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IDENTIFICATION AND CHARACTERIZATION OF A CHROMOSOMAL ARSENIC/ANTIMONY INDUCIBLE OPERON IN GRAM-NEGATIVE BACTERIA

by

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ABSTRACT

In searching for genetically-programmed responses to arsenic oxyanion exposure in plasmid-free bacterial strains, an arsenate-inducible *Escherichia coli* chromosomal operon was identified using a Mud I (*lac* Ap^r) transposable bacteriophage. The operon is induced by cellular exposure to arsenic and antimony oxyanions. The operon contains three cistrons that are homologous to the *arsR*, *arsB*, and *arsC* genes of plasmid-borne *ars* operons, coding for an arsenite/antimonite-inducible repressor, an inner membrane arsenite transporter, and an arsenate reductase, respectively. The *E. coli* chromosomal *ars* operon is functional in protection against arsenic toxicity, as mutants of this operon are hypersensitive to arsenic. To elucidate regulation of *ars* operon expression, two luciferase transcriptional gene fusions, *arsR::luxAB* and *arsB::luxAB*, were constructed and analysed. The *E. coli* chromosomal *ars* operon was demonstrated to be regulated by the *trans*-acting repressor, ArsR, in a manner similar to that of the plasmid R773 *ars* operon. The operon is transcribed as a single transcription unit, and regulated by arsenic mainly, though not exclusively, at the mRNA level. The individual gene products of the operon were visualized in both *in vivo* and *in vitro* expression systems. The chromosomal *ars* operon is conserved in the chromosomes of a number of Gram-negative bacterial species.

To monitor the toxicity and bioavailability of arsenic compounds, the potential of the *E. coli arsB::luxAB* fusion strain as a bacterial biosensor has been evaluated. This luciferase gene fusion strain was shown to selectively detect the presence of arsenic in a mixed wood preservative, chromated copper arsenate (CCA).

To further elucidate the evolutionary relationship of chromosomal *ars* operons between different bacterial species, a *Pseudomonas aeruginosa* PAO1 chromosomal *ars* operon homolog was cloned and found to confer resistance to arsenite and antimonite, and to a lesser extent, to arsenate, in an *E. coli ars*$^-$ mutant strain. Three cistrons were identified that share homology with the *arsR*, *arsB*, and *arsC* genes of other known *ars* operons. Expression of the *P. aeruginosa ars* operon is induced by arsenite as shown by RNA dot blots. Homologous sequences of this operon have also been detected in other strains and species of the genus *Pseudomonas*.

RÉSUMÉ

La recherche de réponses génétiques à l'arsenic chez des souches bactériennes dépourvues de plasmides, a mené à l'identification d'un opéron chromosomique répondant à les ions de arsenic chez *Escherichia coli*. Cet opéron, qui a pu être identifié en utilisant un bactériophage transposable MudI (*lac* Ap^r), est activé par le contact de cellules bactériennes à des sels d'arsenic ou d'antimoine. Il comprend trois cistrons homologues aux gènes *arsR*, *arsB*, et *arsC* des opérons *ars* de plasmides, et qui codent respectivement un répresseur activé par l'arsénite ou l'antimonite, un transporteur pour l'arsénite dans la membrane interne, et une enzyme réductrice d'arséniate. Les *E. coli* mutants de l'opéron *ars* sont hypersensibles à l'arsenic, ce qui suggère un rôle fonctionnel de cet opéron dans la protection contre la toxicité de l'arsenic.

Deux fusions de transcription avec la luciférase, *arsR*::*luxAB* et *arsB*::*luxAB*, ont été construites et analysées. Il a été démontré que l'opéron chromosomique *ars* d'*E. coli* est controlé par le répresseur ArsR agissant en *trans*, similairement à l'opéron *ars* du plasmide R773. Cet opéron est transcrit en un seul brin, et le contrôle de son expression par l'arsenic se fait principalement, mais non exclusivement, au niveau de l'ARN messager. Chacun des produits de l'opéron a pu être vu à l'aide de systèmes d'expression *in vivo* et *in vitro*. L'opéron *ars* est conservé chez les chromosomes de plusieurs espèces de bactéries Gram-négatives.

Afin d'analyser la toxicité et la biodisponibilité de composés d'arsenic, la capacité de la souche de fusion d'*E. coli arsB*::*luxAB* à fonctionner comme biosonde bactérienne a été évaluée. Il a été démontré que cette souche de fusion génique de luciférase peut détecter la présence d'arsenic dans un composé de présenvation du bois, l'arsenate de cuivre chromé (ACC). Dans le but d'élucider les liens évolutionnaires des opérons *ars* chromosomiques chez diverses espéces bactériennes, un homologue chromosomique d'*ars* chez *Pseudomonas aeruginosa* PAO1 a été cloné. Cet homologue d'*ars* confère de la résistance à l'arsénite et à l'antimonite et, à un moindre degré, à l'arséniate chez une souche d'*E. coli* mutante (*ars*$^-$). Trois cistrons, qui présentent une homologie aux gènes *arsR*, *arsB* et *arsC* d'autres opérons *ars*, ont été identifiés. L'opéron *ars* de *P. aeruginosa* est activé par l'arsénite au niveau de l'ARN messager. Des séquences

homologues à cet opéron ont aussi été détectées chez d'autres souches et espèces du genre *Pseudomonas*.

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Sincerest gratitude is expressed to my thesis supervisor, Dr. Michael S. DuBow, who guided me from an entry-level molecular biologist to the completion of this thesis. His enthusiasm and earnestness for science have always been a source of inspiration. His continuous support and guidance throughout the years of my graduate studies, made my scientific pursuit a success. His friendship and encouragement have greatly eased my way through times of difficulty.

I would like to thank all members of the DuBow lab, past and present, for their help and friendship, which made my experience here an unforgettable one. A deep thank you to Caroline Diorio and Gina Macintyre, who have been particularly helpful in many aspects of this research. I sincerely thank Angelina Guzzo for her help and suggestions, Felix Sieder for his excellent computer aid, Angelina Guzzo, Doris Fortin, and Kirsty Salmon for generously providing me with plasmids. I thank Kirsty Salmon for her selfless help during the final stages of the work.

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PREFACE TO THE THESIS

In accordance with the guidelines concerning thesis preparation, and with the approval of the Department of Microbiology and Immunology, I have opted to present the experimental portion of this thesis (Chapters 2-5, inclusive) in the form of original papers. A provision in the guidelines concerning thesis preparation reads as follows:

> The candidate has the option, subject to the approval of their department, of including as part of the thesis, the text of one or more papers submitted or to be submitted for publication, or the clearly-duplicated text of one or more published papers. These texts must be bound as an integral part of the thesis.
>
> -If this option is chosen, connecting texts that provide logical bridges between the different papers are mandatory. The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.
>
> -The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation". The thesis must include: a Table of Contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.
>
> -Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgment to be made of the importance and originality of the research reported in this thesis.
>
> -In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. Supervisors must attests to the accuracy of such statements at the doctoral oral defense. Since the task of examiners is made more difficult in these cases. It is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers.

With regard to these conditions, I have included as chapters of this thesis, four original papers, of which two have been published, one is in press, and one is submitted for publication. Chapters 2 to 5, inclusive, each contain an Abstract, Introduction, Materials and Methods, Results, and Discussion section. Chapters 3 to 5 contain prefaces that serve as the connecting texts to bridge the manuscripts. A General Introduction (Chapter 1) and a Summary (Chapter 6) have also been included, and references for all chapters are

collected alphabetically at the end of the thesis. A detailed Table of Contents, List of Figures and Tables, and Abbreviations are included at the beginning of the thesis.

