process of transferring the metalloid from a high to a low-affinity site is overcome. While the details of metalloid transfer from ArsD to ArsA are unknown at this time, the mechanism of transfer of copper from the metallochaperone CCS to the superoxide dismutase SOD1 is instructive (Lamb et al., 2001; Torres et al., 2001). The cysteine residues of CCS project into the active site of SOD1. By doing so, Cu(I) bridges the donating cysteine residues of CCS and the receiving histidine residues, stabilizing the heterodimer and facilitating metal transfer. ArsD is highly efficient in its ability to transfer metalloids to the lower affinity site on ArsA, in contrast to other metallochaperones. For example, the bacterial metallochaperone CopZ has lower affinity for Cu(I) than its cognate receptor protein, CopY, so that Cu(I) transfer is simply from a low- to a high-affinity site and is thermodynamically favorable (Cobine et al., 2002). In the case of human metallochaperone Hah1, NMR studies indicate that Cu(I) is slowly transferred to the isolated metal-binding

domains of the Menkes protein that have a 5-10-fold higher affinity (Banci et al., 2005b). In *Saccharomyces cerevisiae*, the metallochaperone Atx1 appears to serve the simple role of protecting Cu(I) from non-specific interactions (Huffman and O'Halloran, 2000). Cu(I) transfer between Atx1 and the Ccc2p ATPase is unaffected by the presence of Cu(I) chelators such as glutathione, and transfer between the sites, which have similar affinities, is reversible and rapid. It is thought that providing a pathway for the transfer process lowers the activation barrier and accelerates transfer. In effect, transfer of copper between the Atx1 and Ccc2p is 'catalysed' by the metallochaperone.

Metalloid transfer studies of the transfer of As(III) or Sb(III) from ArsD to ArsA are consistent with a mechanism in which this metallochaperone accelerates the rate of transfer to its partner. However, for ArsD and ArsA, this transfer process is thermodynamically unfavorable, suggesting that ArsD not only serves the role of protecting the cell from free metalloid but also in loading ArsA for metalloid extrusion. Indeed, because ArsD has a considerably higher affinity for metalloid than ArsA, it can 'scavenge' the cytosol for free metalloid for delivery to ArsA, allowing the ArsAB pump to confer resistance at significantly lower concentrations of As(III). The K_m of ArsB as a secondary carrier is 140 μM (Kuroda et al., 1997), but natural waters range in concentration of total inorganic arsenic from 7 nM to 70 μM , and concentrations of arsenic in drinking water in worst arsenic-contaminated wells in West Bengal and Bangladesh are approximately 40 μM (Smedley and Kinniburgh, 2002). By lowering the concentration of substrate at which the pump functions efficiently, ArsD and ArsA

provide cells with a mechanism to respond to environmental concentrations of metalloid.

Moreover, we have established that ArsD not only delivers metalloids to ArsA but also enhances its ATPase activity at low metalloid concentrations, the first report of a metallochaperone that also activates its cognate efflux pump. As(III) or Sb(III) enhance the ATPase activity of ArsA, so the increase in affinity of ArsA for metalloids in the presence of ArsD can simply be attributed to the higher affinity of ArsD in the ArsD-ArsA complex, that is, ArsD allows for activation of ArsA ATPase at lower metalloid concentrations. As a result, cells expressing only *arsA* and *arsB* were quickly replaced with the cells expressing *arsD*, *arsA* and *arsB* at a subtoxic concentration of As(III). Even though ArsA and ArsB are sufficient to form a functional pump, all *ars* operons with an *arsA* gene have an adjacent *arsD* gene, suggesting that their gene products co-evolved before association with ArsB. In summary, the increase in ArsA efficiency resulting from interaction with ArsD leads *in vitro* to augmented activity of the ArsAB extrusion pump, to greater resistance in organisms with the *arsDAB* genes, and finally to increased fitness for growth in the low but ubiquitous levels of environmental arsenic (Figure 28).

Understanding the nature of the metalloid binding sites on metallochaperones is crucial to a molecular description of the metalloid transfer process. Previous studies suggested that the three vicinal cysteine pairs in ArsD form three independent metalloid binding sites per monomer (Li et al., 2001; Li et al., 2002). For being a transcriptional repressor, ArsD required both the first pair

Cys12-Cys13 and second pair Cys112-Cys113 but not the third pair Cys119-Cys120 to respond to metalloids inducers. In this study we investigated the metalloid binding capacity of ArsD using ICP-MS, in which we directly measured the amount of ArsD-bound Sb(III) or As(III). Not surprisingly, the wild type ArsD has a metalloid binding capacity of three per monomer, and the capacity was reduced when one or more vicinal cysteine pairs were eliminated. Yet Cys18, which is conserved in all ArsD homologues, was found to be required to the site 1 (MBS1) which also includes Cys12 and Cys13. Since ArsD is a native dimer and Cys18 is at a distance from Cys12 or Cys13 in the primary sequence, Cys18 could coordinate with Cys12-Cys13 in the same subunit or between different subunits. In a preliminary bBBr crosslinking experiment, we found that Cys18 on a His-tagged ArsD was able to crosslink to Cys12 or Cys13 on an MBP-ArsD (Lin, Yang, unpublished), suggesting that Cys18 from one subunit of the ArsD dimer may coordinate with Cys12-Cys13 from the other subunit to form a metalloid binding site. As(III) and Sb(III) form three-coordinate complexes with thiols, so a three-cysteine metalloid binding site was expected. The other two sites have cysteine pairs, but the third ligand in these metalloid binding sites is not known.

Considering that ArsD dimer possesses multiple metalloid binding sites, at least one of which is formed between the cysteines on adjacent subunits of the dimer, the binding sites could fill and empty sequentially (Li et al., 2002). Giving credence to our proposal; the crystal structure of the Hah1 metallochaperone revealed that Cu(I) is coordinated by cysteines from the adjacent subunits of the

dimer, with one subunit contributing two bonds and the other one bond (Wernimont et al., 2000). However, solution studies indicate that Cu(I) can be bound by pairs of cysteines within the Hah1 monomer (Banci et al., 2005a): leading to the proposal that dimers form transiently, due to Cu(I) bridging of the two subunits, and that these intermediates form with quite low energy loss that permits rapid metal transfer between subunits. It is thought that metals are transferred between the metallochaperone and target protein by an analogous mechanism; in which the two proteins first form a heterodimer due to the interaction of complementary electrostatic surfaces, bringing the binding sites into close enough proximity to allow the metal to become coordinated by a cysteine on the target protein and subsequently transferred (Arnesano et al., 2004; Wernimont et al., 2000). This would suggest that it is the monomer that interacts and delivers the metal to the target protein. In contrast, our studies indicate that it is the ArsD dimer that interacts with ArsA, excluding any mechanism in which the ArsD dimer dissociates before interacting with ArsA.

Although the cysteines in MBS1 and in MBS2 are required for metalloid-inducibility of expression of the *ars* operon (Li et al., 2001), only those in MBS1 appear responsible for the delivery of metalloids to ArsA. Under these circumstances, one might expect these cysteine residues to interact with a single site on ArsA. Structural studies have revealed that ArsA is arranged into two halves, composed of its homologous N-terminal (A1: residues 1-282) and C-terminal (A2: residues 321-583) halves that are connected by a flexible 25-residue linker (residues 283-320); and that it has two nucleotide and three

metalloid binding sites, which are formed from residues contributed from both halves, that are located at subunit interfaces in close proximity to one another (Zhou et al., 2000). In addition to cysteines, histidines and a serine are also used to co-ordinate antimonite in the metalloid binding-sites; with one Sb(III) bound to His148 (A1) and Ser420 (A2), one to Cys113 (A1) and Cys422 (A2) and one to Cys172 (A1) and His453 (A2) (Zhou et al., 2000). Studies have revealed that these three metalloid binding-sites have different affinities for Sb(III) (Ruan et al., 2006); so that if the Sb(III) was delivered to the low affinity site, it could then be transferred to another site with higher-affinity. It is notable that the three cysteine residues are all within a distance of 5Å of one another, and all the residues are within 6Å, which would facilitate the transfer of metalloids between the three sites. Indeed, while Sb(III) is bound between Cys113 and Cys422 in the crystal structure; Cys422 is actually closer to Cys172, which are within 4Å of one another. It seems plausible that these residues work in conjunction with one another, increasing the number of residues co-ordinating the metalloid, to facilitate its 'extraction' from ArsD. A similar mechanism has been proposed for the cyanobacterial metallochaperone Atx1, which is involved in trafficking copper between the CtaA and PacS ATPases in the plasma and thylakoid membranes, respectively (Tottey et al., 2005). In addition to being co-ordinated by a pair of cysteines, the copper is also co-ordinated by a histidine in Atx1 (Banci et al., 2004; Borrelly et al., 2004). The histidine is located at the end of a loop that is proposed to be flexible, bringing the histidine into contact with the copper to stabilize its acquisition from CtaA, but moving away from the copper to enable its

transfer to PacS. For ArsA, one Sb(III) is bound within 4Å of Cys113, Cys172, Ser420 and Cys422; whilst Cys113, Ser420 and Cys422 are positioned within α -helices, Cys172 is positioned within a more flexible loop that could allow its repositioning depending upon whether ArsA is acquiring or releasing the metalloid. Mutation of these residues caused an increase in the concentration of Sb(III) required for half-maximal activation for metal-stimulated ATPase hydrolysis; with the C113S and C172S proteins giving a 20-fold increase and the Cys422-Ser protein a 200-fold increase (Bhattacharjee et al., 1995). This behaviour is consistent with a role for these residues in receiving metalloids from ArsD and subsequently activating the ArsA ATPase. Docking of the yeast Atx1 metallochaperone onto the soluble copper-binding domain of the Ccc2 ATPase suggests that the interaction is mainly electrostatic with a number of lysines in Atx1 and aspartic and glutamic acid residues in Ccc2, located close to the metal binding-sites, forming H-bonds (Arnesano et al., 2004). It is notable that there are a number of electronegative residues, such as Glu415, Glu416, Asp417, Glu444 and Glu445, clustered around the metalloid binding-sites of ArsA that could interact with complimentary lysines in ArsD.

The yeast two-hybrid results from the assays with truncated versions of ArsD suggested a core region on ArsD for both dimerization and interaction with ArsA. It seems likely that the last 77 residues in ArsD were not required for protein-protein recognition; however when we attempted to purify these truncated ArsDs, they were degraded before or during the purification and had reduced or no metallochaperone activity in ArsA ATPase assays (data not shown). The

involvement of these residues in ArsD in its stability or activity remains uncertain.

TABLES

Table I. Strains

Strain	Genotype	Reference
<i>E. coli</i> strains		
JM109	F' <i>traD36 lacIq</i> $\Delta(lacZ)M15$ <i>proA⁺B⁺</i> / e14 ⁻ (McrA ⁻) $\Delta(lac-$ <i>proAB)</i> <i>thi gyrA96</i> (Nal ^r) <i>endA1</i> <i>hsdR17</i> (r _K ⁻ m _K ⁺) <i>recA1 relA1 supE44</i>	(Sambrook et al., 1989)
JM110	<i>rps</i> (Str ^r) <i>thr leu thi-1 lacY galK galT ara tonA</i> <i>tsx dam dcm supE44</i> $\Delta(lac-proAB)$ [F' <i>traD36</i> <i>proAB lacIqZ</i> Δ M15]	Stratagene
BL21(DE3)	<i>hsdS gal</i> (λ clts857 <i>ind1 Sam7 nin5 lacUV5-</i> T7gene/	(Sambrook et al., 1989)
AW3110	K-12 F ⁻ IN(<i>rrnD-rrnE</i>) Δ <i>ars::cam</i>	(Carlin et al., 1995)
<i>S. cerevisiae</i> strain		
AH109	<i>MATa</i> , <i>trp1-901</i> , <i>leu2-3, 112</i> , <i>ura3-52</i> , <i>his3-</i> <i>200</i> , <i>gal4</i> Δ , <i>gal80</i> Δ , <i>LYS2::GAL1_{UAS}-GAL1_{TATA}-</i> <i>HIS3</i> , <i>GAL2_{UAS}-GAL2_{TATA}-ADE2</i> , <i>URA3::MEL1_{UAS}-MEL1_{TATA}-lacZ</i>	Clontech

Table II. Plasmids.**a. plasmids acquired commercially or from collaborators**

Plasmid	Description	Reference
pET28a	<i>E. coli</i> cloning and expression vector (Kan ^r)	Novagen
pSE380	Expression vector offering <i>trc</i> promoter, <i>lacO</i> operator, <i>lacI^q</i> repressor (Amp ^r)	Invitrogen
pBAD/Myc-His A	Expression vector with PBAD promoter (Amp ^r)	Invitrogen
pACYC184	Cloning vector (Cm ^r and Tc ^r)	(Chang and Cohen, 1978)
pGBT9	GAL4 ₍₁₋₁₄₇₎ DAN-binding domain, <i>TRP1</i> (Amp ^r)	Clontech
pACT2	GAL4 ₍₇₆₈₋₈₈₁₎ activation domain, <i>LEU2</i> (Amp ^r)	Clontech
pMAL-c2X	A vector for expression of MBP-fusion proteins (Amp ^r)	New England Biolabs
pAlterAB1	3.2-kilobase pair <i>HindIII-KpnI</i> fragment containing C-terminal his-tagged <i>arsA</i> and wild type <i>arsB</i> genes cloned into pALTER-1 vector (Amp ^r)	(Bhattacharjee et al., 1995)
pAlterAB2	An additional <i>HindIII</i> site after the end of non-tagged <i>arsA</i> gene in pAlterAB1 (Tc ^r)	(Zhou and Rosen, 1997)
pAlter-ArsC	553-bp <i>NdeI-HindIII</i> fragment of entire <i>arsC</i> gene on pALTER-Ex2 (Tc ^r)	(Shi et al., 2003)
pArsD6HΔ119-120 and series	<i>arsD</i> residues 119 and 120 are replaced by six histidine codons with indicated mutations of cysteine codons in plasmid pALTER1 (Amp ^r)	(Li et al., 2001)
pMAL-ArsR	354-bp <i>BamHI-HindIII</i> fragment of entire <i>arsR</i> gene on pMAL-c2X	Jun Ye, unpublished

Table II. Plasmids (continued)**b. plasmids constructed in this study**

Plasmid	Description	Reference
pACBAD	380-bp <i>Bcl</i> I- <i>Eco</i> RI fragment containing P _{BAD} promoter on pBAD/Myc-His A cloned into pACYC184 (Tc ^r)	This study
pACBAD-D	Entire <i>arsD</i> gene in pAC-BAD (Tc ^r)	This study
pGBT-X series	<i>arsA</i> , <i>C</i> , <i>D</i> , and <i>R</i> genes cloned in pGBT9 (Amp ^r)	This study
pGBT-D_x series	Various deletions and/or mutations of <i>arsD</i> gene cloned in pGBT9 (Amp ^r)	This study
pACT-X series	<i>arsA</i> , <i>C</i> , <i>D</i> , and <i>R</i> genes cloned in pACT2 (Amp ^r)	This study
pACT-A_x series	Mutations of <i>arsA</i> gene in pACT9 (Amp ^r)	This study
pSE-X series	<i>arsD</i> , <i>arsAB</i> or <i>arsB</i> genes in pSE380 (Amp ^r)	This study
pMAL-D_x series	Deletions and/or mutations of <i>arsD</i> gene in pMAL-c2X (Amp ^r)	This study

Table III. Oligonucleotide primers

The added restriction sites are underlined. Altered nucleotides from original sequence are written in lowercase letters.

a. primers used for sequencing or vector plasmid modification

Primer	Sequence (5'-3')	Target/ Direction
<i>Sequencing</i>		
T7 promo	TAATACGACTCACTATAGGG	T7 promoter region/ Forward
Tac promo	GACAATTAATCATCCGGCTCG	Tac promoter region/ Forward
GAL4 AD	CCACTACAATGGATGATGTATA TAAC	GAL4 activation domain/ Forward
GAL4 BD	CATCGGAAGAGAGTAGTAACA AAGG	GAL4 DNA-binding domain/ Forward
<i>Vector plasmid modification</i>		
ET XbaHind(+)	CAATTCCCCTCTAGAA <u>gct</u> TTT TGTTTAAC	+319 to +349 on pET28a/ toward MCS
ET StuI(-)	GCTCGAGTGCGGCCGCA <u>gGCc</u> <u>I</u> GTCGACGG	+157 to +186 on pET28a/ toward MCS
BAD pro	GCCATACT <u>gaTC</u> ATACTCCCGC	-31 to -9 on pBAD/Myc-His
BclI(+)	C	A/ toward promoter region
BAD Myc(-)	CAGATCCTCTTCTGAGATGAG	Myc epitope on pBAD/Mys-His A/ Reverse

Table III. Oligonucleotide primers (continued)**b. primers used for cloning of *ars* genes**