The manuscripts, in order of their appearance, in the thesis are:

1) Diorio, C., Cai, J., Marmor, J., Shinder, R., and DuBow, M.S. 1995. An *Escherichia coli* chromosomal *ars* operon homolog is functional in arsenic detoxification and is conserved in Gram-negative bacteria. J. Bacteriol. 177: 2050-2056.

2) Cai, J., and DuBow, M.S., 1996. Expression of the *Escherichia coli* chromosomal *ars* operon. Can. J. Microbiol. 42: 662-71.

3) Cai, J., and DuBow, M.S., 1997. Use of a luminescent bacterial biosensor for biomonitoring and characterization of arsenic toxicity of chromated copper arsenate. Biodegradation, in press.

4) Cai, J., and DuBow, M.S. Identification and characterization of a chromosomal *ars* operon homolog in *Pseudomonas aeruginosa*. Submitted to J. Bacteriol.

As has been attested by the thesis supervisor (Dr. M.S. DuBow), I was responsible for all the research described in Chapters 2-5 inclusively, with the following exceptions:

1) Rowen Shinder isolated the *arsB::lacZ* gene fusion strain, LF20001 (Chapter 2).

2) Joy Marmor mapped the junction region between the MudI left end and the adjacent chromosomal sequence in the strain LF20001, and constructed the plasmid pJS29 (Figure 1, Chapter 2) and the strain LF20018.

3) Joy Marmor and I equally contributed to the ß-galactosidase assay in Figure 3, Chapter 2.

4) Caroline Diorio performed DNA sequencing and computer analysis of the *E. coli* chromosomal *ars* operon (Figure 2, Chapter 2), the majority of the Southern blotting analysis (Figure 5, Chapter 2), and mapping of the *E. coli* chromosomal *ars* operon using the Kohara library.

CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

1. An *E. coli* chromosomal *ars* operon was discovered by random transposition mutagenesis using a MudI (*lac* Ap^r) transposable bacteriophage.

2. I have cloned the wild type *E. coli* chromosomal *ars* operon, which was mapped to the 77.5 min region in the *E. coli* genetic map.

3. Sequencing analysis showed that this operon contains three open reading frames, which share homology with the *arsR*, *arsB*, and *arsC* open reading frames of plasmid-based *ars* operons.

4. I have shown that the *E. coli* chromosomal *ars* operon plays a protective role against arsenic toxicity. Disruption of the coding sequence resulted in decreased cell resistance to arsenic salts, while increased copy number of the operon conferred increased resistance to arsenic salts.

5. Expression of the chromosomal *arsB::lacZ* transcriptional gene fusion was shown to be induced by arsenite (III), arsenate (V), and antimonite (III), to which the operon confers resistance.

6. The operon was shown to be conserved in the chromosomes of many Gram-negative bacterial species.

7. I have constructed two *E. coli* chromosomal transcriptional luciferase gene fusions, *arsB::luxAB* and *arsR::luxAB*, and I have shown that expression of the *arsB::luxAB* gene fusion was arsenic-inducible in a concentration-dependent manner while expression of the *arsR::luxAB* gene fusion was constitutive.

8. I have cloned the individual genes of the *E. coli* chromosomal *ars* operon into an expression vector, and visualized the gene products in both *in vitro* and *in vivo* expression systems.

9. I have demonstrated that the *E. coli* chromosomal *arsR* gene product functions as a *trans*-acting repressor that negatively regulates the expression of the *ars* operon, and its function can be replaced by the *E. coli* plasmid R773 *arsR* gene.

10. I have shown that the operon was transcribed as a single transcription unit of approximately 2.1 kb in size under arsenite induction conditions, and the full length transcript was probably processed into two smaller mRNA species.

11. I have determined the transcriptional initiation site of the *E. coli* chromosomal *ars* operon by primer extension analysis, located 27 nt upstream of the initiation codon of the *arsR* gene.

12. I have shown that the *E. coli* chromosomal *arsB::luxAB* gene fusion strain has potential as a living luminescent bacterial biosensor in monitoring bioavailable levels and toxicity of arsenic compounds of environmental concern.

13. I have cloned an *ars* operon homolog from the genomic DNA of *Pseudomonas aeruginosa* strain PAO1.

14. I have sequenced the *P. aeruginosa* chromosomal *ars* operon, and identified three open reading frames that share significant homology with the ArsR, ArsB and ArsC proteins encoded by other known *ars* operons.

15. I have demonstrated that the *P. aeruginosa* chromosomal *ars* operon is functional in protecting cells from arsenic/antimony toxicity.

16. I have shown that expression of the *P. aeruginosa* chromosomal *ars* operon is induced by arsenite at the mRNA level.

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ABBREVIATIONS

Ap^r: ampicillin resistance

As: arsenic

As(III): trivalent arsenic

As(V): pentavalent arsenic

ATP: adenosine triphosphate

ATPase: adenosine triphosphatase

bp: basepair

BSA: bovine serum albumin

DEPC: diethyl pyrocarbonate

dNTP: deoxyribonucleoside triphosphate

DTT: dithiothreitol

EDTA: ethylenediaminetetraacetic acid disodium salt

IPTG: isopropylthio-ß-D-galactoside

kb: kilobase

kD: kilodalton

LB: Luria-Bertani medium

µM: micromolar

NaCl: sodium chloride

nt: nucleotide

ORF: open reading frame

PAGE: polyacrylamide gel electrophoresis

ppb: parts per billion

ppm: parts per million

Sb: antimony

SDS: sodium dodecyl sulfate

Tc^r: tetracycline resistance

Tn: transposon

CHAPTER 1

GENERAL INTRODUCTION

1.1 OCCURRENCE AND DISTRIBUTION OF ARSENIC

Arsenic is an abundant element in the environment, coming from both natural and anthropogenic sources. It has been estimated that there are 155,000 metric tons of arsenic released worldwide into the environment each year, of which approximately 30% is derived from weathering of natural materials and 70% is released from anthropogenic sources [Moore and Ramamoorthy, 1984]. Major natural sources of arsenic include volcanoes, geothermal wells, and weathering rocks [Vahter, 1986a; Ferguson and Gavis, 1972]. These sources liberate mostly inorganic compounds, such as arsenic trioxide, arsenite, and arsenate [Tamaki and Frankenberg, 1992]. High levels of arsenic are found in minerals, where they occur as arsenides of copper, lead, or gold, or as sulfide. Major arsenic-containing sulfides are arsenopyrite (FeAsS), realgar (As_4S_4), and orpiment (As_2S_3) [Vahter, 1986a]. High levels of arsenic are also present in some coals and ground water. Over the past 100 years, many commercially-produced arsenic compounds have been deliberately added to the biosphere. Anthropogenic sources of arsenic occur mainly as a result of smelting or roasting of sulfide minerals, combustion of fossil fuels, leaching of exposed waste from mining activities and coal-fired power plants, and production and use of arsenic biocides and wood preservatives [Vahter, 1986a; Chilvers and Peterson, 1987]. The level of arsenic in the earth's crust is 1.5-2 mg/kg (ppm), ranking twentieth in crust abundance [WHO, 1981]. Various concentrations of arsenic are present in soils, depending on the geographical characteristics, the type of soils, and the influence of human activity. The average concentration of arsenic in soil, as measured in various countries, ranges from 2 to 20 ppm [Huang, 1994]. High levels of arsenic (several hundred ppm) were found in soils overlying sulfide-ore deposits [IPCS, 1992]. Soils having a high clay or organic matter content tend to bind more arsenic than sandy soils [IPCS, 1992; Huang, 1994]. Precipitation from the atmosphere accounts for another major source of arsenic influx into soil. The total deposit of arsenic from the atmosphere into the soil is estimated to be 63,600 metric tons per year [Tamaki and Frankenberg, 1992].

Transportation of arsenic in the environment takes place mainly through water. Speciation of arsenic in aquatic systems depends largely on redox potential and pH [For

review, see Cullen and Reimer, 1989]. Thermodynamically, As(V) should be the predominant species in oxygenated water, whereas As(III) becomes the predominant species in reduced water [Cullen and Reimer, 1989]. Methylated forms of arsenic, mainly monomethylarsonate (MMAA) and dimethylarsinate (DMAA), are frequently detected in freshwater and marine systems [Braman and Foreback, 1973; Anderson and Bruland, 1991]. Arsenic concentrations in seawaters range from 1.5 to 5 μg/kg (ppb), with an average of 1.7 ppb [Sanders, 1980; Chilvers and Peterson, 1987]. However, the levels of arsenic in fresh waters depend on the presence of nearby mineral sources, and can vary dramatically. Most freshwater systems have arsenic concentrations ranging from 1.0 to 10 ppb [Sanders, 1980]. In aquatic environments, arsenic partitions preferentially to sediments, where arsenic is associated largely with iron and aluminum compounds [Brannon and Patrick, 1987; Cullen and Reimer, 1989]. Arsenic speciation and potential mobility in sediments is largely influenced by environmental conditions, including the variety of ligands present, pH, redox potential, and aerobic/anaerobic conditions [Brannon and Patrick, 1987; Cullen and Reimer, 1989; Aurilio et al., 1994].

Airborne arsenic results from non-biological and biological volatilization. Non-biological volatilization processes include both natural events, such as volcanism, and anthropogenic processes. Copper smelting and coal combustion account for 60% of the total anthropogenic sources, with nonferrous metal production, agricultural uses and burning accounting for the remaining load. Biological volatilization results mainly from biotransformation reactions, which lead to aerobic and anaerobic releases of alkyl-arsenic compounds into the atmosphere [Johnson and Braman, 1975; Cullen and Reimer, 1989]. Approximately 15% of the total arsenic in ambient air is in the form of methylarsines [Johnson and Braman, 1975]. The ratio of natural to anthropogenic sources of atmospheric arsenic is estimated to be approximately 1.5 [Cullen and Reimer, 1989]. Concentrations of airborne arsenic are generally less than a few nanograms per cubic meter, except when measurements are taken near points of emissions [Chilvers and Peterson, 1987; WHO, 1981].

Various forms of arsenic have been found in living matter. Marine organisms contain large amounts of organic arsenicals, which are mostly water soluble methylated forms [Benson, 1989; Cullen and Reimer, 1989; Shiomi, 1994]. Arsenobetaine (AB), a trimethylated arsenic compound, is the major form of arsenic in marine animals, whereas algae contain mainly arsenosugars, which are dimethylated arsenic compounds. Small amounts of arsenocholine (AC), trimethylarsine (TMA), and trimethylarsine oxide (TMAO) have also been found in some marine organisms [Cullen and Reimer, 1989; Shiomi, 1994]. An exception to the prevalence of organic arsenic species in marine organisms is the brown alga, *Hizikia fusiforme*, in which approximately 60% of total arsenic is in the form of inorganic arsenic [Shiomi, 1994]. High levels of arsenic in marine organisms result from food chain accumulation. Arsenobetaine is bioaccumulated mainly in the higher trophic levels of aquatic food chains and is metabolically very stable [Vahter et al., 1983]. Arsenocholine is likely to be the immediate metabolic precursor of arsenobetaine, since arsenocholine administered to experimental fish as part of their diet was rapidly and almost completely converted into arsenobetaine and retained in fish tissues [Francesconi et al., 1989]. Food chain accumulation of arsenic compounds is also observed in marine zooplankton and phytoplankton [Lindsay and Sanders, 1990; Shibata, et al., 1996]. Zooplankton contained arsenobetaine as the dominant arsenic species, whereas phytoplankton contained arsenic-containing ribofuranosides in a species-specific manner [Shibata et al., 1996]. The concentration of total arsenic in marine organisms is very high compared to most terrestrial organisms [Shiomi, 1994]. Unicellular algae were able to accumulate arsenic at concentrations 3000 times higher than that of the surrounding water [IPCS, 1992]. In addition to marine organisms, some terrestrial species have also been found to contain high levels of arsenic. For example, several higher fungi have been reported to contain very high levels of organic forms, including MMAA, DMAA, AB, as well as toxic inorganic arsenate and arsenite [Stijve et al., 1990; Stijve, 1995; Stijve and Bourqui, 1991; Byrne et al., 1995]. Furthermore, some arsenic-accumulating species of fungi are able to convert poisonous inorganic arsenic into organic

derivatives of considerably lower toxicity. The concentration of total arsenic in these fungal species ranges from 0.9 to 2115 ppm dry weight [Byrne et al., 1995; Stijve, 1995].

1.2 USES

Arsenic is probably the most popular element in human history. Its notorious reputation stems from its prominent use as a poison in homicidal and suicidal events. Arsenic is one of the ingredients in the famous warfare agent, Lewisite, developed during World War I. However, arsenic has also long been used as a remedy and tonic. Recognition of the many beneficial properties of this element has led to the use of arsenic compounds in a wide variety of fields. Over the last two centuries, this element has made major contributions to science, medicine, and technology. For instance, Salvarsan 606 (arsphenamine) was the first man-made antimicrobial chemotherapeutic agent. Synthesized early this century by Paul Erlich, Salvarsan 606 enabled an unprecedented efficacy of treatment for syphilis in humans. Nearly 40 years passed before it was replaced by the more efficient and less toxic antibiotic, penicillin [Doak and Freedman, 1970]. Inorganic trivalent arsenic (e.g. Fowler's solution, which contains 1% potassium arsenite) has been used for the treatment of leukemia, psoriasis, and asthma, and doses can be up to several milligrams daily [Pershagen, 1986]. Although more potent and selective drugs are now available for treatment of most infections, aromatic arsenicals (e.g. Melarson oxide) and drugs containing the related metal, antimony (e.g. Pentostam), are still used to treat some protozoan infections caused by *Trypanosome* spp. (e.g. African sleeping sickness) or *Leishmania* spp. (e.g. espundia, kala azar).

In addition to its medical uses, arsenic compounds have been employed in other fields, including industry, agriculture, and forestry. Agricultural arsenicals are mainly used as biocides, sheep dips, feed additives, and cotton desiccants. Arsenate and arsenite have been used largely as wood preservatives (e.g. chromated copper arsenate) and debarking agents. Gallium arsenide (GaAs) and indium arsenide (InAs) have been employed in the semiconductor industry. Industrial use of arsenicals also occurs in glass manufacturing, leather tanning, metal alloy manufacturing, and many other industries. Many arsenic

compounds are now commercially available for human use. The primary man-made arsenic product is arsenic trioxide, also known as white arsenic, which can be used for the production of other arsenic compounds. Arsenic trioxide is a by-product in the smelting of copper, lead, cobalt, and gold ores. It has been estimated that world production of arsenic, as arsenic trioxide, is approximately 60,000 tons per year [IPCS, 1992; Azcue and Nriagu, 1994], about one third of which is used for wood preservation [IPCS, 1992]. Although arsenic is no longer used mainly with evil intents, its prevalence and toxicity still make it one of the top four elements of environmental concern (the other three are lead, mercury, and cadmium).