Primer	Sequence (5'-3')	Target/ Direction
D PstI(-)	GCATGCCTGCAGGTCGACTCTA G	+8 to +30 downstream of <i>arsD</i> on pArsD6HΔ119- 120/ Reverse
D HindIII(-)	GGCAGACCTTCTGCCCCGGAAG C	+119 to +221 on <i>arsD</i> / Reverse
D XbaI(-)96(+)	GGGCGAATTCTaGaTTCAGTTAA C	-96 to -73 on <i>arsD</i> gene/ Forward
Dwt(-)	AGCTCGAATTCGGATCCTTAGTG GTGGTGtTaGcaGcaAGAAGTATT ACC	His-tag region on pArsD6HΔ119-120/ Reverse
D NcoI(+)	GGTATTTA <u>ccATGg</u> AAACGTTAAT GG	-10 to +16 on <i>arsD</i> gene/ Forward
D EcoR(-)	CTGTAAGAATT <u>c</u> CATATGTCCTC C	+9 to +32 downstream of <i>arsD</i> gene on pSE-DAB/ Reverse
R EcoNco(+)	GGATTT <u>CAGAATTC</u> Ac <u>caTGg</u> AAC TAACACC	<i>arsR</i> start codon region on pMAL-ArsR/ Forward
R BamH(-)	CGACGGCC <u>gGatCC</u> AAGCTTTTA GATGC	<i>arsR</i> end codon region on pMAL-ArsR/ Reverse
A NcoI(+)	acatg <u>ccatg</u> ATATGCAATTCTTACA GAATATCCC	<i>arsA</i> start condon region on pAlterAB1
A KpnI(-)	tcgggT <u>ACCC</u> AGCAAGTTGTTTGA GTTTGTC	<i>arsA</i> end condon region on pAlterAB1
C NcoI(+)	ACTGCAGCGGCC <u>cCATGg</u> ATATG AGC	<i>arsC</i> start codon region on pAlter-ArsC/ Forward
C BamH(-)	CGTTACCATC <u>GGATc</u> CAGGCATG	+44 to +69 downstream of <i>arsC</i> on pAlter-ArsC/ Reverse

Table III. Oligonucleotide primers (continued)**c. primers used for cloning of *arsD* truncations**

Primer	Sequence (5'-3')	Target/ Direction
D P109Bam(-)	ACCACAG <u>gAtCCTTAAGGCGCTA</u> ATC	+317 to +342 on <i>arsD</i> / Reverse
D E93Bam(-)	GCCAAACCAG <u>gGAtCCTACTCAG</u> CGCGTTTC	+267 to +297 on <i>arsD</i> / Reverse
D L77Bam(-)	CACTGTTTCG <u>ggATCCTATAACAA</u> CAATGGCAG	+217 to +249 on <i>arsD</i> / Reverse
D I65Bam(-)	CTGCCCC <u>GGAtcCTTAAATAAAC</u> GCTTTG	+188 to +261 on <i>arsD</i> / Reverse
D I43Bam(-)	CGCAAGATT <u>GGatCcCTAAATTTG</u> TACACCGC	+116 to +147 on <i>arsD</i> / Reverse
D S14Eco(+)	GCGATG <u>gaaTtCAGCACCGGCGT</u> CTGC	+28 to +54 on <i>arsD</i> / Forward
D C12Eco(+)	TTTGACCCG <u>GaatTcTGTTGCAGC</u> ACC	+19 to +45 on <i>arsD</i> / Forward
D D8Eco(+)	ACGTTAATGG <u>GaATTcGACCCGGC</u> GATGTG	+7 to +36 on <i>arsD</i> / Forward
D M5Nco(+)	GAAAACGT <u>ccATGGTATTTGACC</u> CG	+3 to +27 on <i>arsD</i> / Forward
D Xmal(+)	GCGAGGTATT <u>cccGggGAAAACGT</u> TAATGGTATTTGACCC	-14 to +26 on <i>arsD</i> / Forward

Table III. Oligonucleotide primers (continued)**d. primers used for site-directed mutagenesis (cysteine to alanine)**

Primer Name	Sequence (5'-3')	Target/ Direction
D C12A(+)	GGTATTTGACCCGGCGATGgcT TGCAGCACCGG	+15 to +47 on <i>arsD</i> / Forward
D C12A(-)	CCGGTGCTGCAAgcCATCGCC GGGTCAAATACC	+15 to +47 on <i>arsD</i> / Reverse
D C13A(+)	ATTTGACCCGGCGATGTGTgcC AGCACCGGCG	+18 to +49 on <i>arsD</i> / Forward
D C13A(-)	CGCCGGTGCTGgcACACATCG CCGGGTCAAAT	+18 to +49 on <i>arsD</i> / Reverse
D C12/13A(+)	GGTATTTGACCCGGCGATGgcT gcCAGCACCGGCGTC	+15 to +51 on <i>arsD</i> / Forward
D C12/13A(-)	GACGCCGGTGCTGgcAgcCATC GCCGGGTCAAATACC	+15 to +51 on <i>arsD</i> / Reverse
D C18A(+)	GCACCGGCGTCgcCGGTACAG ATGTTGATCAGGC	+41 to +74 on <i>arsD</i> / Forward
D C18A(-)	GCCTGATCAACATCTGTACCGg cGACGCCGGTGC	+41 to +74 on <i>arsD</i> / Reverse

Table III. Oligonucleotide primers (continued)

e. primers used for site-directed mutagenesis (cysteine to serine)

Primer Name	Sequence (5'-3')	Target/ Direction
D C12S(+)	GGTATTTGACCCGGCGATGTcT TGCAGCACCGGCG	+15 to +49 on <i>arsD</i> / Forward
D C12S(-)	CGCCGGTGCTGCAAgACATCG CCGGGTCAAATACC	+15 to +49 on <i>arsD</i> / Reverse
D C13S(+)	GGTATTTGACCCGGCGATGTG TTcCAGCACCGGCG	+15 to +49 on <i>arsD</i> / Forward
D C13S(-)	CGCCGGTGCTGgAACACATCG CCGGGTCAAATACC	+15 to +49 on <i>arsD</i> / Reverse
D C12/13S(+)	GGTATTTGACCCGGCGATGTcT TcCAGCACCGGCG	+15 to +49 on <i>arsD</i> / Forward
D C12/13S(-)	CGCCGGTGCTGgAAgACATCG CCGGGTCAAATACC	+15 to +49 on <i>arsD</i> / Reverse
D C18S(+)	CAGCACCGGCGTCTcCGGTAC AGATGTTGATC	+39 to +70 on <i>arsD</i> / Forward
D C18S(-)	GATCAACATCTGTACCGgAGAC GCCGGTGCTG	+39 to +70 on <i>arsD</i> / Reverse

Figure 1. Multiple alignment of ArsD homologues.

Representative ArsD homologues are from: *E. coli* plasmid R773 (accession number U13073); *Salmonella typhimurium* plasmid R64 (U38947); *Klebsiella oxytoca* plasmid pMH12 (AF168737); *Acidiphilium multivorum* plasmid pKW301 (AB004659); *E. coli* plasmid R46 (AY046276); *Shewanella putrefaciens* CN-32 (AALB01000006); *Leptospirillum ferriphilum* transposon TnLfArs (DQ057986); *Alcaligenes faecalis* NCIB 8687 (AY297781); *Methylobacillus flagellatus* KT (CP000284); *Azoarcus* sp. (CR555306); *Dechloromonas aromatica* RCB (CP000089); *Lactobacillus plantarum* plasmid pWCFS103 (CR377166); *Rhodoferrax ferrireducens* DSM 15236 (CP000267); *Rhodococcus erythropolis* PR4 plasmid pREL1 (AP008931); *Rhodococcus erythropolis* plasmid pBD2 (AY223810); *Magnetospirillum magneticum* AMB-1 (AP007255); *Rhodospirillum rubrum* (CP000230); *Burkholderia vietnamiensis* G4 (AAEH02000012); *Rhodopirellula baltica* SH 1 (CAD76333); *Listeria innocua* plasmid pLI100 (AL592102); *Staphylococcus saprophyticus* plasmid pSSP1 (AP008935); *Mycobacterium flavescens* PYR-GCK (AAPA01000005); *Syntrophomonas wolfei* (AAJG01000050); *Staphylococcus epidermidis* RP62A (CP000029); *Acidithiobacillus caldus* transposon TnAtcArs (AY821803); *Photobacterium profundum* 3TCK (AAPH01000002); *Psychromonas* sp. CNPT3 (AAPG01000022); *Alkalilimnicola ehrlichei* MLHE-1 (AALK01000009); *Geobacter uraniumreducens* Rf4 (AAON01000041); *Bacillus cereus* plasmid pBc10987 (AE017195); *Bacteroides thetaiotaomicron* VPI-5482 (AE015928); *Desulfitobacterium hafniense* Y51 (AP008230); *Bacillus* sp. CDB3 (AAD51848); *Halobacterium* sp. NRC-1 megaplasmid pNRC100 (AF016485); *Alkaliphilus metalliredigenes* QYMF (AAKU01000003). Cysteine residues are indicated. The multiple alignment was calculated with CLUSTAL W (Thompson et al., 1994).

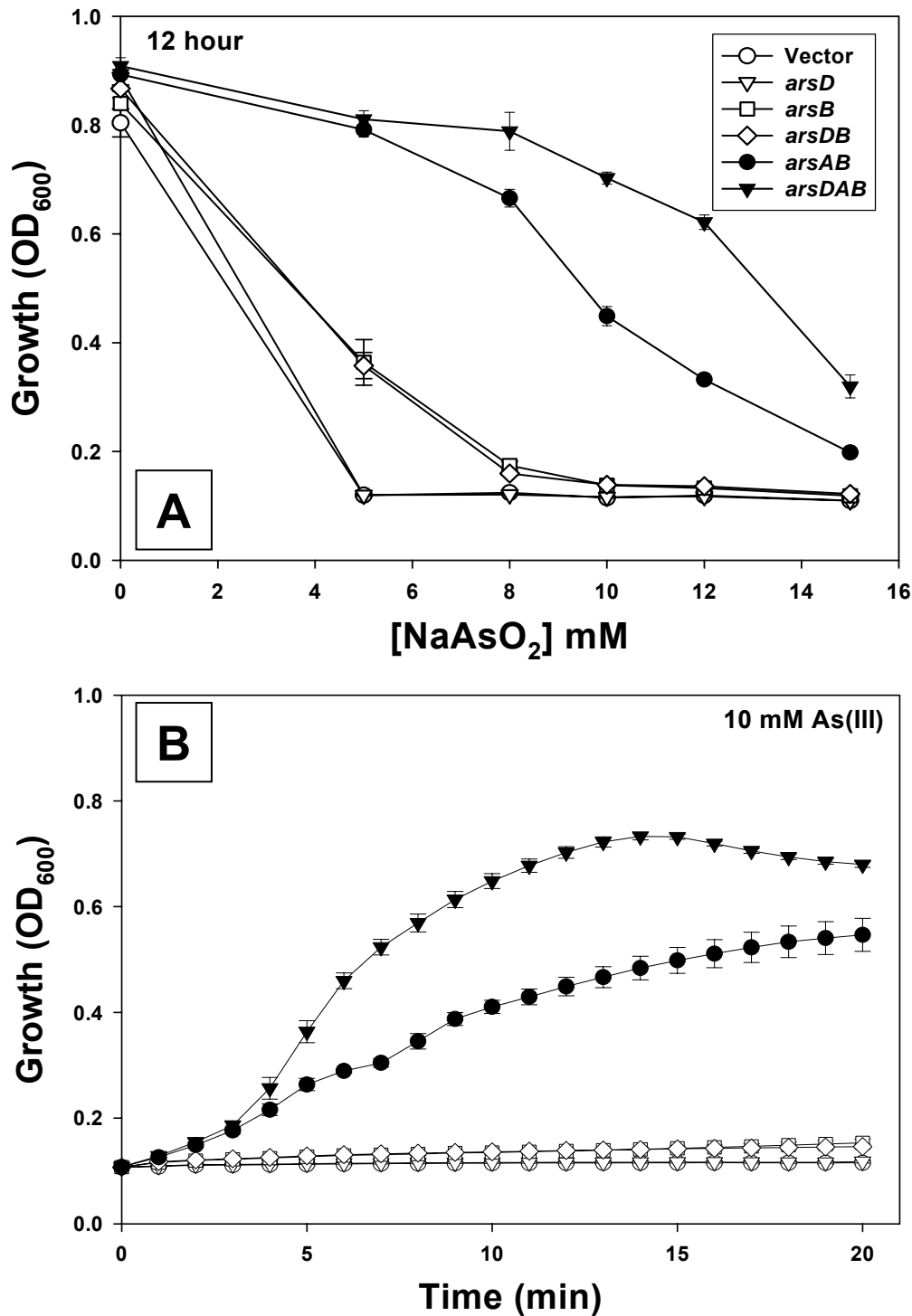


Figure 2. Contributions of *ars* genes to arsenite resistance.

Cells of *E. coli* strain AW3110 ($\Delta arsRBC$) harboring vector plasmids pSE380 and pACBAD (vectors, ○) or pSE380 and pACBAD-D (*arsD*, ▽), pSE-B and pACBAD (*arsB*, □), pSE-B and pACBAD-D (*arsDB*, ◇), pSE-AB and pACBAD (*arsAB*, ●) or pSE-AB and pACBAD-D (*arsDAB*, ▼) were grown in LB medium overnight. The cells were then diluted 50-fold into LB medium at 37 °C and the absorbance at 600 nm were monitored. The values are the mean of three separate experiments.

A: at different concentrations of sodium arsenite after 12 hr of growth.

B: at 10 mM sodium arsenite.

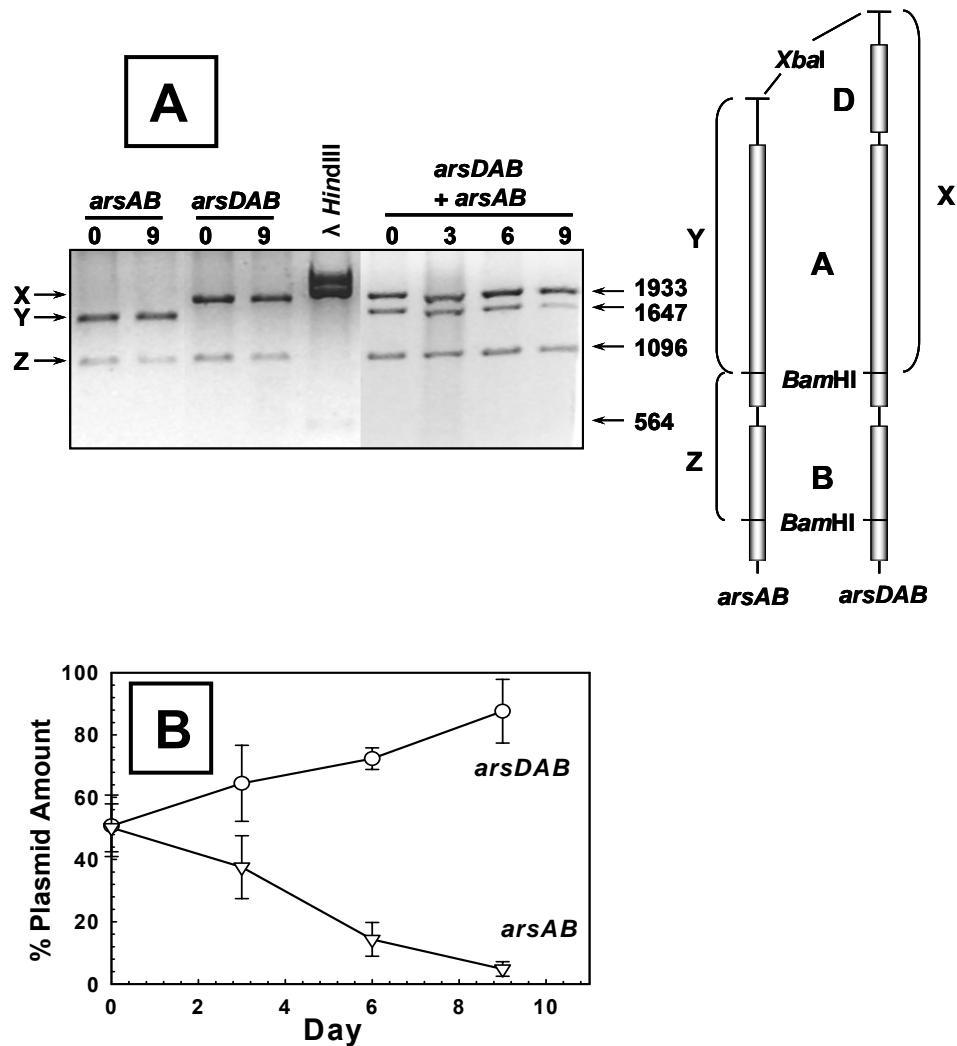


Figure 3. Molecular competition in an arsenic-containing environment.

A: Molecular competition between cells with *arsDAB* and *arsAB*. Assays were performed as described in MATERIALS AND METHODS. Mixed cultures of cells of *E. coli* strain AW3110 bearing either pSE-AB or pSE-DAB were allowed to grow for 9 days with daily dilutions into fresh medium. The plasmids were extracted and analyzed by restriction enzyme digestion with *XbaI* and *BamHI*. Both plasmids produced a large fragment (not shown) of vector DNA, and both produced a small *arsB*-containing *BamHI* fragment of 1.1 kb (fragment Z). Digestion of pSE-AB also produced a fragment of 1.6 kb containing most of *arsA* (fragment Y), and digestion of pSE-DAB yielded the 1.9 kb fragment X containing *arsD* and most of *arsA*.

B: Cells with only *arsAB* are lost from the population. The fraction of each plasmid was calculated by quantifying bands X, Y and Z by densitometry. The percentage of the cells with each plasmid was calculated as following: *arsDAB*: $X/((\text{vector} + Z)/2)$; *arsAB*: $Y/((\text{vector} + Z)/2)$. The data are the mean of values from five separate gels representing three independent experiments.