1.3 HUMAN EXPOSURE AND METABOLISM

Because arsenic is ubiquitous in the environment, human exposure to arsenic is a common occurrence, and thus it is of great concern to the public. The general population is exposed to arsenic compounds predominantly through ingestion of arsenic-containing food and drinking-water and, to a lesser extent, through inhalation. For example, seafood is known to contain relatively high levels of organic arsenic. The amount of seafood ingested greatly influences the daily intake of arsenic by humans. However, the majority of arsenic present in seafood is found in less toxic, organic forms [IPCS, 1992], such as arsenobetaine and arsenosugar. In humans, ingested arsenobetaine is rapidly excreted through urine without metabolic changes, whereas ingested arsenosugars are thought to be enzymatically degraded via microbial activity in the human body [Le et al., 1994]. Accumulation of arsenic in tissues of poultry and swine, due to the use of some organic arsenic compounds as feed additives, also contributes to human exposure through food ingestion. In some regions of the world, ingestion of arsenic-containing drinking-water is a major source of exposure. The standard for arsenic in drinking-water is 50 μg/l, as set by the US Environmental Protection Agency (EPA), and 10 μg/l, as set by WHO (1993). Although most major drinking-water supplies contain levels of arsenic lower than 5 μg/l, a significant portion of the population likely drink water containing arsenic exceeding these standards. Particularly in some areas in Taiwan, Mexico, Chile, Argentina, and India, high

levels of arsenic have been found in drinking-water sources [for review, see WHO, 1981; Cebrián et al., 1994]. For example, in southwest Taiwan, up to 671±149 μg of total dissolved arsenic per liter, on average, have been found in ground waters, which have been used as drinking-water sources for a subpopulation of the inhabitants. The predominant species of arsenic in these water sources was As(III), with an average As(III)/As(V) ratio of 2.6 [Chen et al, 1994]. These high concentrations of arsenic in the daily water source are thought to be the major cause of an endemic peripheral vascular disease, known as blackfoot disease (BFD), among the local population [Tseng et al., 1968]. In Mexico, high levels of inorganic arsenic, predominantly As(V), were found in well waters in some rural areas. Populations chronically exposed to arsenic through drinking these well waters have typical signs and symptoms of arsenic poisoning and an increased incidence of skin cancer [Cebrián et al., 1994]. In addition to ingestion of food or water containing high arsenic, above-average levels of exposure can occur among patients treated with arsenic-containing medicine, such as Fowler's solution. Arsenic exposure through inhalation is low, usually less than 0.1 μg per day in urban areas, among the general population. However, markedly elevated levels of arsenic exposure can occur among workers in, and residents nearby, copper and other metal smelters, and among persons manufacturing or using arsenic-containing pesticides and herbicides. In these populations, the daily intake of arsenic through air may be as high as 20 μg [WHO, 1981].

Acute toxicity of inorganic arsenic compounds for man is a source of potential danger. Ingested doses of 50-300 mg of arsenic oxide have been reported to be fatal in humans [Chen and Lin, 1994]. Long term exposure to a daily oral intake above 200μg may also cause significant toxic effects [IPCS, 1992; Cebrián et al., 1994]. Due to the obvious toxicity of inorganic arsenicals, *in vivo* metabolism of inorganic arsenic has been studied extensively in mammals. The metabolic pathway has been elucidated and can be divided temporally into four stages: uptake, distribution, biotransformation and elimination. It is known that most ingested or inhaled arsenic is readily absorbed from either the gastrointestinal or respiratory tract, where it rapidly enters the bloodstream and is transported to a large number of tissues and organs [Hunter et al., 1942]. Arsenic

compounds absorbed into the body undergo biochemical transformation processes, mainly methylation and reduction/oxidation reactions. In terms of methylation, most mammals methylate inorganic arsenic to less toxic organic metabolites, such as MMAA and DMAA. Both MMAA and DMAA may undergo further methylation, giving rise to dimethylated and trimethylated compounds. All administered arsenic compounds, with or without biotransformation, tend to be eliminated in a relatively short period of time. Methylated arsenicals have been shown to be excreted more rapidly than inorganic arsenic compounds. The excretion of arsenic occurs mainly through urine [For review see Vahter and Marafante, 1988].

Based on the accumulated information on arsenic metabolism, the levels of arsenic in whole blood and urine are often examined clinically as biological indicators of human exposure. In persons with no known source of exposure, the whole blood arsenic levels are in the range of a few micrograms per liter, and the urine arsenic levels range from 10 to 50 μg/l. While in subjects exposed to water containing high levels of arsenic, the whole blood arsenic can reach concentrations as high as 50 μg/l [WHO, 1981]. Furthermore, the urine arsenic levels in smelter workers exposed to inorganic arsenic may reach a few hundred micrograms per liter [WHO, 1981]. Major species differences exist in mammals with respect to arsenic methylation [Vahter, 1994]. For example, a significant amount of MMAA is found only in human urine following exposure to inorganic arsenic [Vahter and Marafante, 1988]. A typical pattern of arsenic speciation in human urine is 10 - 15% inorganic arsenic, 10 - 15% MMAA, and 60 - 80% DMAA [Le et al., 1994; Vahter, 1986b; Buchet et al., 1981a; b; Fao et al., 1984]. More efficient methylation of inorganic arsenic was found in rodents and dogs, and this methylation occurred mainly to DMAA. MMAA accounts for less than a few percent of urinary arsenic. Excretion of arsenic is also faster in dogs and rodents than in humans. [Vahter and Marafante, 1988; Vahter, 1994]. Marmoset monkeys [Vahter et al., 1982; Vahter and Marafante, 1985] and chimpanzees [Vahter et al., 1995b] are the only mammals reported not to be able to methylate inorganic arsenic due to the lack of methyltransferases that catalyze the methylation of inorganic arsenic [Zakharyan et al., 1996]. As a result, extensive tissue and blood retention of

arsenic and a low rate of excretion have been observed [Vahter and Marafante, 1985; 1988].

Recently, a unique pattern of inorganic arsenic metabolism was observed in native Andean women living in four villages in northern Argentina, where elevated levels of arsenic were found in drinking-water (2.5, 14, 31, and 200 μg/l, respectively) and food (9-427 μg As/kg wet weight) [Vahter et al., 1995a]. Elevated arsenic concentrations, ranging between 2.7 to 18 μg/l, were found in the blood of the subjects consuming the highest arsenic concentration (200 μg/l) in drinking-water. The average concentrations of the sum of inorganic As, MMAA and DMAA metabolites in the urine samples ranged from 14 to 256 μg/l, whereas the total urine arsenic concentrations were only slightly higher (18 to 258 μg/l), indicating that inorganic arsenic was the main form of As ingested. A striking finding was the extremely low level of MMAA (overall median 2.2%, ranging 0-11%) in the urine samples, suggesting that genetic polymorphism exists for the control of the methyltransferase activity involved in arsenic methylation [Vahter et al., 1995a]. Moreover, a significantly higher percentage of urinary DMAA was found in the subjects consuming water containing the highest level of arsenic (200 μg As/l), indicating an induction of DMAA formation by this high arsenic exposure [Vahter et al., 1995a].

Although total urine and whole blood arsenic levels are often used as biological indicators of exposure, limitations exist for both of these measurements in evaluating the extent of exposure. For example, the total urine and blood arsenic levels can be readily affected by the intake of arsenic-containing food, unless the subjects under study refrain completely from ingestion of seafood before their urine is sampled. To overcome the interference from the seafood arsenic, selective methods have been developed for monitoring exposure to inorganic arsenic [Braman and Foreback, 1973; Braman et al., 1977; Norin and Vahter, 1981], as will be discussed later. Another limitation for the use of whole blood arsenic concentration is its time-dependence, since arsenic in blood is rapidly cleared following exposure. Other biochemical indicators and genetic markers, such as changes in the ratio of coproporphyrin:uroporphyrin associated with urinary excretion [Cebrián et al., 1994], the frequency of chromosomal aberrations and sister-chromatid

exchanges [Conner et al., 1993; Cebrián et al., 1994], and the levels of gene expression and enzymatic activities [Craig et al., 1996], have been sought for monitoring arsenic exposure and toxicity. These will be discussed in later sections.