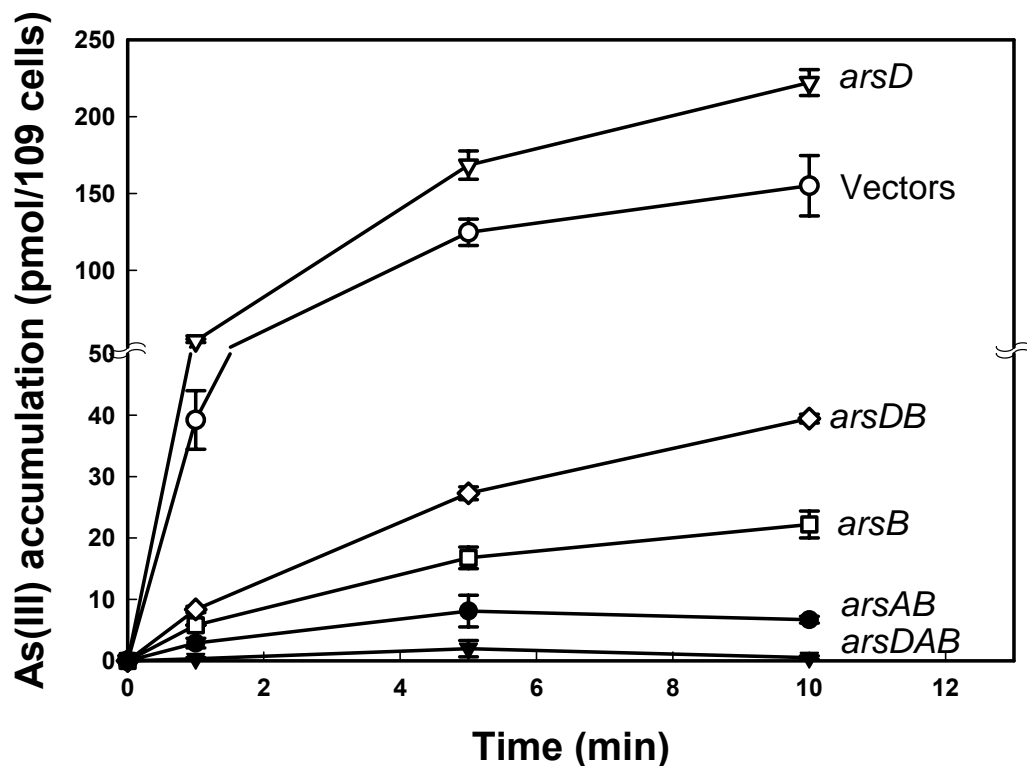


Figure 4. Contributions of *ars* genes to As(III) accumulation in cells.

Transport of As(III) in AW3110 bearing vector plasmids pSE380 and pACBAD (○) or plasmids with *arsD* (▽), *arsB* (□), *arsDB* (◇), *arsAB* (●) or *arsDAB* (▼) was assayed as described in MATERIALS AND METHODS. The values are the average of two independent assays.

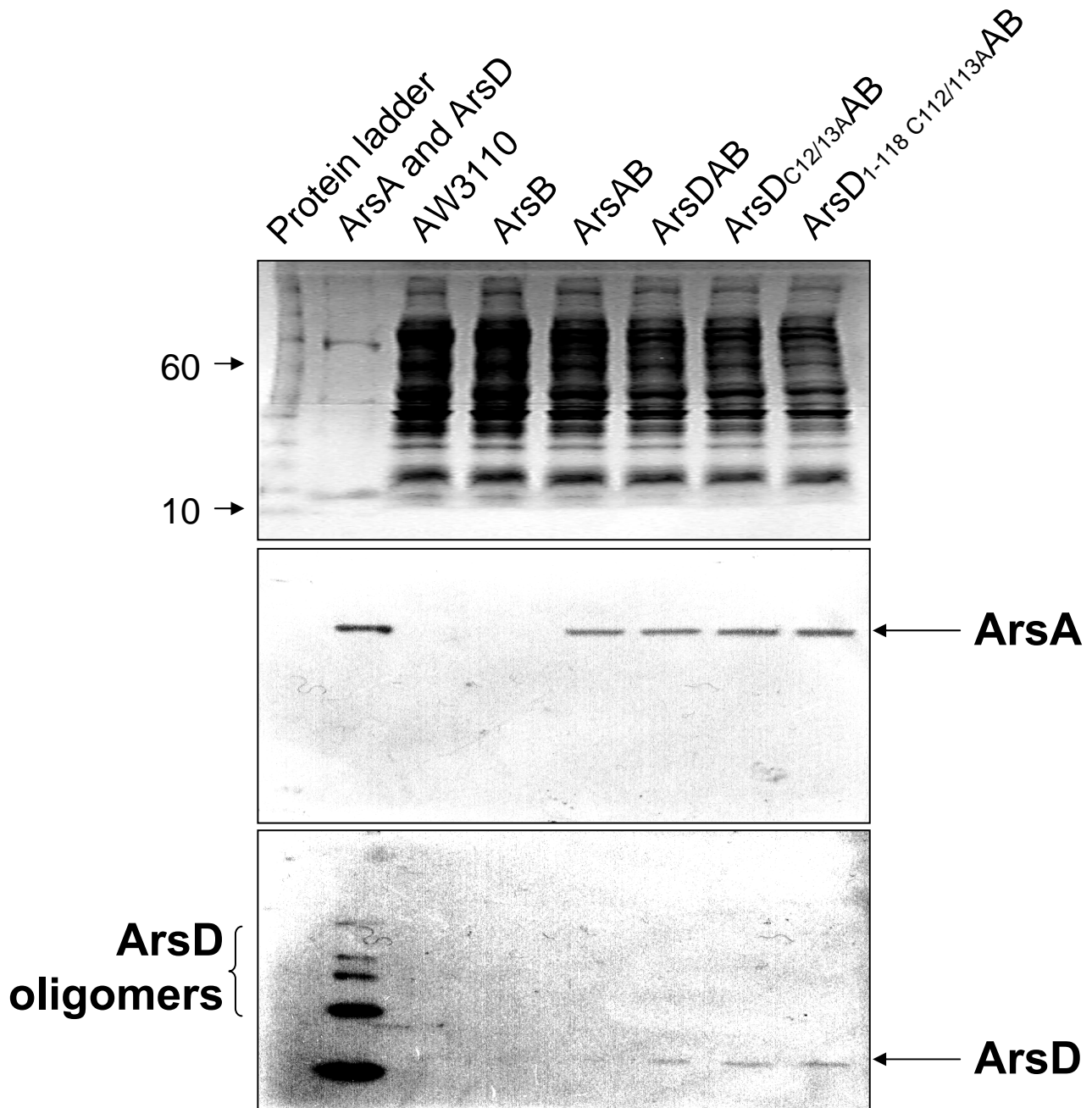


Figure 5. Protein expression levels in As(III) resistance and transport assays.

Harvested cell cultures (100 μ l) from resistance or transport assay were boiled with loading buffer and analyzed on a 10%-16% step gel.

Top: Coomassie blue staining

Middle: Immunoblotting with anti-ArsA antibody

Bottom: Immunoblotting with anti-ArsD antibody

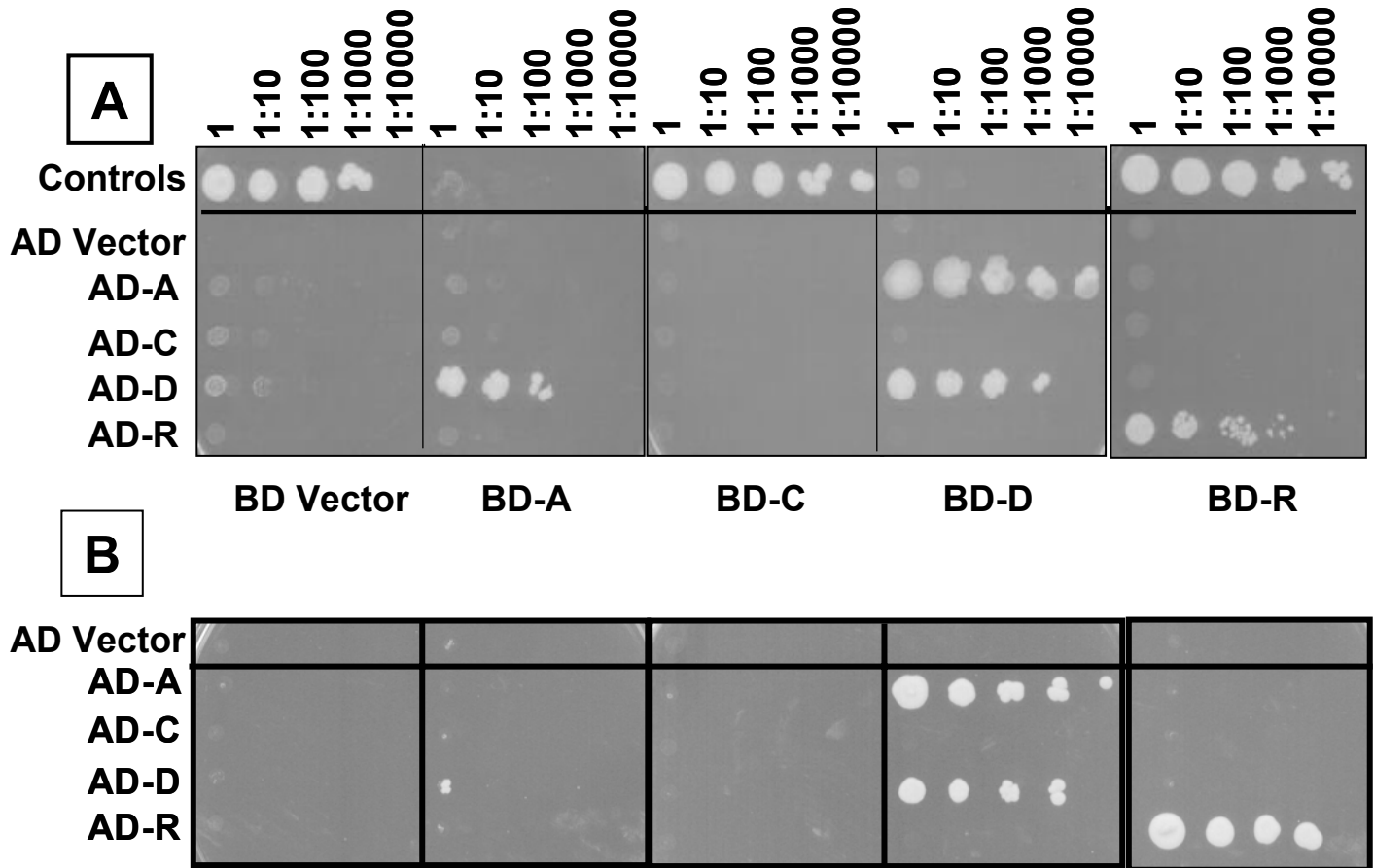


Figure 6. Yeast two-hybrid assays with wild type *ars* genes.

Yeast strain AH109 bearing both GAL4 AD and BD fusion plasmids was grown in SD medium overnight and then inoculated on agar plates with SD lacking histidine with 10-fold serial dilutions. The plates were incubated at 30° for 2-3 days. As a positive control, pVA3 (BD-p53) was expressed with pTD1 (AD-T antigen); as a negative control, vector plasmid pGBT9 was expressed with pACT2 (alternating in the top row).

A: In the absence of metalloids.

B: In the presence of 100 μM potassium antimonyl tartrate (Sb(III)).

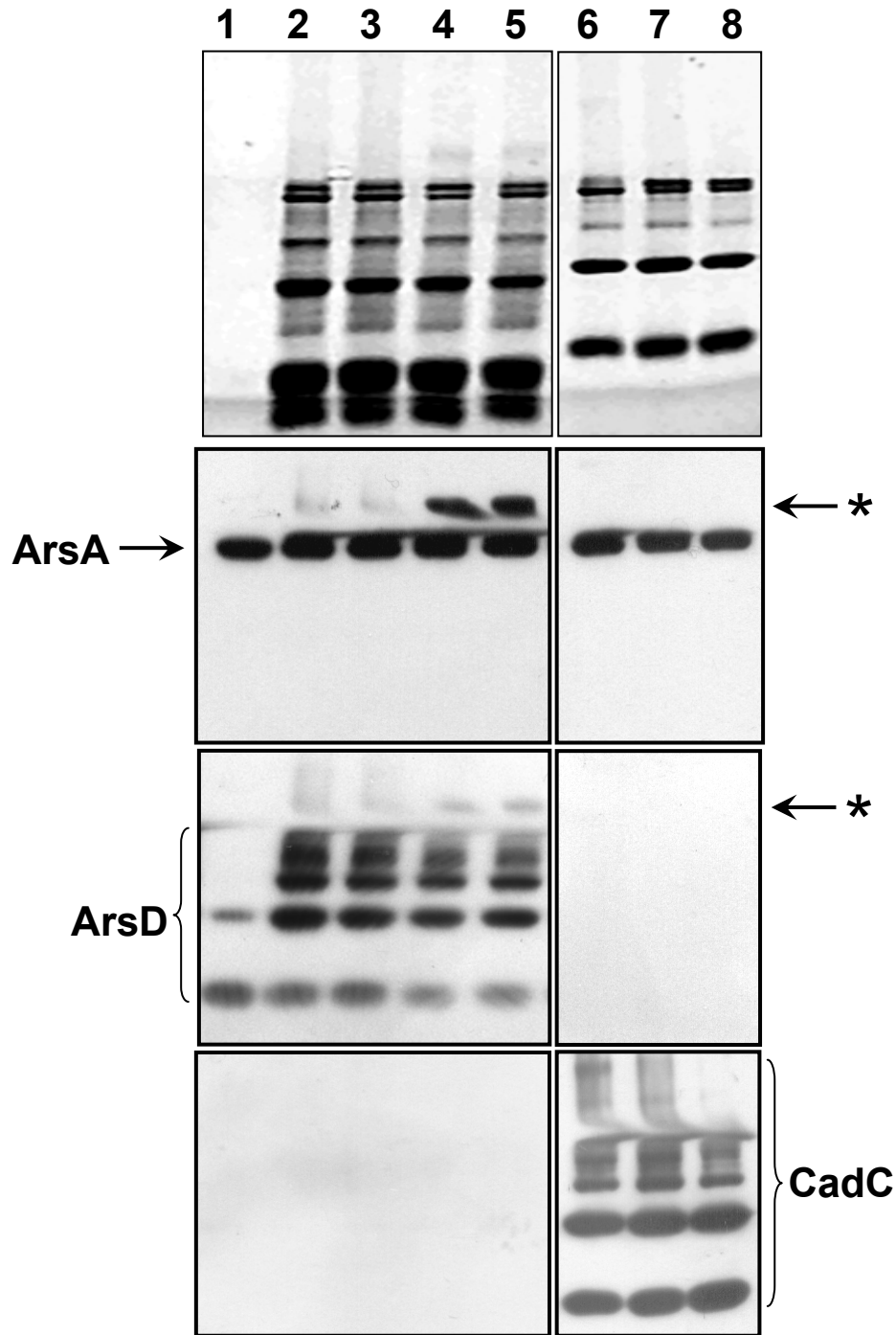


Figure 7. bBBR crosslinking.

The indicated proteins (16 μ M each) were incubated with 0.5 mM bBBR and/or 1 mM each of potassium antimonyl tartrate, MgCl_2 and ATP. Samples were analyzed by SDS-PAGE and visualized at UV 365 nm (**top panel**) or immunoblotting with anti-ArsA (**second panel**), anti-ArsD (**third panel**) or anti-CadC (**bottom panel**). Lane 1: ArsA + ArsD; Lane 2: ArsA + ArsD + bBBR; Lane 3: ArsA + ArsD + Sb(III) + bBBR; Lane 4: ArsA + ArsD + MgATP + bBBR; Lane 5: ArsA + ArsD + Sb(III) + MgATP + bBBR; Lane 6: ArsA + CadC + bBBR; Lane 7: ArsA + CadC + MgATP + bBBR; Lane 8: ArsA + CadC + Sb(III) + MgATP + bBBR. The positions of individual proteins are indicated by arrows, and (*) shows the location of the ArsD-ArsA adduct.