1.4 CHEMICAL PROPERTIES

Arsenic is a member of the nitrogen group, situated between phosphorus and antimony in the periodic table, and thus classified as a transitional element or metalloid. The transitional status of arsenic between metals and nonmetals reflects the fact that it can form alloys with metals, as well as covalent bonds with carbon, sulfur, hydrogen, and oxygen [Malachowski, 1990]. Therefore, the chemistry of arsenic is very complex. Arsenic may exist as a free element [As (0)], which is extremely rare in nature, or in various combined inorganic or organic forms, in which arsenic appears mainly in three oxidation states, +V, +III, and -III [Tamaki and Frankenberger, 1992]. Elemental arsenic is a gray crystalline material with atomic number 33 and atomic weight 74.92, and is formed by the reduction of arsenic oxides. Arsines and alkylarsines, which are characteristic of arsenic in the -III oxidation state [As(-III)], are generally unstable in air. Arsenate [As(V)] and arsenite [As(III)] are the prevalent forms of inorganic arsenic found in natural environments. The toxicity of arsenic compounds to living organisms varies with oxidation state, organometalloidal form, dose, and duration of exposure. In general, arsine gas (H_3As) is the most toxic form to mammals, followed in order of decreasing toxicity by inorganic trivalent arsenic compounds, organic trivalent arsenic compounds, inorganic pentavalent arsenic compounds, organic pentavalent arsenic compounds, and finally, elemental arsenic [Malachowski, 1990; Gorby, 1994]. Some organic arsenic compounds are not found to be toxic at any doses, such as arsenobetaine [Vahter et al., 1983], the major form of arsenic compound in seafood. In biological systems, arsenicals are readily converted between oxidation states and organometalloidal forms, thus altering their relative toxicity, and complicating the understanding of their intoxication and detoxification mechanisms.

1.5 TOXICITY AND BIOCHEMISTRY

Existing knowledge on the biological toxicity of arsenicals to humans is far from complete. Our current understanding of the mechanisms of arsenic toxicity comes mainly from the early work on arsenic toxicity in animals, the development of organic arsenical drugs during the late 1800s and the early 1900s, and from the work stimulated during the 1940s by the need to find effective antidotes to arsenical warfare agents. These studies revealed that the toxicity of arsenic is primarily due to its ability to form covalent bonds with sulfur and its similarity to phosphorus, an essential element to living organisms [For review, see Squibb and Fowler, 1983; Malachowski, 1990; Tamaki and Frankenberger, 1992]. Trivalent arsenic is highly reactive with sulfhydryls in biological systems, resulting in the inhibition of critical sulfhydryl-containing enzymes. Many enzymes are susceptible to deactivation by arsenic. In most cases, the enzyme activity can be protected or restored by addition of excess amounts of monothiols, such as glutathione, suggesting that inhibition is due to the interaction of arsenic with a single thiol group in the enzyme molecule [Squibb and Fowler, 1983]. An important exception to this finding is that arsenite can inhibit the pyruvate dehydrogenase multi-enzyme complex, whose activity cannot be protected against arsenic toxicity by addition of excess monothiols. Further studies found that inhibition of pyruvate dehydrogenase activity is mediated by binding of arsenic to a lipoic acid moiety, which has a dithiol group that can form a stable ring structure with arsenite [Aposhian, 1989]. This finding stimulated the search for dithiol compounds that are able to antagonize the warfare agent, Lewisite, and led to the discovery of a 2',3'-dimercaptopropanol, also known as British anti-Lewisite (BAL), which eventually became widely used as an antidote for arsenic poisoning. The interaction between trivalent arsenic and thiols also accounts for the efficacy of the aromatic arsenical drug, melarson oxide [p-(4,6-diamino-s-triazinyl-2-yl) aminophenyl arsenoxide], in the treatment of African sleeping sickness [Fairlamb et al., 1989; Cunningham et al., 1994]. The primary target for melarson oxide is trypanothione, which is a dithiol of trypanosomatids. Interaction of trivalent arsenic with trypanothione results in the formation of a stable adduct, which is a competitive inhibitor of the flavoprotein

trypanothione reductase, an enzyme that is essential in regulating the thiol/disulfide redox balance in the parasite and absent from the host [Fairlamb et al., 1989; Cunningham et al., 1994]. A similar mechanism may also be responsible for the efficacy of Salvarsan 606, the aromatic trivalent arsenic compound used in the treatment of syphilis.

On the other hand, arsenate (H_3AsO_4), the inorganic pentavalent form, is an analogue of phosphate and is taken up via phosphate transport systems by most organisms. Arsenate interferes with phosphate metabolism by competition and substitution for phosphate, resulting in the formation of unstable arsenate esters. These arsenate esters readily undergo hydrolysis with a concomitant loss of high-energy phosphate bonds to the cells, resulting in uncoupling of oxidative phosphorylation [Valee et al., 1960; Summers and Silver, 1978; Malachowski, 1990]. This toxic process is termed arsenolysis and appears to be limited to inorganic pentavalent arsenate. Pentavalent MMAA and DMAA, and trivalent arsenite, did not perturb phosphate metabolism [Delnomdedieu et al., 1995], probably because of their chemical structures differing from that of phosphate. In addition, arsenate can be reduced to arsenite within cells, as will be discussed below, exerting its toxic effects via interaction with sulfhydryl groups of critical cellular enzymes.

Volatile arsine gases, such as arsenic hydride (AsH_3), are the most toxic forms to mammals and induce lysis of red blood cells [Malachowski, 1990; Tamaki and Frankenberger, 1992]. However, the mechanism of arsine toxicity remains to be elucidated. Further tests are also needed to determine the toxicity of alkylarsines.

Arsenic species in natural environments are subject to oxidation, reduction, and methylation reactions, most of which are biochemically mediated [Summers and Silver, 1978; Cullen and Reimer, 1989; Tamaki and Frankenberger, 1992]. Arsenic oxidation/reduction occurs in various living organisms [Green, 1918; Turner, 1949; Phillips and Taylor, 1976; Bencko et al., 1977; Vahter and Envall, 1983] and is involved in many metabolic and toxicological processes. Oxidation of arsenite to less toxic arsenate was found in arsenite tolerant bacteria isolated from cattle-dipping solutions [Green, 1918; Turner, 1949]. The process was effected by a soluble enzyme, whose activity was inducible, and appeared to be coupled to electron transport via cytochromes to oxygen

[Turner, 1949]. Thirteen arsenite-oxidizing strains of *Alcaligenes faecalis* were isolated in another study [Phillips and Taylor, 1976]. In one particularly active strain, YE56, the arsenite-oxidizing ability was found to be induced in the presence of arsenite [Phillips and Taylor, 1976]. Understanding of the mechanism of arsenite oxidation in *Alcaligenes faecalis* has progressed with the purification and characterization of an arsenite oxidase [Anderson et al., 1992]. The enzyme is an 85 kDa monomer containing one molybdenum, five (or six) irons, and inorganic sulfide cofactors, and was found to be located on the outer surface of the inner membrane. Azurin and cytochrome *c* were found to be reduced in the presence of arsenite and arsenite oxidase. Moreover, reduced azurin also reduces cytochrome *c*, suggesting the possible formation of a periplasmic electron transfer pathway for arsenite detoxification, and indicating that the electron transfer pathway for arsenite oxidation may involve both azurin and cytochrome *c* [Anderson et al., 1992]. Since arsenite is more toxic than arsenate, this oxidation reaction may represent a detoxification mechanism evolved in these organisms. Arsenite oxidation has also been found in mixed thermophilic bacterial cultures used in mining industries for biooxidation of gold-bearing arsenopyrite, giving rise to Fe(III), S(VI) and As(V) [Barrett, et al., 1993]. Arsenic oxidation and reduction are also involved in the release of arsenic from sulfidic ores via microbial activities [Cullen and Reimer, 1989; Tamaki and Frankenberger, 1992].

Reduction of arsenate to arsenite has been observed in a variety of organisms, and implicated in many biochemical and detoxification processes. In mammalian cells, reduction of arsenate to arsenite is usually a prerequisite for arsenate methylation. In bacteria, arsenate reduction occurs mostly through detoxification mechanisms, in which arsenate is reduced to arsenite before being exported from the cells via an energy-dependent process [Silver and Keach, 1982]. Reduction of arsenate has also been observed in the aquatic bacterium, *Pseudomonas fluorescens*, under aerobic conditions, in activated sewage sludge under anaerobic conditions, and in wine yeast [Cullen and Reimer, 1989]. Recently, an arsenate utilizing microorganism, MIT-13, has been identified which acquires energy for growth through reduction of arsenate to arsenite [Ahmann et al., 1994].

In nature, biological methylation of arsenic is a ubiquitous phenomenon [Cullen and Reimer, 1989]. Since inorganic arsenate and arsenite are known to be more toxic than their corresponding methylated compounds to humans and animals [Braman and Foreback, 1973; Peoples, 1974; WHO, 1981], arsenic methylation in living organisms may account for a natural defense mechanism against inorganic arsenic toxicity. Alternatively, arsenic detoxification may simply be an additional consequence of methylation reactions involved in arsenic metabolism. Due to the complexity of arsenic chemistry, however, the mechanism of arsenic methylation is not fully understood and thus attracts much research interest. It has been shown that trivalent arsenicals are preferred substrates for methylation reactions, and that arsenate must be reduced to arsenite in order to be methylated [Cullen et al., 1984; Buchet and Lauwerys, 1985; 1987; 1988]. Arsenate is methylated to DMAA via a sequential series of reactions, which include the formation of arsenite and MMAA [Challenger, 1945]. Dithiols are also involved in arsenic methylation [Buchet and Lauwerys, 1988; Georis et al., 1990]. Methylation reactions occur enzymatically via oxidative addition of a methyl group to arsenic with S-adenosyl-methionine (SAM) as the methyl donor [Challenger et al., 1954; Cullen et al., 1977; 1995]. Finally, mono- and dimethylation reactions are catalyzed by distinct methyltransferases, a monomethyltransferase and a dimethyltransferase [Buchet and Lauwerys, 1985; Georis et al., 1990].

Given the high reactivity of trivalent arsenicals with thiols, the role of glutathione (GSH), a major intracellular non-protein thiol, has been shown to be important in arsenic biotransformation and toxicology. GSH is involved in both reduction of As(V) to As(III) and methylation [Vahter and Envall, 1983; Vahter and Marafante, 1983; Buchet and Lauwerys, 1988; Delnomdedieu et al., 1994a]. Cellular toxicity of arsenicals is inversely related to intracellular levels of glutathione, and can be enhanced by glutathione depletion [Chang et al., 1991; Huang et al., 1993; Ochi et al., 1994, Oya-Ohta et al., 1996]. Winski and Carter (1995) showed that removal of thiols by pre-treating cells with the sulfhydryl derivatizing agent, N-ethylmaleimide (NEM), severely inhibited the formation of As(III). The interaction of arsenic with thiols is not limited to the free thiols, since the concurrent

increase in glutathione disulfide (GSSG) did not account for the total loss of GSH during arsenate reduction, suggesting the formation of mixed disulfides with proteins (ProSSG). This is supported by the observation of a mixed complex of As(III) with GSH and hemoglobin in red blood cells [Winski and Carter, 1995]. *In vitro* assays with rabbit erythrocytes exposed to arsenite or arsenate showed that the accumulation of As(III) in the cells reached a quasi-plateau at 78% of the total As after a 1 h incubation and 88% of the total As after a 24 h incubation [Delnomdedieu et al., 1994b; 1995]. Approximately 20% of the total erythrocyte As(III) was associated with proteins, particularly with hemoglobin, and 68% is bound to GSH. The main fraction of the As(V) enters the phosphate metabolic pathway, depletes ATP, and increases free phosphate (Pi) [Delnomdedieu et al., 1994b; 1995].

During the methylation process, GSH is required mainly for the reduction of arsenate to arsenite, and MMAA(V) to MMAA(III) [Cullen et al., 1984]. The role of GSH in the reduction of As(V) and DMAA(V) and subsequent binding to As(III) and DMAA(III) was examined using proton and ^{14}C NMR [Delnomdedieu et al., 1994a], and the results suggest that As(V) must be reduced to As(III) before complexing with GSH. Stoichiometrically, two GSH molecules are required as electron donors for the reduction of one As(V) to As(III), and three GSH molecules are required for the subsequent complexing with one As(III), forming a $(GS)_3As(III)$ complex [Delnomdedieu et al., 1994a]. Omission of GSH from an *in vitro* assay system nearly abolished the methylation of arsenite [Styblo et al., 1996a]. In addition, GSH also plays a role in stabilizing the reductive cellular environment, allowing arsenic methylation to occur. On the other hand, cellular levels of GSH are susceptible to arsenic exposure. For example, a dose-dependent decrease in GSH has been observed in the liver, kidney, and heart of rats following arsenite treatment [Ramos et al, 1995]. Arsenate was also able to decrease non-protein thiols, mainly GSH, in rat red blood cells in a time- and dose-dependent manner, probably through a redox reaction with subsequent formation of arsenite and oxidized glutathione (GSSG) [Winski and Carter, 1995]. GaAs, a superior semiconductor with extremely low solubility, caused a decrease in the blood GSH level following treatment of male rats

[Flora and Kumar, 1996]. This was probably due to the liberation of inorganic arsenic *in vivo*, as was previously observed in hamsters [Yamauchi et al, 1986]. At an equimolar concentration of 0.5-10 mM arsenic, As(III) is more potent in depleting thiols than As(V) [Winski and Carter, 1995]. This can be explained by a higher uptake rate of As(III) into cells than As(V) [Delnomdedieu et al., 1994b; 1995], and the direct interaction of As(III) with cellular thiols. A model for the interaction between arsenic and thiols has been proposed based on the observation that the affinity of arsenite for dithiols is greater than that for monothiols, and the binding affinity is inversely related to the distance between the two thiol groups [Delnomdedieu et al., 1993].

In summarizing the mechanism of biomethylation in higher eukaryotic systems, a chemical hypothesis was proposed which consists of two mechanisms (I and II) differing in the involvement of dithiol cofactors for arsenic methylation [Thompson, 1993]. It is hypothesized that arsenate is reduced to arsenite using GSH as an electron donor. Arsenite binds to a dithiol, which could be a methylation cofactor (in Mechanism I) or a monomethyltransferase (in Mechanism II). The arsenic-dithiol complex may remain bound through the dimethylation step or the dithiol cofactor may be hydrolyzed from the arsenic complex after the monomethylation step is completed. Most likely, the dithiol cofactor could be the mono- or dimethyltransferase. The As(III) species bind to mono- or dimethyltransferase and are then released upon oxidative methylation, because the organoarsenic (V) compounds have a lower affinity for dithiols than the corresponding As(III) species [Thompson, 1993]. This is the underlying principle of arsenic detoxification. However, there are exceptions to this methylation detoxification mechanism. For example, as noted earlier, marmoset monkeys and chimpanzees are unable to methylate inorganic arsenic due to the lack of methyltransferase activity [Vahter et al., 1982; 1995b; Vahter and Marafante, 1985; Zakharyan et al., 1996]. Therefore, a different mechanism may be involved in arsenic detoxification in these organisms.