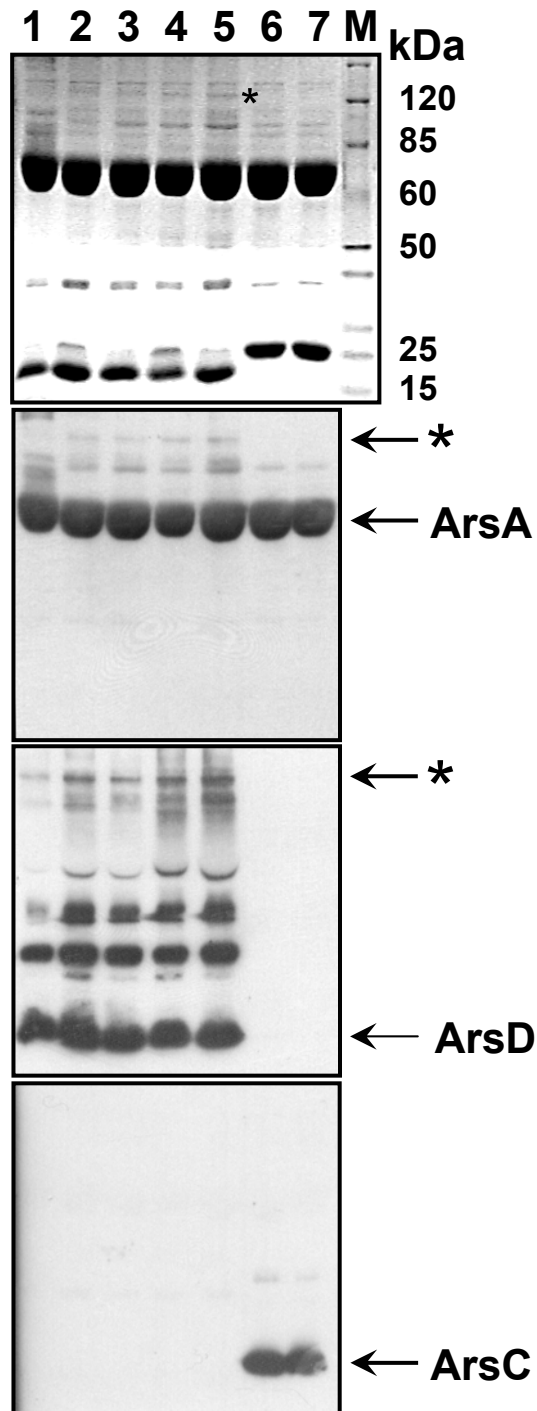


Figure 8. DMA crosslinking.

ArsD1-109, ArsA and ArsC, each with an N- or C-terminal six-histidine tag (each at $30 \mu\text{M}$) were incubated with 10 mM DMA. The samples were analyzed by SDS PAGE using a step gradient gel of 10% and 16% acrylamide followed by Coomassie Blue staining (**top panel**); immunoblotting with anti-ArsA (**second panel**); anti-ArsD (**third panel**); or anti-ArsC (**bottom panel**). Lane 1: ArsA+ArsD; lane 2: ArsA + ArsD + ATP; lane 3: ArsA + ArsD + MgATP; lane 4: ArsA + ArsD + MgATP + Sb(III); lane 5: ArsA + ArsD + MgATP + As (III); lane 6: ArsA + ArsC + MgATP + Sb (III); lane 7: ArsA + ArsC + MgATP + As(III); lane M: molecular weight marker proteins. The positions of individual proteins are indicated by arrows, and (*) shows the location of the ArsD-ArsA adduct.

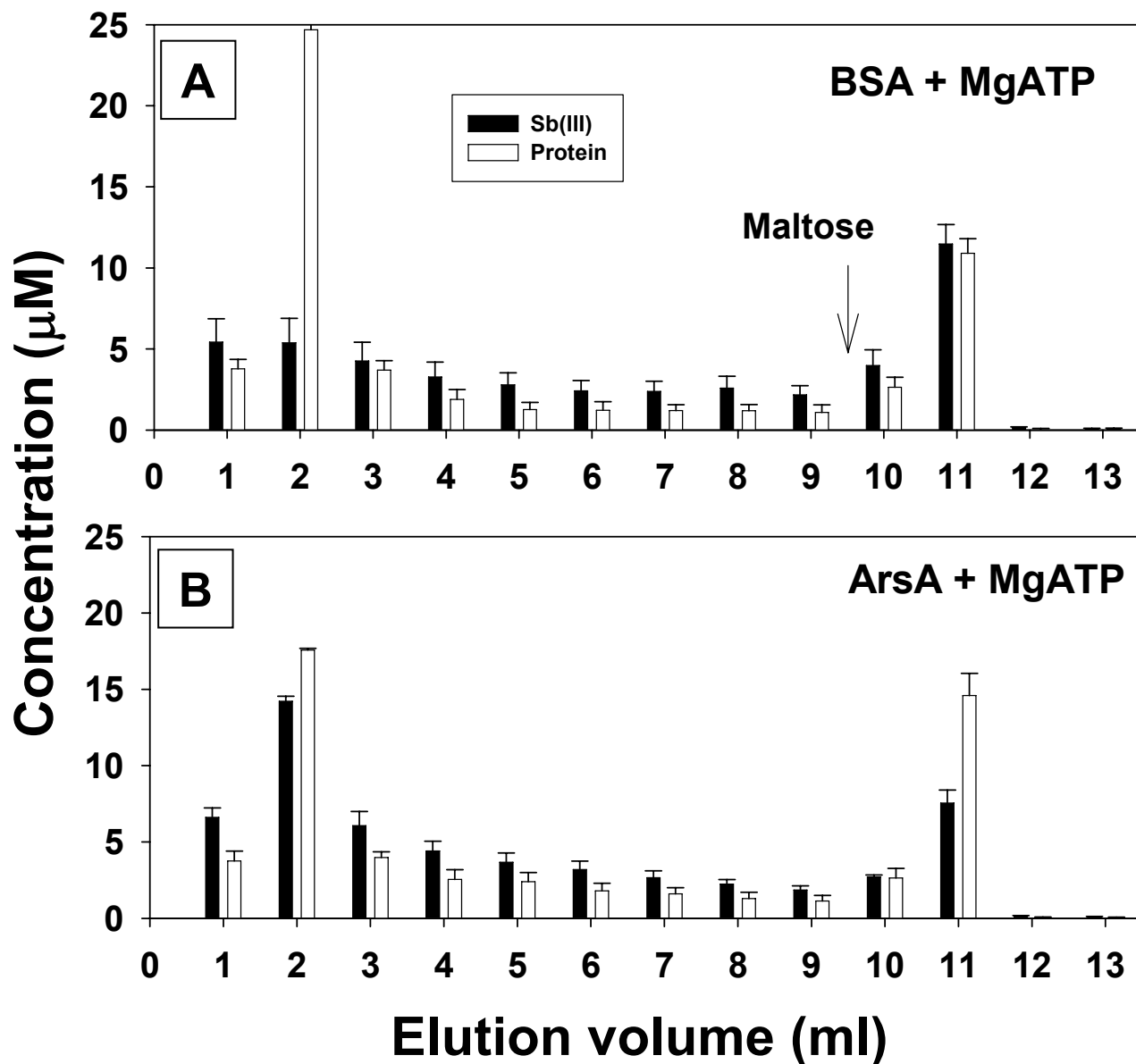


Figure 9. ArsA releases Sb(III) from ArsD.

Sb(III)-MBP-ArsD was bound to a 2-ml amylose column, as described in MATERIALS AND METHODS. 1 ml of either 20 μM BSA (**A**) or ArsA (**B**) incubated with 1 mM MgATP was then applied to the column. The column was washed with 8 ml of column buffer, following which MBP-ArsD was eluted with 2 ml of 10 mM maltose. The molar concentration of each protein in fractions was estimated from the absorption at 280 nm (white bars), and amount of Sb(III) by ICP-MS (black bars).

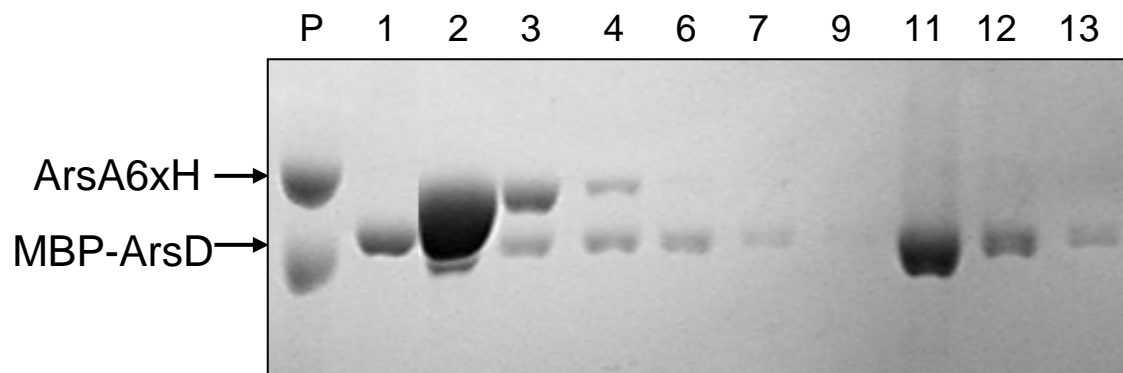


Figure 10. SDS PAGE with elution fractions from Sb(III) transfer assay.

The identity of the protein in each fraction was determined by SDS-PAGE. BSA or ArsA eluted primarily in fraction 2, and most of the MBP-ArsD eluted in fraction 11.

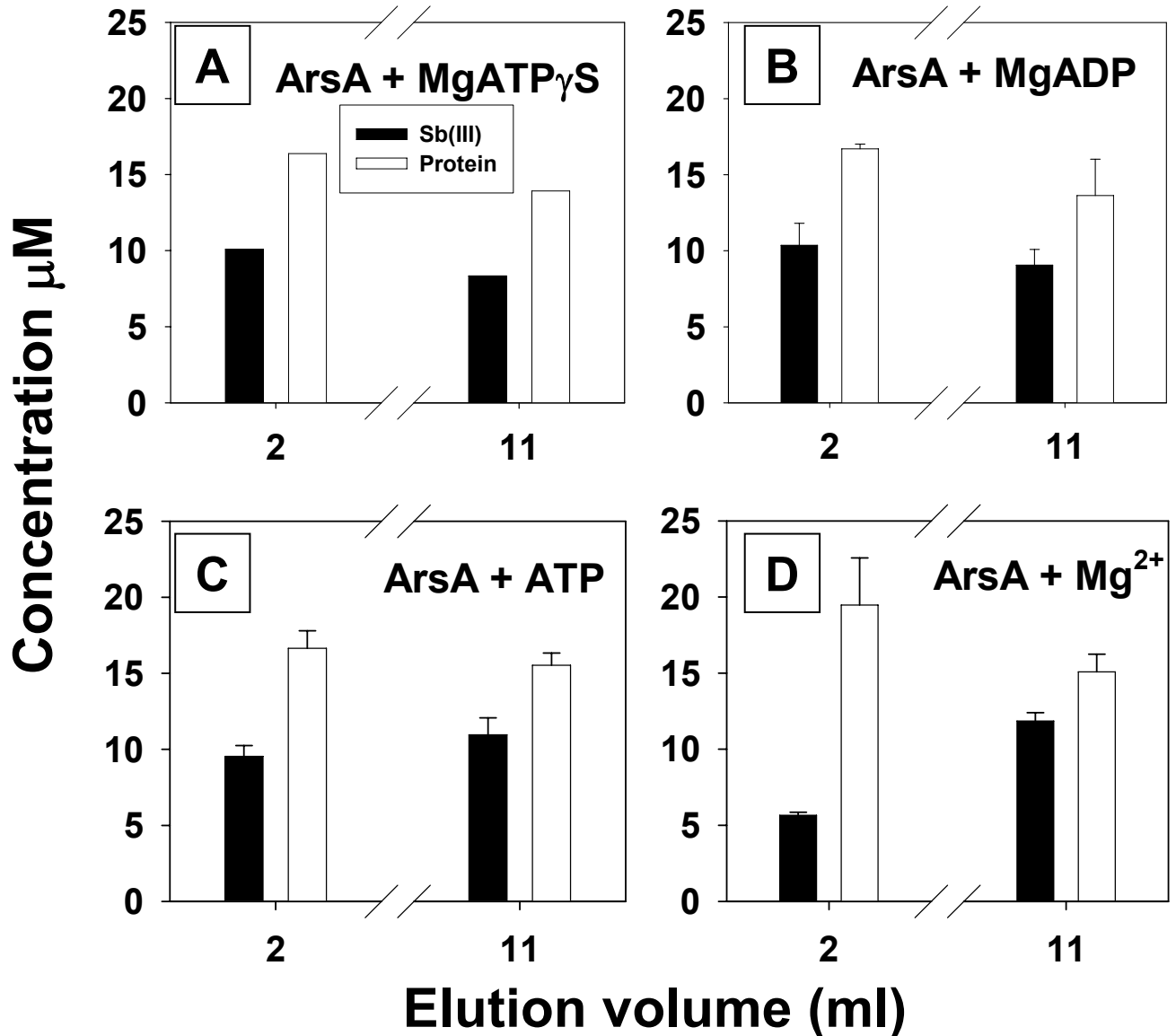


Figure 11. Effects of nucleotides on the ArsA-induced release of Sb(III) from ArsD.

As described in METHODS AND MATERIALS, 1 ml of ArsA incubated with either MgATP γ S (A), MgADP (B), ATP (C) or MgCl₂ (D) was applied to the Sb(III)-MBP-ArsD-bound amylose column. After washing and elution, the molar concentration of each protein in fractions and amount of Sb(III) was estimated.

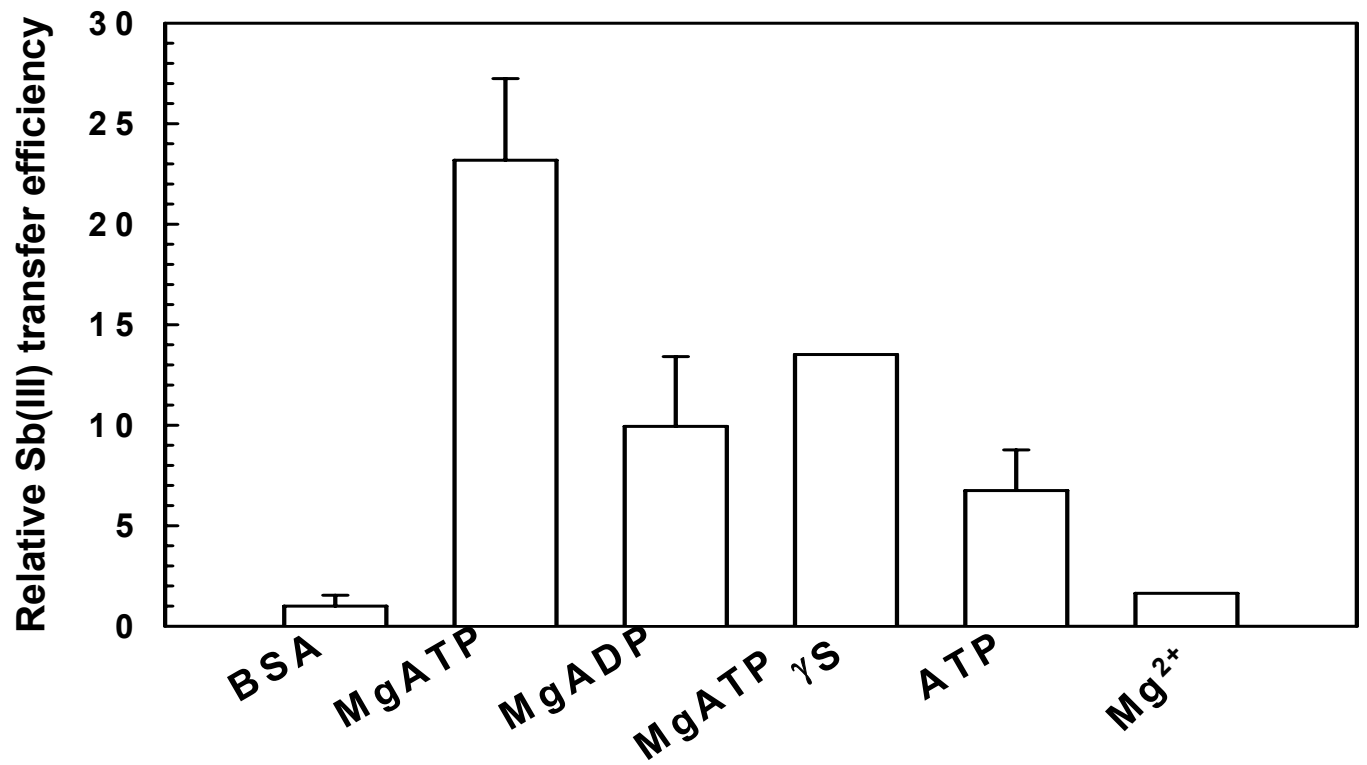


Figure 12. Sb(III) transfer efficiency in different conditions.

Sb(III) transfer efficiency was calculated as $([\text{Sb(III)ArsA}]/[\text{ArsA}])/([\text{Sb(III)ArsD}]/[\text{ArsD}])$. The values were expressed relative to the value with BSA. These are the average of two independent assays.

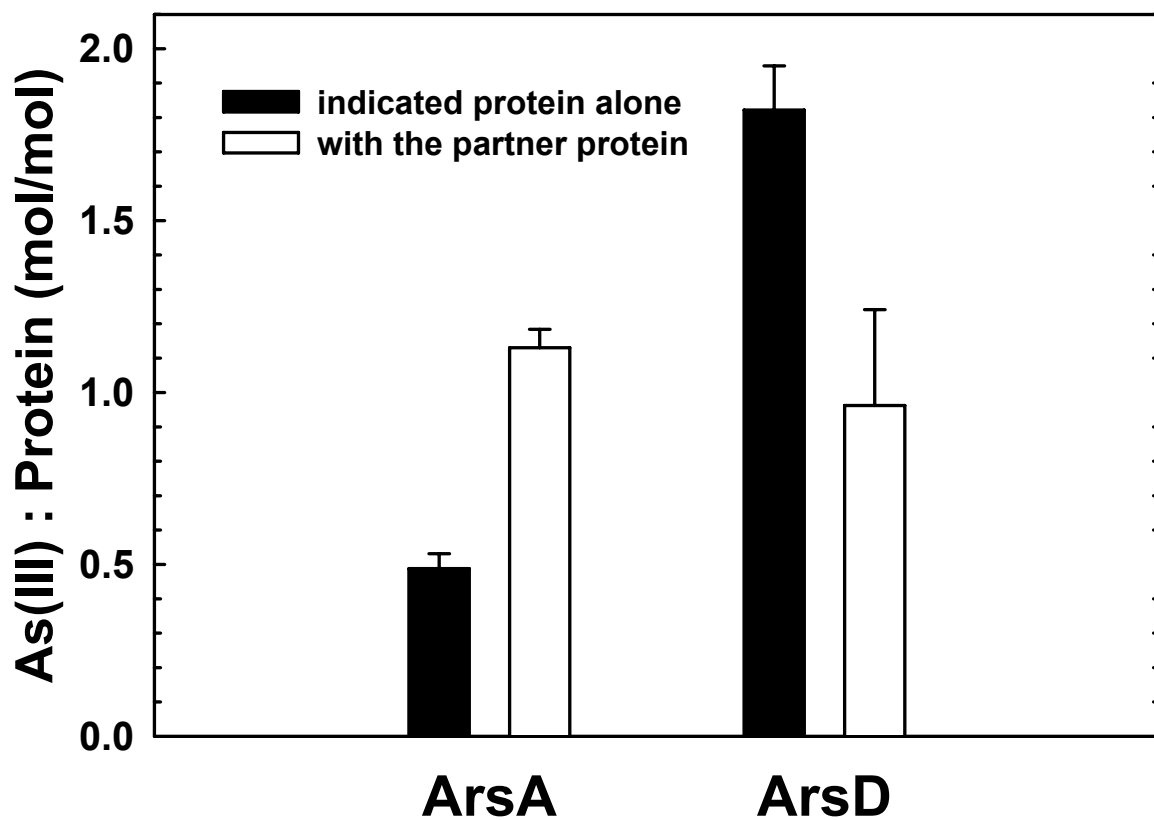


Figure 13. As(III) transfer from ArsD to ArsA.

The molar ratio of As(III) to either ArsA or ArsD monomer was measured with either protein alone (black bars) or in the presence of the partner protein (white bars) using the metalloid transfer assay as described. The values are the mean of three independent assays.

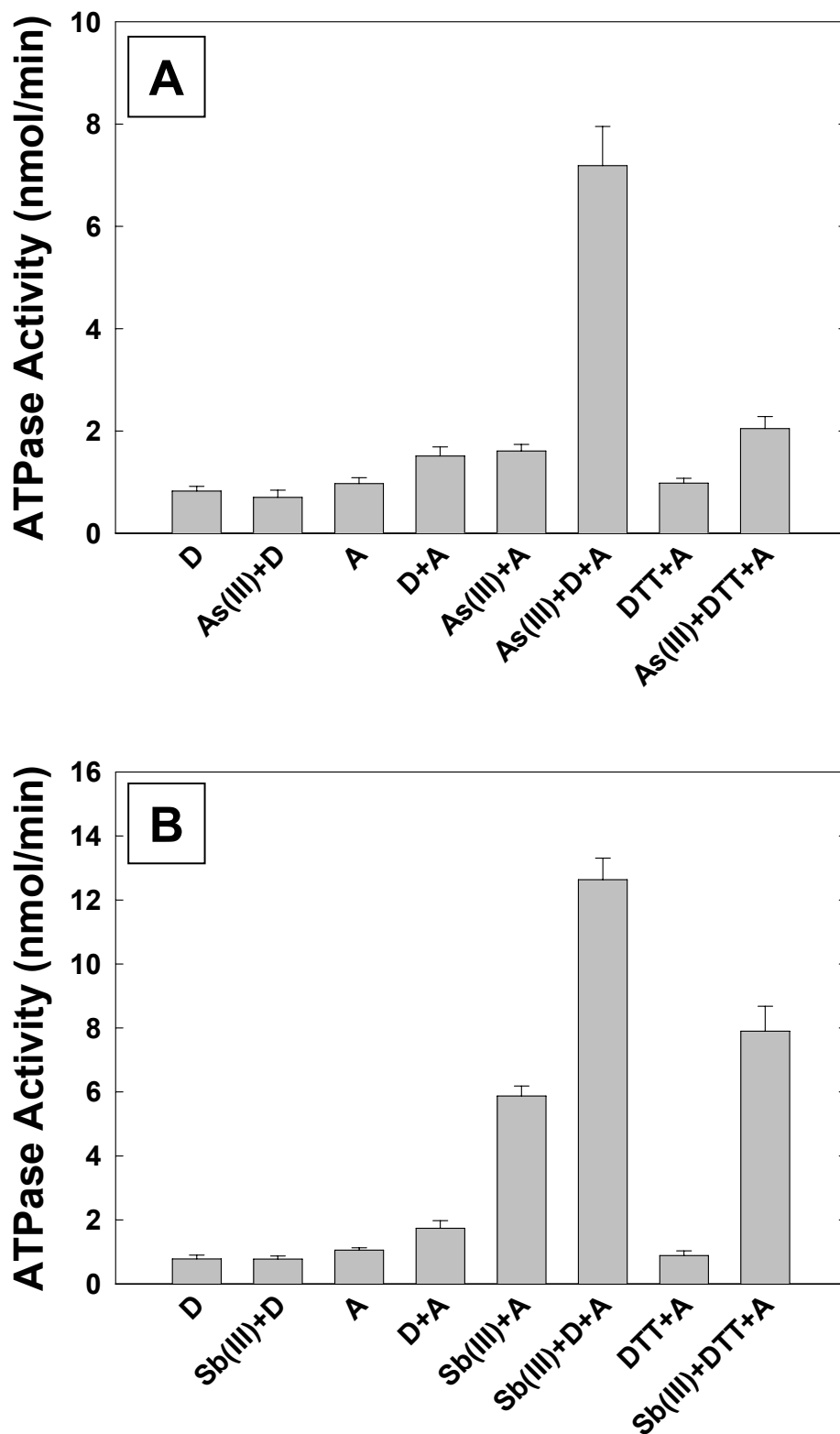


Figure 14. Stimulation of ArsA ATPase activity.

ATPase activities were measured with 0.3 μ M ArsA in the absence or presence of 3 μ M Arsenite or DTT at 100 μ M sodium arsenite (A) or 10 μ M Potassium antimonyl tartrate (B).

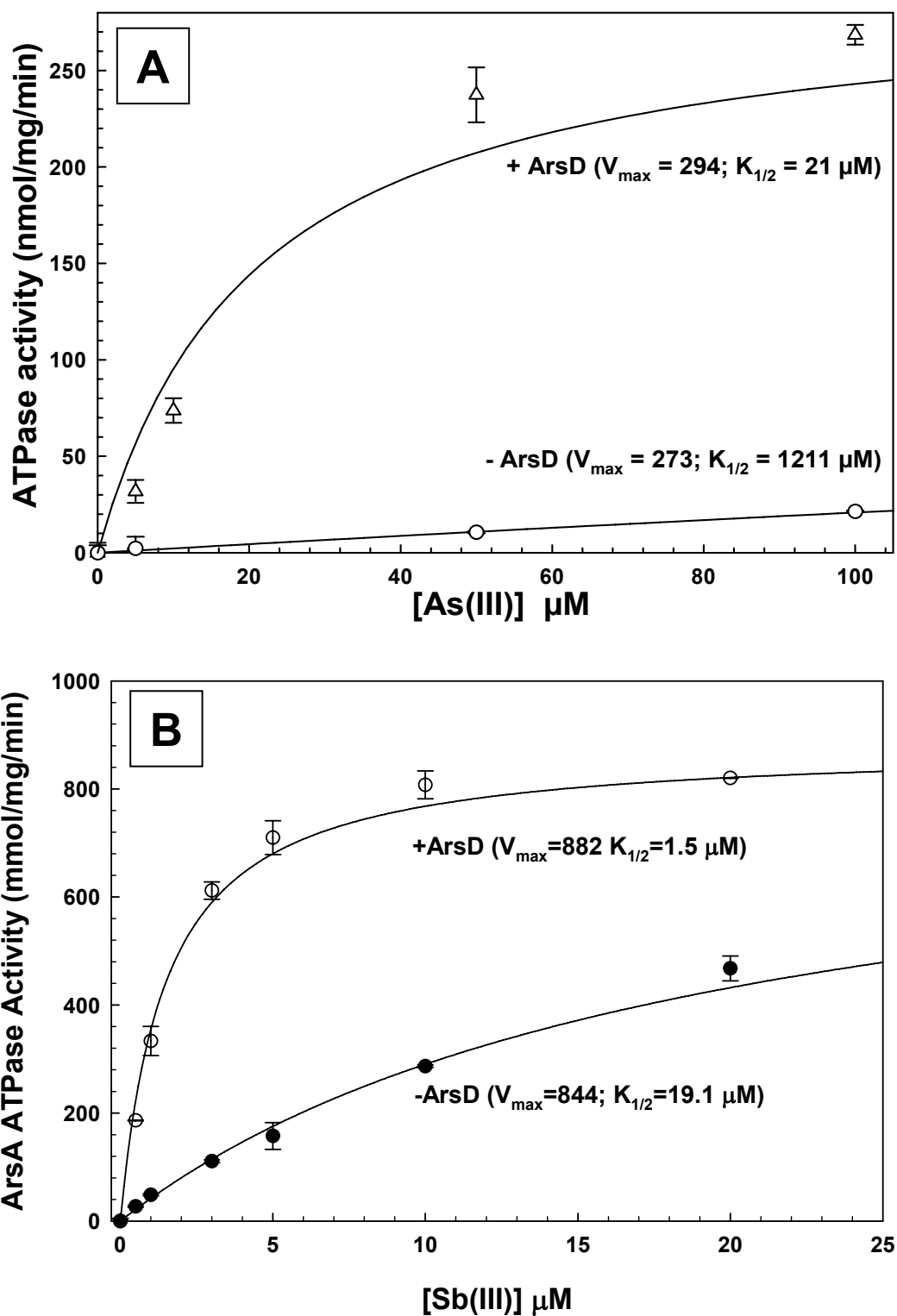


Figure 15. Effect of ArsD on the affinities of the ArsA ATPase for metalloids.

ArsA ATPase activities were measured at various concentrations of sodium arsenite (**A**) or potassium antimonyl tartrate (**B**) in the presence or absence of ArsD. All the values shown were after the subtraction of the basal values. The values in each plot are the mean of three independent assays. V_{\max} and K_m values were calculated from the best fit using SigmaPlot 9.0.

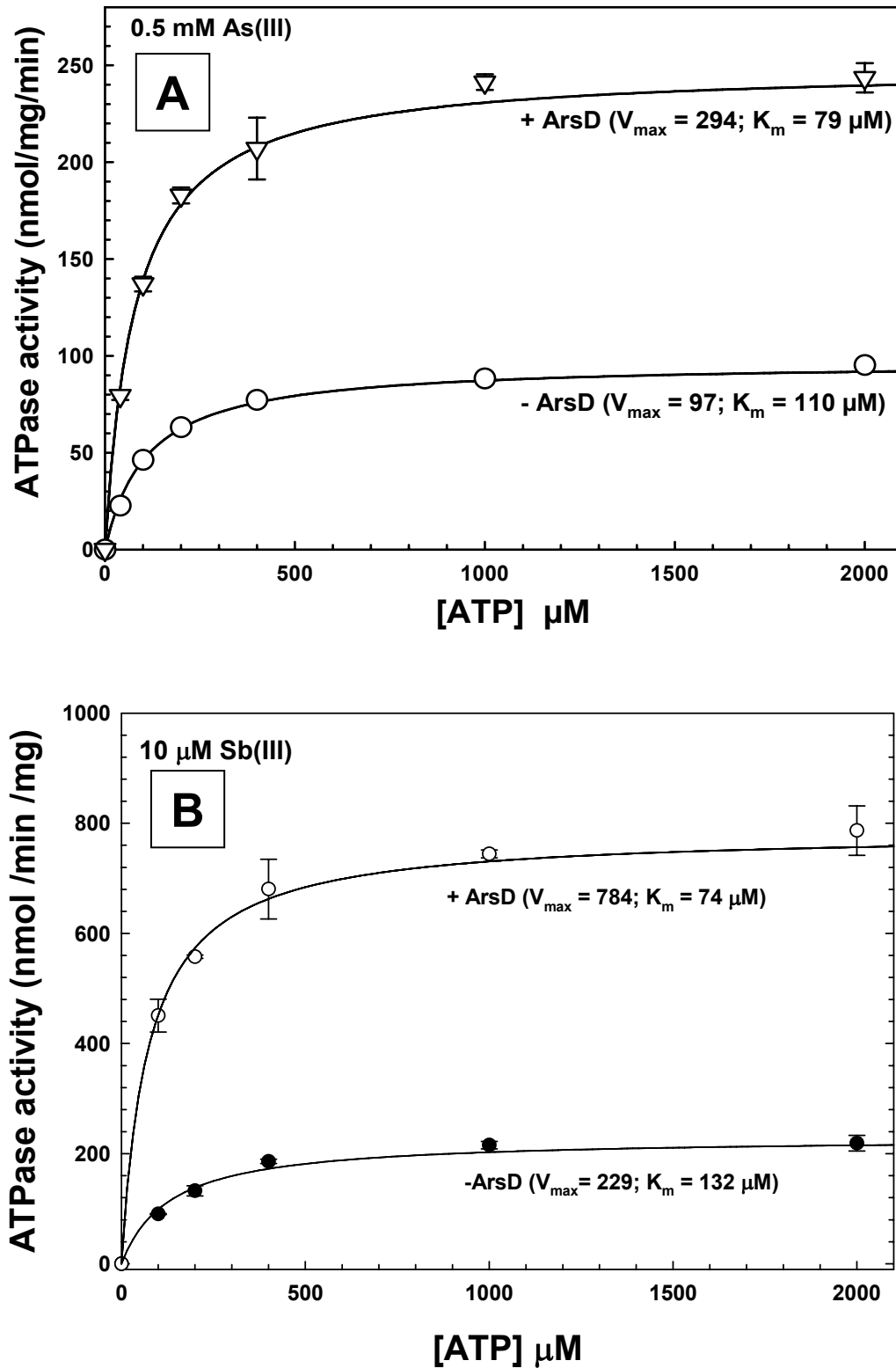


Figure 16. Effects of ArsD on the affinity of ArsA for ATP.

ArsA ATPase activities were measured at varying concentrations of ATP in the presence of 0.5 mM sodium arsenite (**A**) or 10 μM potassium antimonyl tartrate (**B**) and the presence or absence of ArsD. The values in each plot are the mean of three independent assays.

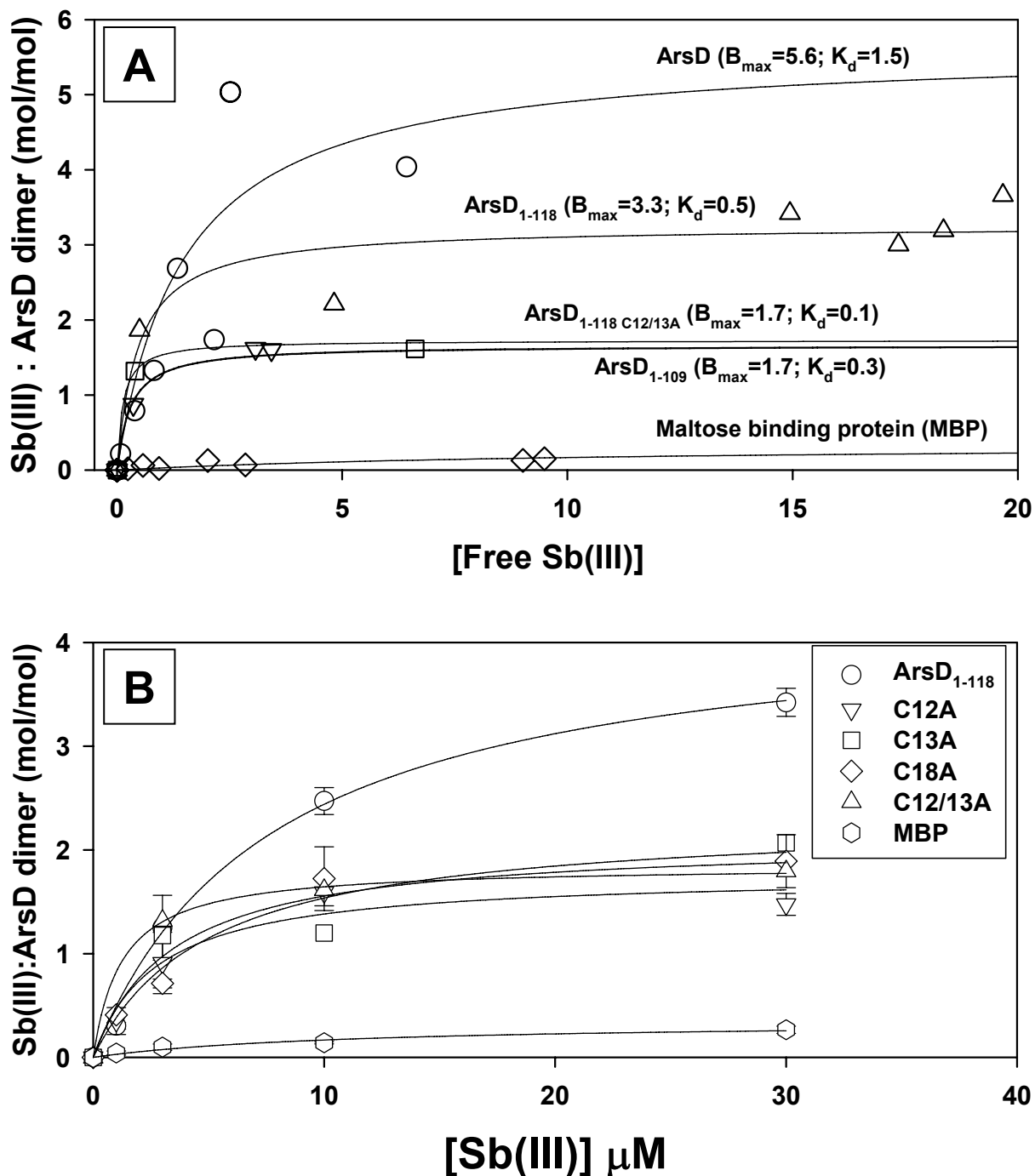


Figure 17. Binding of Sb(III) to ArsD.