Microorganisms (e.g. fungi and bacteria in soil, algae in water) have the capacity to methylate inorganic arsenic to the much less acutely toxic compounds, MMAA and DMAA; the latter is readily converted in soil to the volatile methylarsines [Summers and

Silver, 1978; Cullen and Reimer, 1989]. The formation of methylarsines is an important part of the environmental cycle of arsenic. The mechanism of arsenic methylation in *Methanobacter* spp. was found to be associated with methane biogenesis, and effected by a coenzyme M, 2,2'-dithiodiethane sulfonic acid [McBride and Edwards, 1977]. Moldy fungi are also able to methylate arsenic, through a process that involves S-adenosylmethionine [Cullen et al., 1977]. Algae actively take up naturally occurring arsenate, which is reduced and transformed into a variety of organic arsenic compounds [Takimura et al., 1996]. On the other hand, demethylation of methyl arsenic compounds by microorganisms has also been reported [Hanaoka et al., 1996; Chen et al., 1996; Hasegawa, 1996]. For instance, sedimentary microorganisms degrade trimethylarsine oxide and tetramethylarsonium salts to inorganic As(V), and arsenocholine to arsenobetaine [Hanaoka et al., 1996]. In the natural waters of Tosa bay and Uranouchi inlet, methylarsenic(V) was found to be produced through bacterial decomposition of organic matter [Hasegawa, 1996]. Both methylation (into trimethylarsine oxide) and demethylation (into arsenite) of DMAA have been observed in rats following chronic oral exposure, and this demethylation of DMAA may be associated with intestinal bacteria [Chen et al., 1996].

1.6 PHYTOTOXICITY

Arsenic is toxic to all living organisms, including bacteria, plants, animals, and human. Studies on the phytotoxicity of arsenic indicate that the margin between background and toxic concentrations of arsenic in soils is very narrow [Sheppard, 1992]. Phytotoxicity primarily depends on the source of arsenic. In contrast to animals and humans, plants are more sensitive to organic arsenicals than to inorganic forms [Sheppard. 1992]. When inorganic forms of arsenic are considered, the type of growth medium, such as the soil content, is the only other significant variable factor for arsenic phytotoxicity [Sheppard, 1992]. For instance, different levels of phytotoxicity of arsenic were observed in Canola when grown in hydroponic solution or in soil containing similar amounts of arsenic. High phosphorus concentrations in the growth medium (the hydroponic solution)

appear to protect against arsenic toxicity in the plant [Cox et al., 1996], indicating a competition of phosphate with arsenate for uptake. The amount of arsenic taken up by plants is proportional to the concentration of arsenic in the soil, except at very high concentrations [IPCS, 1992]. In the mineral and arsenic enriched soils surrounding a former smelter, marked modifications to the native vegetation community were observed, including loss of evergreen forests, species impoverishment, and reduction in the vertical complexity of the habitat [Galbraith et al., 1995]. Substantial phytotoxicity was typically represented by the reduction in root growth. The causes of phytotoxicity in mineral soils are positively correlated with the high concentrations of As, Cu, and Zn and, to a lesser extent, Pb and Cd [Kapustka et al., 1995]. Resistance to arsenic toxicity has been found in plants growing on mine or smelter wastes [Meharg et al., 1993; IPCS, 1992]. These plants may contain high levels of arsenic and poison animals consuming them. Arsenic is distributed to all tissues in plants [IPCS, 1992]. The toxicity of arsenic to aquatic plants and invertebrates varies with pH and usually decreases with increasing pH, reflecting a change in oxidation states [Tamaki and Frankenberger, 1992].

1.7 DETRIMENTAL EFFECTS OF ARSENIC TO HUMANS AND ANIMALS

The toxic effects of arsenic to humans and animals are numerous. Acute symptoms of arsenic poisoning in humans, who have ingested inorganic arsenic, are characterized by profound gastrointestinal distress, including constriction of the throat, severe nausea with projectile vomiting, gastric pain, profuse watery or bloody diarrhea, dehydration, and also leg cramps, irregular pulse, shock stupor, paralysis and coma [Malachowski, 1990; Landrigan, 1992; Garcia-Vargas and Cebrián, 1996]. Inorganic arsenic also has carcinogenic effects on humans [Smith et al., 1992; Chen and Lin, 1994]. Systemic effects of chronic oral exposure to inorganic arsenic include skin abnormalities, such as hyper-pigmentation, palmer and plantar keratosis, and cutaneous malignancies [Malachowski, 1990; Cebrián et al., 1994; Chen and Lin, 1994; Maloney, 1996]. Internal malignancies have also been found to be associated with long term arsenic exposure [Chen et al., 1992]. Inhalation of inorganic arsenic has a well-documented history of inducing lung cancer

among smelter workers [WHO, 1981]. Although arsenic has been clearly identified as a human carcinogen, there is limited evidence to show the carcinogenicity of arsenic in experimental animals. Arsenic has been shown to induce chromosomal damage and gene amplification. However, it fails to induce mutations at specific gene loci. Arsenic potentiates cytotoxicity and mutagenicity of several chemicals. The mechanisms of arsenic-induced carcinogenicity and co-mutagenicity, however, remain to be elucidated. Arsenic exposure is also associated with embryotoxicity in both humans and animals. In this section, epidemiological observations of arsenic induced carcinogenesis and the cause-effect relationship of arsenic carcinogenicity, genotoxicity and co-mutagenicity, as well as the developmental toxicity of arsenic on humans and experimental animals, will be discussed.

1.7.1 Carcinogenicity

Arsenic is a well-established human carcinogen [IARC, 1980; 1982] with a well-defined dose-response relationship [Chen et al., 1986a; 1988; Chiou et al., 1995]. The cancer risk from ingested arsenic has been reviewed repeatedly [IARC, 1980; Smith et al., 1992; Chen and Lin, 1994]. Populations who consume drinking-water containing high levels of arsenic were found to have high rates of skin cancer [Tseng, et al., 1968; Cebrián, et al., 1983; Chakraborty and Saha, 1987; Zaldivar, 1974]. A causal relationship between ingested arsenic and liver, lung, bladder, and kidney cancers has also been established [Chen et al., 1992; Smith et al., 1992; Chiang et al., 1993]. For example, the rate of bladder cancer in an area of endemic peripheral blackfoot disease (BFD) on the southwest coast of Taiwan is about 10 times higher than that found in other areas in Taiwan [Chiang et al., 1993]. High concentrations of arsenic in artesian well water were identified to be a major factor related to the high rate of bladder cancer [Chiang et al., 1993]. In an 11-year follow-up study, a significant excess of bladder cancer mortality (observed/expected ratio = 5/1.6; $p = 0.05$) was observed in a cohort of 478 patients treated with Fowler's solution [Cuzick et al., 1992]. Increased incidence of liver cancers, such as hepatic angiosarcoma and hepatocellular carcinoma, was found among vintners

who had been exposed to arsenic-containing pesticides [Roth, 1957], among patients treated with Fowler's solution [Popper et al., 1978; Falk et al., 1981; Roat et al., 1982], and among subpopulations drinking high arsenic-containing water [Rennke, 1971; Chen et al., 1985a]. A significantly increased mortality from hepatocellular carcinoma has been reported among copper smelter workers in Japan, with a standardized mortality ratio of 3.4 [Tokudome and Kuratsune, 1976]. High incidence of lung cancers caused by inhalation of arsenic through contaminated air in smelter workers has also been reported [IARC, 1980; Reger and Morgan 1993; Liu and Chen, 1996]. The content of arsenic in the lung tissue of exposed mining workers was found to be 17 times higher than that in the control group. Moreover, the incidence of lung cancer was found to be positively correlated with the amount of arsenic accumulated in the lungs, the length of time of working in the mines, and the ambient arsenic concentration in the mining environment [Liu and Chen, 1996].