Purified ArsD proteins were incubated at 4 °C with varying concentrations of potassium antimonyl tartrate. Sb(III)-Protein complexes were obtained as described in MATERIALS AND METHODS and the molar ratio was calculated.

A: Metalloid binding sites (MBSs). The MBSs on ArsD were eliminated by the truncation of the last 11 residues including the last 4 cysteines, deleting the last 2 cysteines and/or replacing the Cys12 and Cys13 with alanines.

B: Effects of mutations on MBS1. The codons for the last 2 cysteines were deleted and Cys12, Cys13 and Cys18 were changed to alanines individually or in pair.

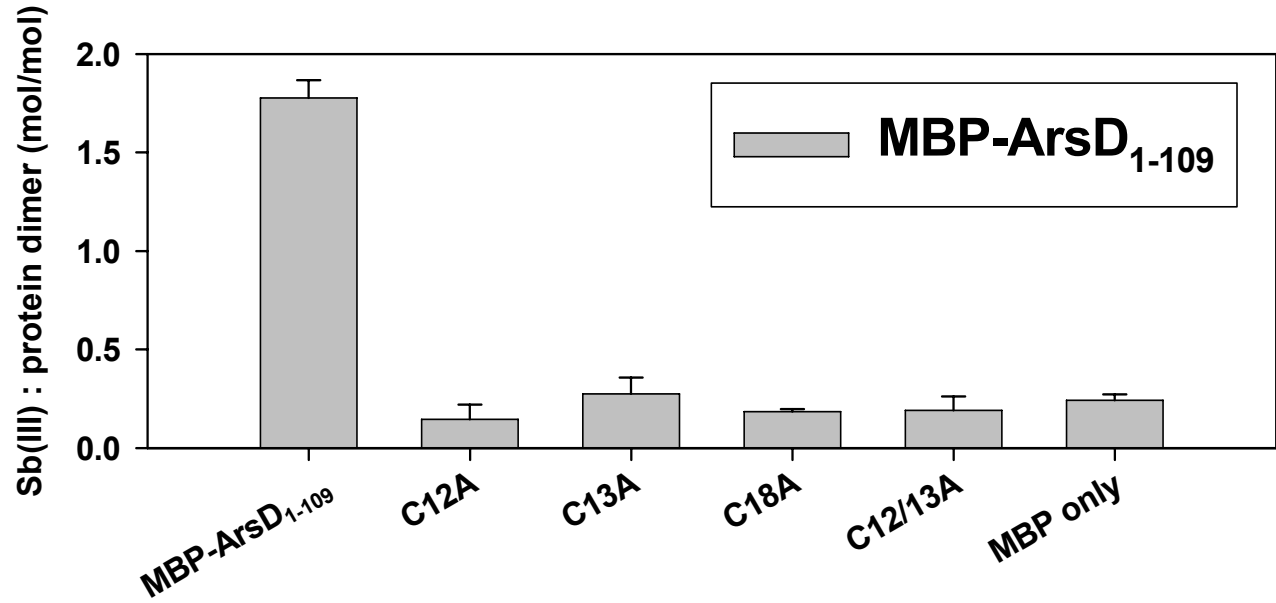


Figure 18. Contributions of the three conserved cysteines in MBS1 of ArsD.

The codons for the last 11 residues were deleted, and the Cys12, Cys13 and/or Cys18 were mutated as indicated.

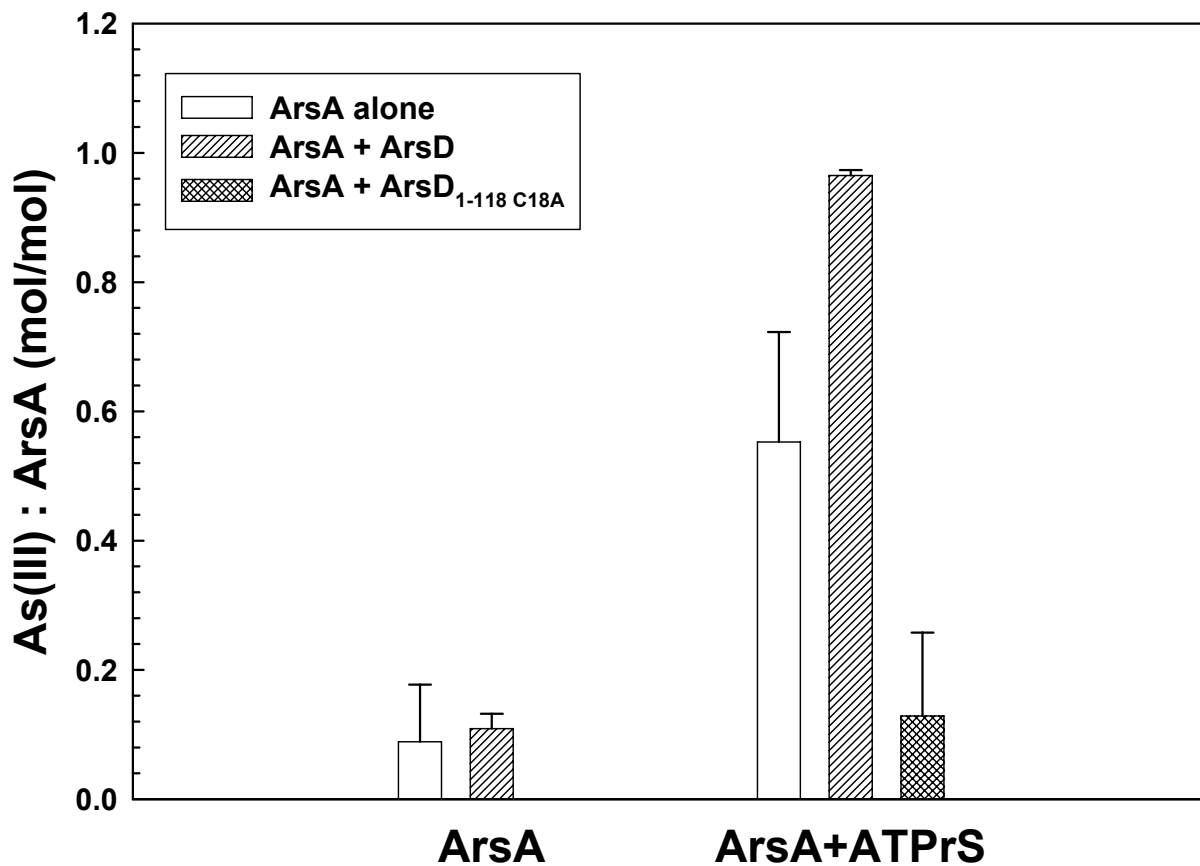


Figure 19. Effect of the MBS1 mutation in ArsD on As(III) transfer.

The molar ratio of As(III) to ArsA was measured with either ArsA alone, in the presence of wild type ArsD or the mutant ArsD_{1-118 C18A} using the metalloïd transfer assay as described. The values are the mean of three independent assays.

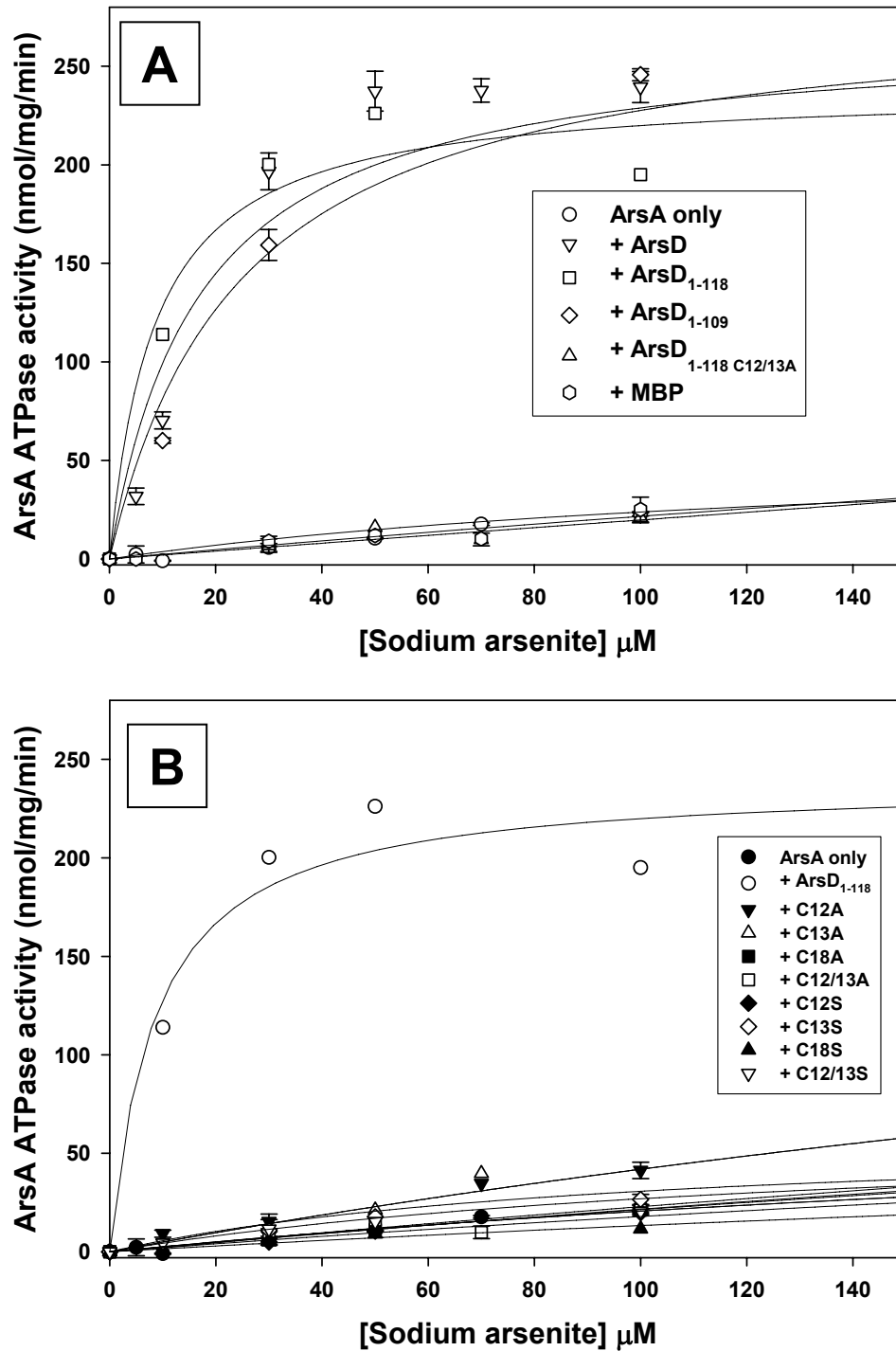


Figure 20. Effects of mutations and truncations in ArsD on activating ArsA ATPase activity.

ArsA ATPase activities were measured at various concentrations of sodium arsenite in the presence or absence of ArsD derivatives.

A: Elimination on the MBSs. The MBS-eliminated ArsD derivatives described in Fig. 15A were also tested with the ability to activate ArsA ATPase activity.

B: Cysteine mutations on MBS1. The Cys12, Cys13 or Cys18 in ArsD MBS1 was changed to alanine or serine. The activities of ArsA ATPase were measured in the presence of these ArsD mutants.

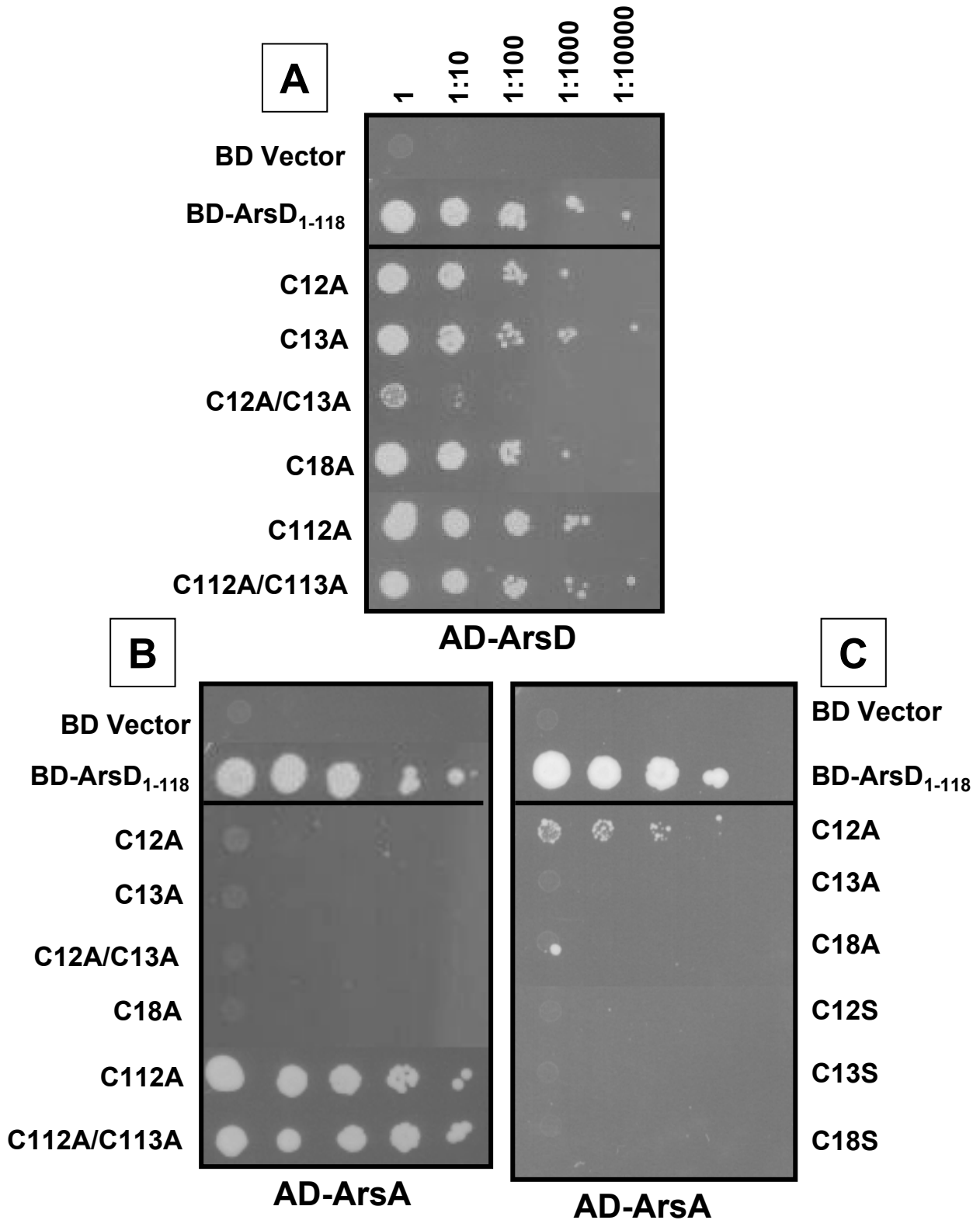


Figure 21. Effects of mutations in *arsD* on protein-protein interactions.

The codons for residues Cys12, Cys13, Cys18, Cys112 and Cys113 in *arsD* gene were changed individually or in pairs to alanines or serines. In each case, the altered *arsD* genes were expressed as BD fusions in *trans* with either AD-ArsD (**A**) or AD-ArsA fusion for 3 days (**B**) or 6 days (**C**) in yeast two-hybrid system.

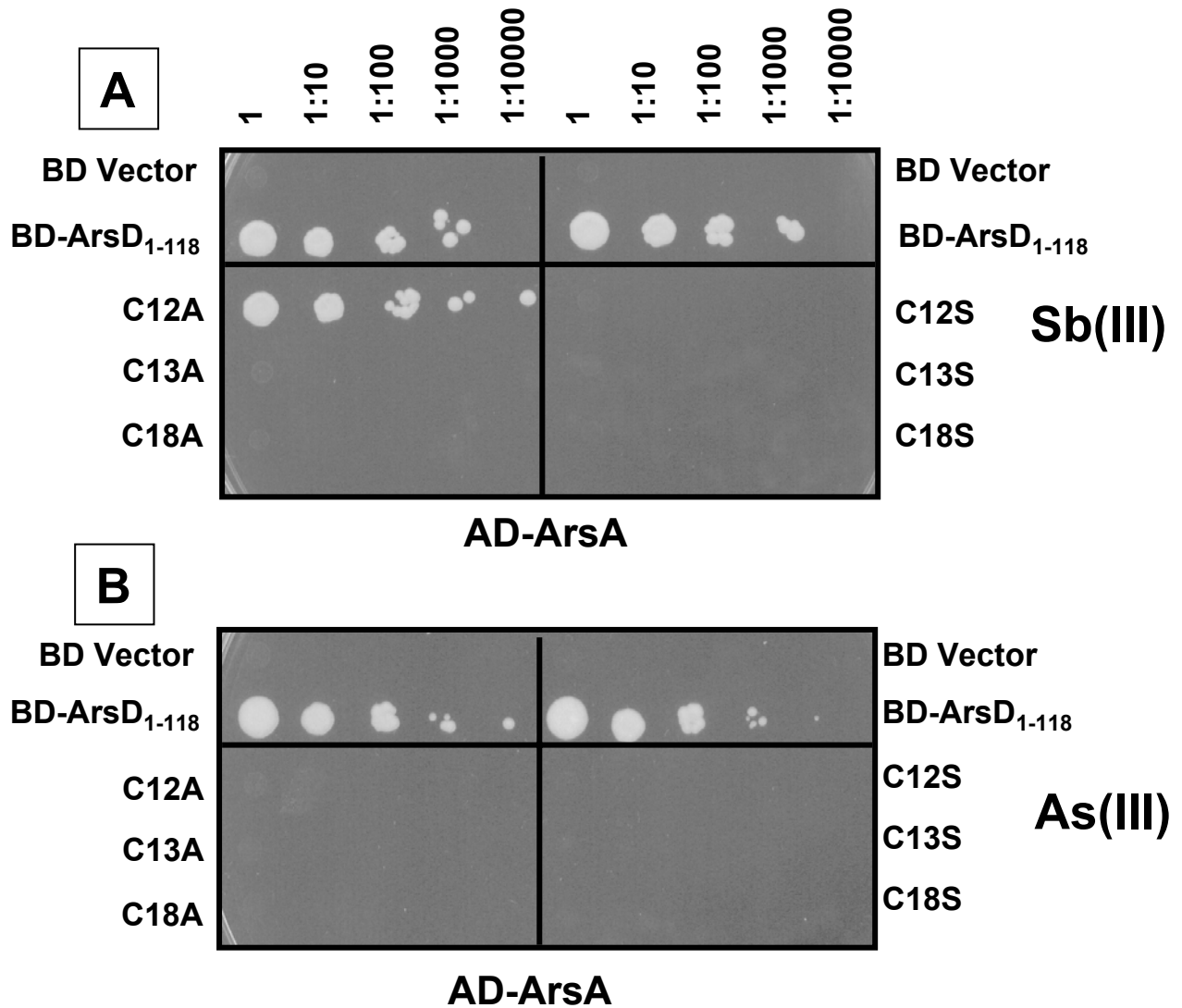


Figure 22. Effects of mutations in *arsD* on ArsD-ArsA interaction in the presence of metalloids.

The ArsD mutants in which the codons for C12, C13 or C18 in *arsD* were changed to alanine or serine were expressed in trans with ArsA in the yeast two-hybrid system.

A. In the presence of 100 μ M potassium antimonyl tartrate.

B. In the presence of 50 μ M sodium arsenite.

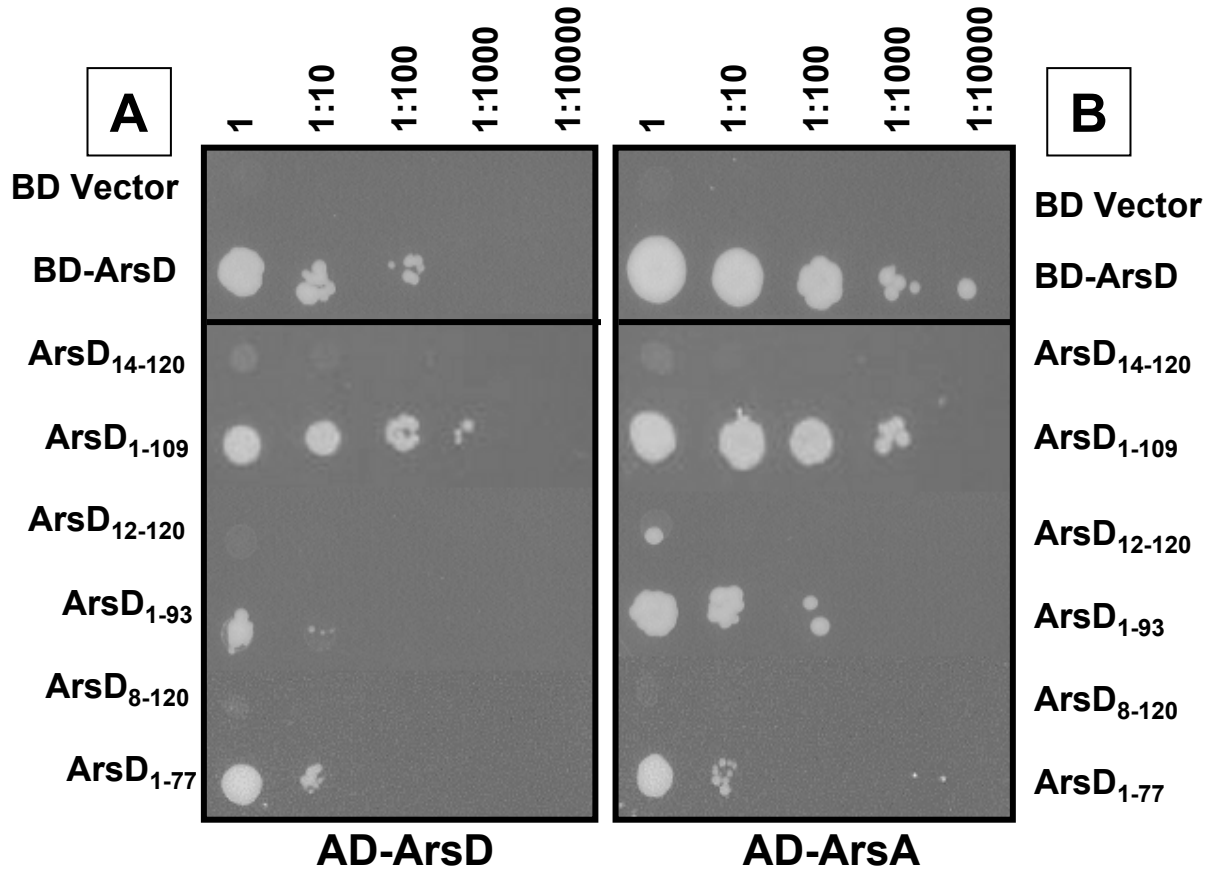


Figure 23. Effects of truncations in ArsD on protein-protein interactions.

The codons for the first 7, 11 or 13, or for the last 11, 27 or 43 residues were deleted. The truncated *arsD* genes were expressed as BD fusions in *trans* with either AD-ArsD (**A**) or AD-ArsA (**B**) fusion.

A	BD-ArsD Truncations									
	3-120	5-120	8-120	12-120	14-120	1-43	1-65	1-77	1-93	1-109
AD-ArsD	+	+	-	-	-	+	+	+	+	+
AD-ArsA	+	+	-	-	-	+	+	+	+	+

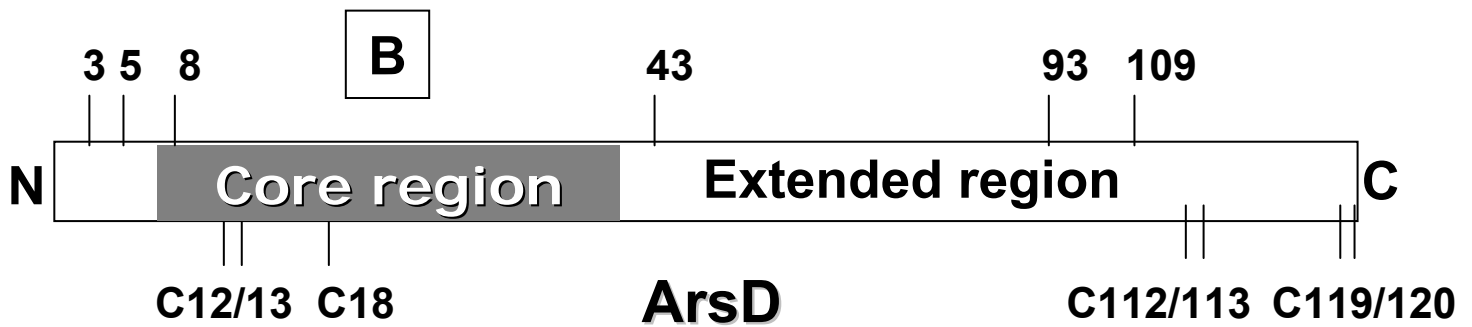


Figure 24. The Core region on ArsD for protein-protein interaction.

All the results were expressed as Positive (+) or Negative (-) in a table (A) or presented as "Core region" (-) or "Extended region" (+) on ArsD sequence (B).

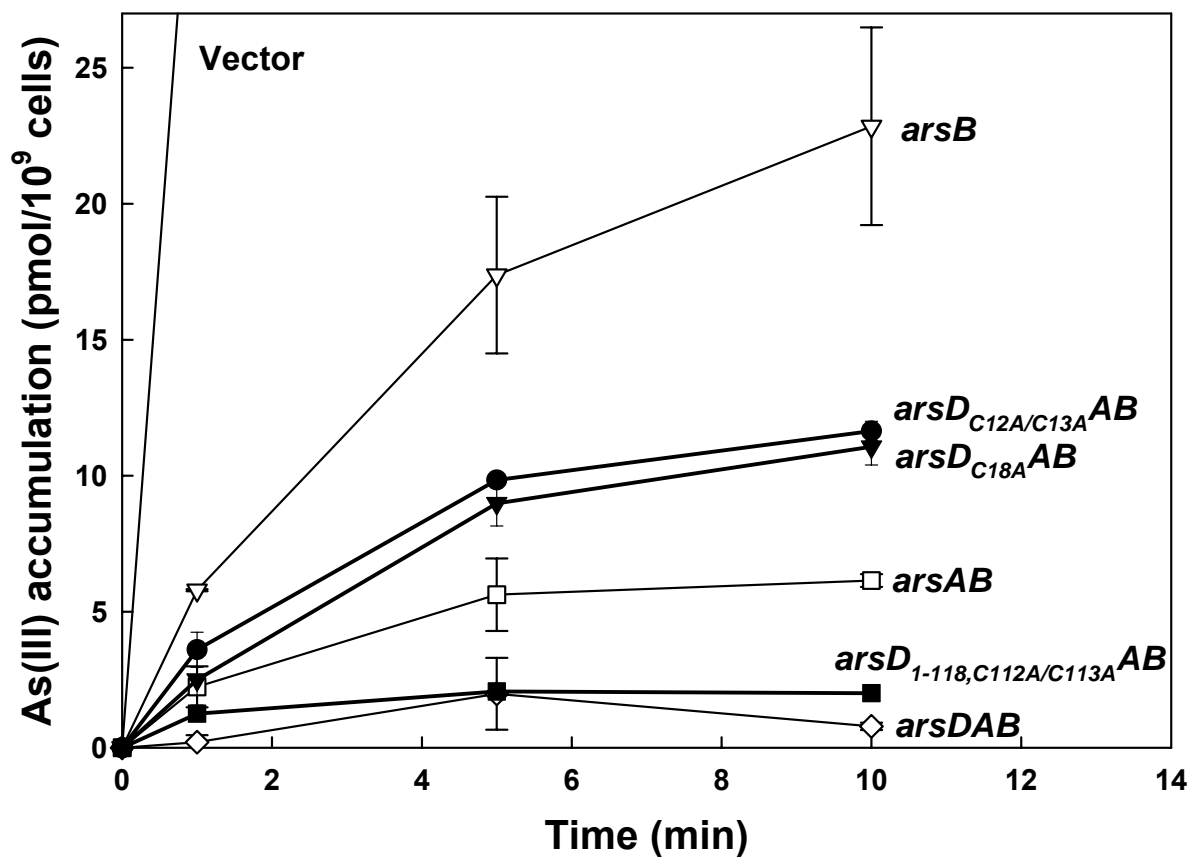


Figure 25. Effects of elimination of MBSs in *arsD* gene on arsenic accumulation.

Accumulation of As(III) in AW3110 bearing plasmids with *arsAB* and either *arsD*₁₋₁₁₈_{C112A/C113A} (■), *arsD*_{C12A/C13A} (●) or *arsD*_{C18A} (▼) was assayed with 10 μ M sodium arsenite, as described in MATERIALS AND METHODS. Each value is the average of two independent assays.

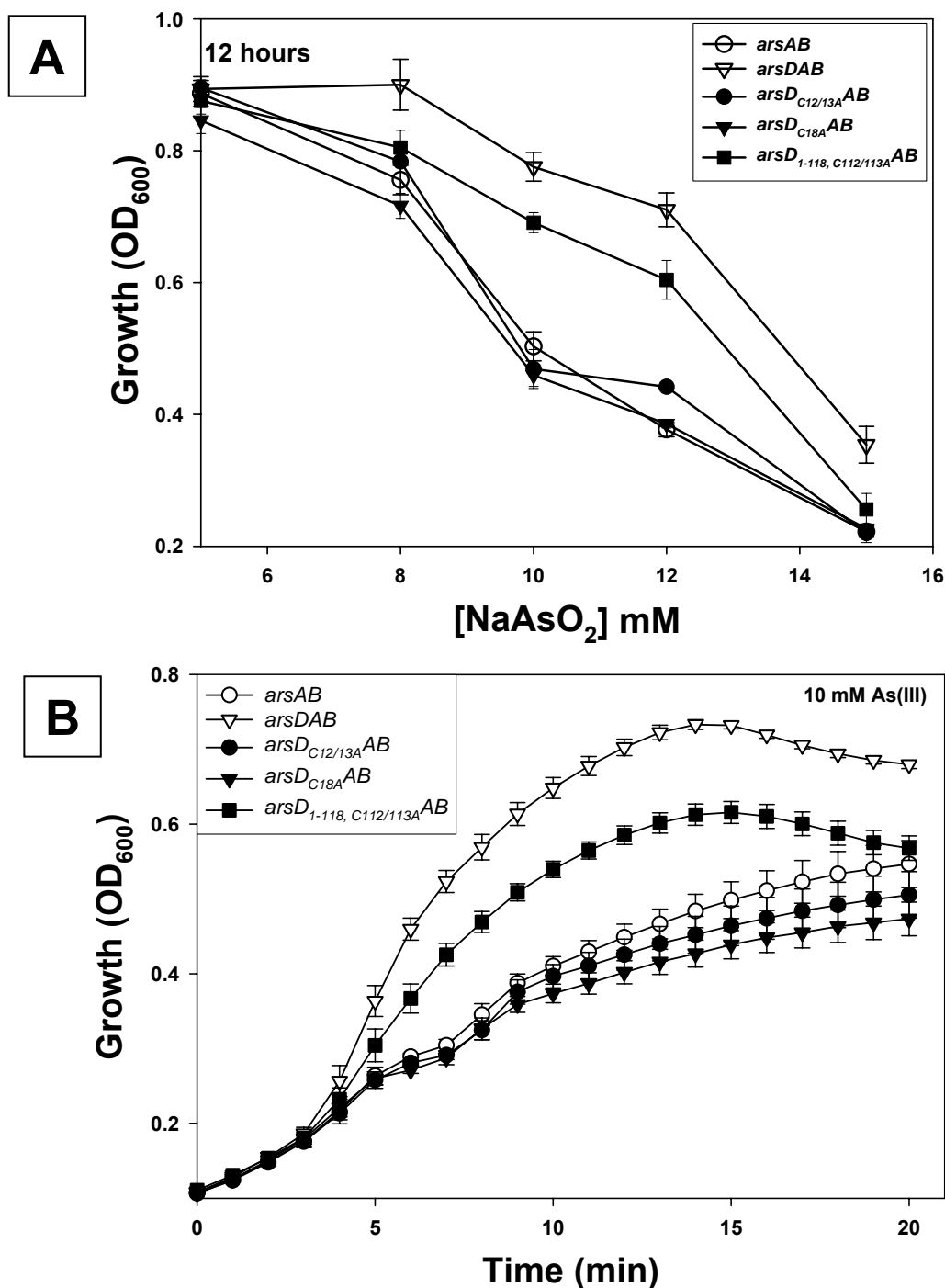


Figure 26. Effects of elimination of MBSs in *arsD* gene on arsenic resistance.

Resistance to As(III) in AW3110 bearing plasmids with *arsAB* and either *arsD_{1-118 C112A/C113A}* (■), *arsD_{C12A/C13A}* (●) or *arsD_{C18A}* (▼) was assayed as described in MATERIALS AND METHODS. The values for *arsAB* (○) and *arsDAB* (▽) are replotted on an expanded scale from (Fig. 3).

A: at different concentrations of sodium arsenite after 12 hr of growth.

B: at 10 mM sodium arsenite.

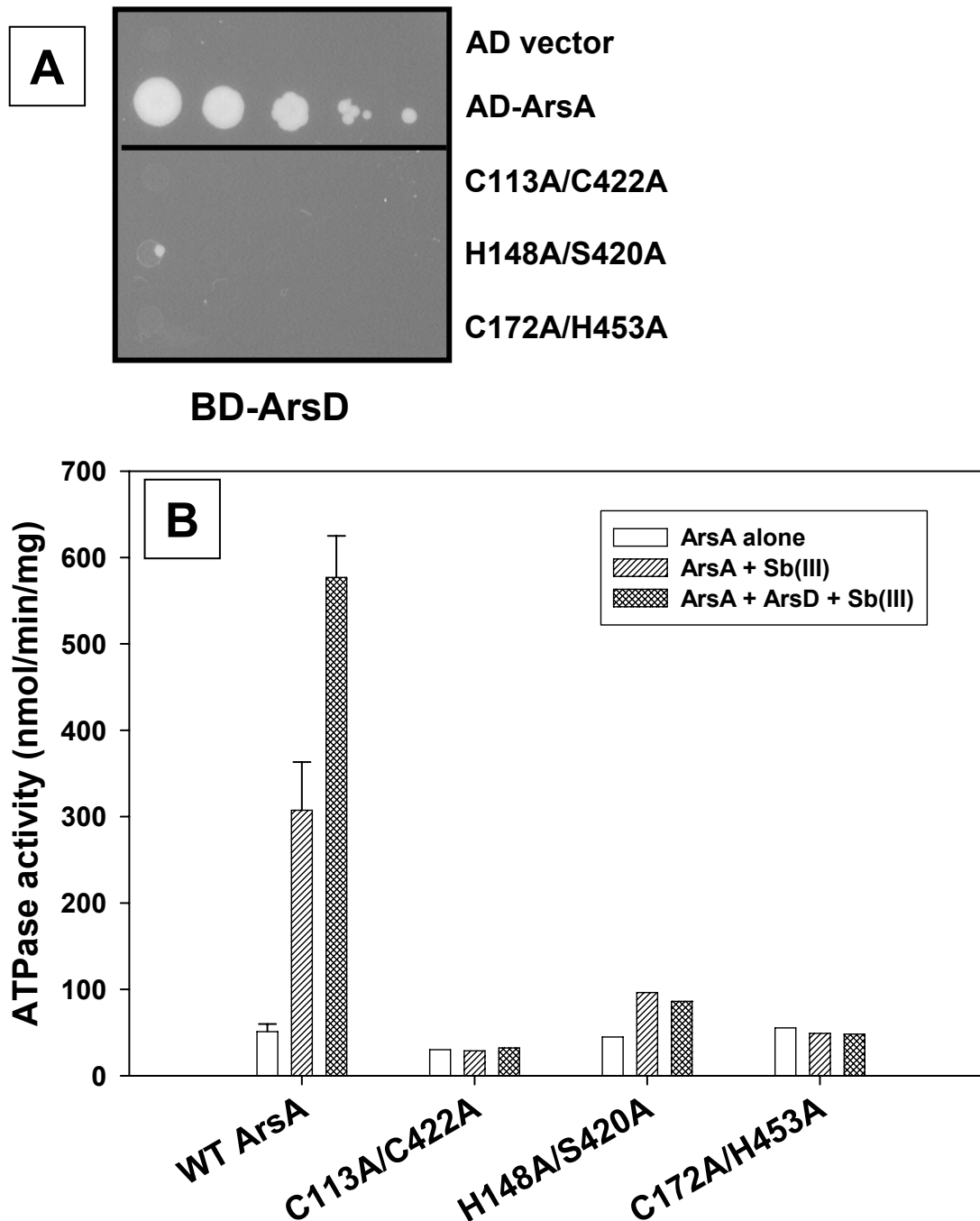


Figure 27. Effects of mutations in ArsA on ArsD-ArsA interaction.

The residues Cys113, Cys422, His148, Ser420, Cys172 and His453 in the three metalloid binding sites of ArsA were substituted to alanines in pair.

A. Yeast two-hybrid analysis. The abilities of these ArsA mutants to interact with wild type ArsD were tested in yeast two-hybrid system.

B. ArsA ATPase activity. The ATPase activities of these ArsA derivatives were measured in the presence or absence of wild type ArsD. The values of the ArsD-containing reactions were corrected by substrating the value of ArsD alone.

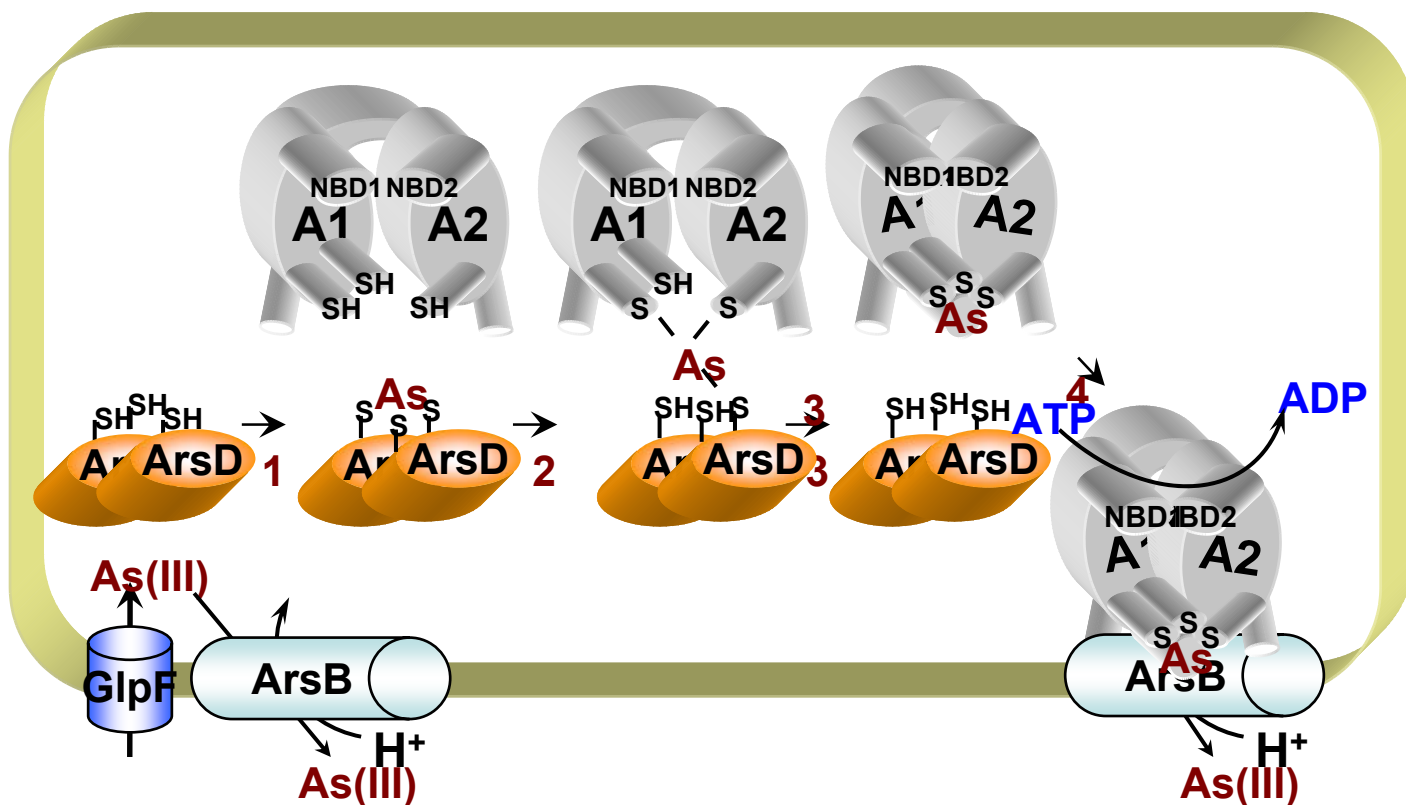

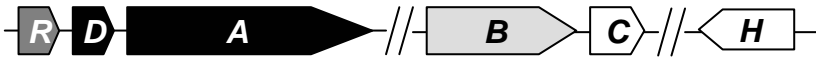




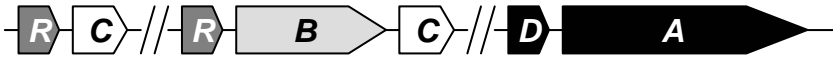
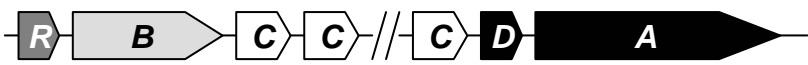
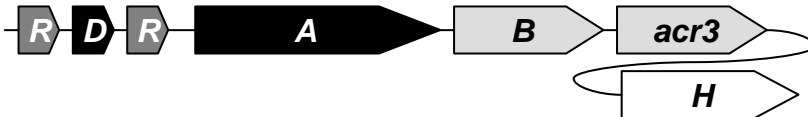





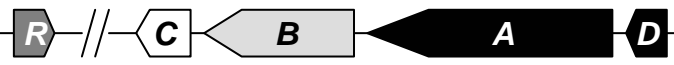
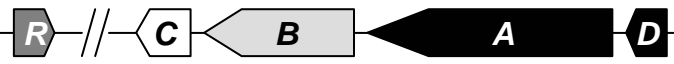






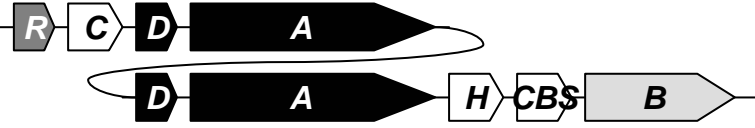



Figure 28. Model of ArsD-ArsA interaction.

As(III) enters cells by aquaglyceroporins such as GlpF, where it is bound by ArsD through two or three cysteine residues (Cys12, Cys13 and/or Cys18). As(III) is then transferred to cysteine residues in the metal binding domain of ArsA in a step-wise manner. ArsD and ArsB are proposed to bind to the same site on ArsA sequentially in a cycle of metal transfer from ArsD to ArsA to ArsB concomitant with ATP binding and then hydrolysis by ArsA.

Appendix A. *ars* operons with *arsD* (**D**) and/or *arsA* (**A**) genes.

Source	<i>ars</i> operon	Swiss-Prot/TrEMBL accession number	
Plasmid-encoded		<i>arsD</i>	<i>arsA</i>
<i>Escherichia coli</i> , R773		P46003	P08690
<i>Salmonella typhimurium</i> , R64		Q8L253	Q8L248
<i>Klebsiella oxytoca</i> , pMH12		Q9KJ14	Q9KJ13
<i>Acidiphilium multivorum</i> , pKW301		O50592	O50593
<i>Escherichia coli</i> , R46		P52148 (Q79SA7)	P52145 (Q79SA6)
<i>Lactobacillus plantarum</i> , pWCFS103		Q6LWG6 Q6LWG2	Q6LWG5
<i>Rhodococcus erythropolis</i> , pREL1		Q3L9K6	Q3L9K5
<i>Rhodococcus erythropolis</i> , pBD2		Q6XN06	Q6XN05
<i>Listeria innocua</i> , pLI100		Q926M4	Q926M2
<i>Staphylococcus saprophyticus</i> , pSSP1		Q49UG6	Q49UG7
<i>Bacillus cereus</i> , pBc10987		Q74NT7	Q74NT8
<i>Halobacterium salinarium</i> , pNRC100		O52028	O52027

Appendix A. (continued) * CBS= Cystathionine Beta Synthase

Source	ars operon	Swiss-Prot/TrEMBL accession number	
		arsD	arsA
<i>Shewanella</i> sp. W3-18-1		Q2X358	Q2X359
<i>Shewanella</i> sp. ANA3		Q366A2 (Q79JC1)	Q366A1 (Q79JC2)
<i>Shewanella</i> <i>putrefaciens</i> CN-32		Q2ZUN1	Q2ZN2
<i>Leptospirillum</i> <i>ferriphilum</i> , TnLfArs		Q2LMN6	Q2LMN5
<i>Alcaligenes</i> <i>faecalis</i> NCIB 8687		Q6WB30	Q6WB29
<i>Methylobacillus</i> <i>flagellatus</i> KT		Q1H177	Q1H178
<i>Azoarcus</i> sp. EbN1		Q5P148	Q5P147
<i>Dechloromonas</i> <i>aromatica</i> RCB		Q47CR3	Q47CR4
<i>Acidithiobacillus</i> <i>calculus</i> TnAtcArs		Q3T561 Q3T559	Q3T560 Q3T558
<i>Rhodospirillum</i> <i>ferrireducens</i> T118		Q21S88	Q21S89
<i>Photobacterium</i> <i>profundum</i> SS9		Q6LRX4	Q6LRX3
<i>Photobacterium</i> <i>profundum</i> 3TCK		Q1Z8S1	Q1Z8S2



Appendix A. (continued)

Source	ars operon	Swiss-Prot/TrEMBL accession number	
		<i>arsD</i>	<i>arsA</i>
<i>Mycobacterium vanbaalenii</i> PYR-1		Q25WE1	Q25WE1
<i>Psychromonas</i> sp. CNPT3		Q1ZFE2	Q1ZFE1
<i>Mycobacterium flavescens</i> PYR-GCK		Q277W2	Q277W3
<i>Burkholderia vietnamiensis</i> G4		Q4BLB4	Q4BLB3
<i>Alkalilimnicola ehrlichei</i> MLHE-1		Q34YA7	Q34YA8
<i>Rhodopirellula baltica</i> SH 1		Q7ULE4	Q7ULE5
<i>delta proteobacterium</i> MLMS-1		Q1NIX3	Q1NPV7
<i>Rhodospirillum rubrum</i> ATCC 11170		Q2RUE6	Q2RUE7
<i>Clostridium</i> sp. OhILAs		Q1EZS8	Q1EZS7
<i>Clostridium beijerincki</i> NCIMB 8052		Q2WLD2	Q2WLD1
<i>Magnetospirillum magneticum</i> AMB-1		Q2W689	Q2W688
<i>Desulfitobacterium hafniense</i> Y51		Q24PV2	Q24PV1 Q24NK5

Appendix A. (continued)

Source	ars operon	Swiss-Prot/TrEMBL accession number	
		arsD	arsA
<i>Desulfitobacterium hafniense</i> DCB-2		Q423V8	Q423V9
		Q18S87	Q18S86
<i>Syntrophomonas wolfei</i> subsp. Goettingen		Q3G9J8	Q3G9J9
<i>Bacteroides thetaiotaomicron</i> VPI-5482		Q8ABJ3 (Q8A9L4)	Q8ABJ4 (Q8A9L3)
<i>Clostridium phytofermentans</i> ISDg		Q1FPR6	Q1FPR4
<i>Staphylococcus epidermidis</i> RP62A		Q5HKC1	Q5HKC0
<i>Staphylococcus epidermidis</i> ATCC 12228		Q8CQF1	Q8CQF2
<i>Bacillus</i> sp. CDB3		Q9RA90	Q9RA89
<i>Bacillus</i> sp. MB24 TnARS1		Q5Q1Q6	Q5Q1Q5
<i>Geobacter uraniumreducens</i> Rf4		Q2DL37	Q2DL38
<i>Staphylococcus haemolyticus</i> JCSC1435		Q4LAA4	Q4LAA5
<i>Haloquadratum walsbyi</i>		Q18H25	Q18H24
<i>Alkaliphilus metalliredigens</i> QYMF		Q3CDN9	Q3CDP0
		Q3C8Z0	Q3CDP1
			Q3C8Z1
			Q3C8Z2

Appendix A. (continued)

Source	ars operon	Swiss-Prot/TrEMBL accession number for <i>arsA</i>
Genomic		
<i>Arthrobacter sp.</i> <i>FB24</i>		Q4NKN0
<i>Clostridium tetani E88</i>		Q893D3

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ABSTRACT**AN ARSENIC METALLOCHAPERONE FOR AN ARSENIC
DETOXIFICATION PUMP**

by

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December 2006

Advisor: Dr. Barry P. Rosen

Major: Biochemistry and Molecular Biology

Degree: Doctor of Philosophy

Arsenic is a metalloid toxicant that is widely distributed throughout the earth's crust and causes a variety of health and environment problems. As an adaptation to arsenic-contaminated environments, organisms have developed resistance systems. *E. coli* plasmid R773 carries the well-studied *arsRDABC* operon. ArsA is an ATPase that is the catalytic subunit of the ArsAB As(III) extrusion pump. ArsD was shown to have weak repressor activity, but this may not be its physiological function. Most *ars* operons contain only three genes, *arsRBC*. Five gene operons have two additional genes, *arsD* and *arsA*, and these are usually adjacent to each other. Obviously *arsD* and *arsA* co-evolved suggesting a related function for the two gene products. Recently metallochaperones have been identified for a number of metals. Metallochaperones prevent inappropriate metal interactions with other cellular components. Thus, these ubiquitous proteins have a critical biological function: to deliver metals in the cytoplasm to the site of utilization or export. In this study,

we report that ArsD is an arsenic chaperone that transfers trivalent metalloids to the ArsA ATPase. Through protein-protein interactions, ArsD increases the affinity of the ATPase for As(III) and results in increased efflux and resistance. This is the first report of an arsenic chaperone and suggests that cells can regulate the intracellular concentration of free arsenite to prevent toxicity. We also determined that the interaction domain on ArsD is in the N-terminus and the three conserved cysteines- 12, 13 and 18 are involved.

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