Carcinogenesis is a multi-step process, including initiation, promotion, and progression stages [For review, seey Weinstein, 1988; Barrett, 1993]. In terms of the role of chemical carcinogens in the temporal development of cancers, they can be grouped as tumor initiators and promoters. In view of the mechanisms of chemical carcinogens, however, they can be classified as genotoxic and nongenotoxic [Cohen and Ellwein, 1990]. Although it is obvious that arsenic is a human carcinogen, little evidence has supported its carcinogenicity in experimental animals [Thorgeirsson et al., 1994]. Rather, increasing evidence appears to support the co-carcinogenic effects of arsenic in animals. Therefore, the mechanism of arsenic carcinogenicity is currently under debate.

Yamamoto and his colleagues (1995) found that when rats were pretreated with multiple known carcinogens, DMAA treatment significantly enhanced tumor induction in the urinary bladder, kidney, liver, and thyroid gland. Induction of preneoplastic lesions, such as glutathione S-transferase placental form-positive foci in the liver and atypical tubules in the kidney, was also significantly increased in DMAA-treated groups. Ornithine decarboxylase (ODC) activity, an enzymatic marker for cell proliferation, was considerably increased in the kidneys of rats treated with 100 ppm DMAA. However, in

rats treated with DMAA alone, no tumors or preneoplastic lesions were observed [Yamamoto et al., 1995]. These results suggest that DMAA, considered to be non-toxic in many situations, is acting as a promoter, but not as an initiator, of urinary bladder, kidney, liver, and thyroid gland carcinogenesis in rats. This is supported by previous findings that accumulation of DMAA occurs particularly in the kidney, liver, and possibly in the thyroid gland [Vahter et al., 1984]. Sodium arsenite was shown to stimulate ODC and heme oxygenase activities in rat liver, but no DNA damage was detected [Brown and Kitchin, 1996], lending further support to the proposition that arsenic is a promoter rather than an initiator of tumorigenesis.

1.7.2 Genotoxicity

Ample evidence has demonstrated that arsenic has genotoxic effects on mammals both *in vivo* and *in vitro*, and the genotoxicity of arsenic appears to be associated with its carcinogenicity [For review, see Snow, 1992]. In addition to hyperkeratosis, melanosis, actinic keratosis, and basal cell carcinoma, a significantly increased frequency of sister-chromatid exchanges (SCEs) in lymphocytes was found in a subpopulation exposed to drinking-water containing more than 0.13 mg/l (0.13 ppm) arsenic in Argentina [Lerda, 1994]. *In vitro* studies indicate that arsenite induces chromatid-type aberrations and SCEs in human lymphocytes [Jha et al., 1992; Wiencke and Yager, 1992], potentiates X-ray- and ultraviolet (UV)-induced chromosomal damage [Jha et al, 1992], and acts synergistically with a DNA crosslinking agent, diepoxybutane (DEB), in the induction of aberrations [Wiencke and Yager, 1992; Yager and Wiencke, 1993]. Both arsenite and arsenate were shown to inhibit human lymphocyte stimulation and proliferation [Jha et al., 1992; Gonsebatt et al., 1992; Wiencke and Yager, 1992]. Induction of SCEs by arsenite correlates with the extent of inhibition of lymphocyte proliferation [Wiencke and Yager, 1992]. The effects of inorganic arsenic on DNA synthesis have been investigated. At very low concentrations, either arsenite or arsenate enhanced DNA synthesis in phytohemagglutinin (PHA)-stimulated human lymphocytes. Whereas inhibition of DNA synthesis was observed when arsenate or arsenite was present at high concentrations

[Meng, 1993]. Animal studies showed that treatment of Chinese Hamster Ovary (CHO) cells with sodium arsenite during their G2 phase induced poorly condensed chromosomes and chromatid breaks during metaphase, doubled the chromosome number by the second division, and delayed the re-entry of mitotic cells into interphase [Gurr et al., 1993]. One speculation on the mechanism of arsenic carcinogenesis suggests that arsenic-induced inhibition of DNA synthesis and delay in lymphocyte proliferation could represent an impairment of the cellular immune response and consequently, may facilitate or eventually lead to malignancy [Ostrosky-Wegman et al., 1991]. Since toxicity varies with the oxidation states of arsenic and its organometalloidal forms, the potency of different arsenicals in the induction of chromosomal aberrations in human fibroblasts has been investigated [Oya-Ohta et al., 1996]. It was found that the rank of clastogenic potency of compounds was in the order arsenite > arsenate > DMAA > MMAA > TMAO (trimethylarsine oxide). Although DMAA did not induce any detectable DNA damage in rat cells [Brown and Kitchin, 1996], it was very potent in causing chromosome pulverization in most metaphases in human fibroblasts when present at doses higher than 7×10^{-3} M. Other organoarsenic compounds, such as arsenosugar, arsenocholine, arsenobetaine, and tetramethylarsonium iodide were less effective [Oya-Ohta et al., 1996]. Depletion of GSH increased the frequency of chromosomal aberrations induced by arsenite, arsenate, and MMAA, but markedly suppressed the clastogenic effects of DMAA. Potent clastogenic effects of DMAA were observed even in GSH-depleted cells with exogenously added GSH [Oya-Ohta et al., 1996], suggesting that GSH may play a role in protecting cells against the clastogenic effects of arsenite, arsenate, and MMAA, but facilitate the expression of the clastogenic effects of DMAA. Together, these observations suggest a possible explanation for the positive carcinogenicity of arsenic compounds in man, but not in animals, that arsenic compounds may affect or cause some, but not all, elements of multi-stage carcinogenesis. The elements of multi-stage carcinogenesis, which arsenic does not cause, may be naturally operative in humans but not in common experimental animals such as rats and mice. Therefore, arsenic may act as an incomplete carcinogen in some circumstances. It is also possible that the clastogenic

effects of arsenic observed in human cells may lead to the loss of tumor suppressor genes or impairment of their function, or the activation of oncogenes, thus contributing to arsenic carcinogenicity.

Although arsenic-induced DNA damage has been widely observed in mammalian systems, the mechanisms of arsenic genotoxicity are not fully understood. However, evidence suggests that the genotoxic effects of arsenic may be mediated by oxygen radicals. For example, arsenite was shown to enhance the production of heme oxygenase, an indicator of oxidative stress [Applegate et al., 1991; Lee and Ho, 1995]. Secondly, the frequency of SCEs induced by arsenite can be reduced by addition of the radical-scavenging enzyme, superoxide dismutase, but not by catalase [Nordenson and Beckman, 1991]. In contrast, an X-ray sensitive CHO cell line, XYS-5, with lower levels of catalase activity, is hypersensitive to arsenite-induced micronucleus formation and cell killing [Wang and Huang, 1994], suggesting that arsenic-induced SCE and micronucleus formation are mediated by different mechanisms. Moreover, catalase could effectively reduce the frequency of arsenite-induced micronuclei, suggesting that arsenite probably induces micronuclei via the overproduction of hydrogen peroxide (H_2O_2) [Wang and Huang, 1994]. Finally, oral administration of DMAA induces lung-specific DNA damage through the formation of active oxygen radicals [Yamanaka et al., 1989; 1990; 1991]. The formation of dimethylarsenic peroxyl radical [$(CH_3)_2AsOO\bullet$] is probably responsible for the lung-specific DNA single-strand breaks (SSB) in mice [Tezuka et al., 1993], and may also be responsible for the formation of DNA-nuclear protein crosslinkings in DMAA-treated cell cultures [Yamanaka et al., 1993]. The linkage was found to occur through a phospho-nitrogen bond at serine and threonine residues of H1 histone and of non-histone proteins [Yamanaka et al., 1993]. To elucidate the mechanism of DMAA-induced DNA damage, Yamanaka and colleagues (1995) assayed alkali-sensitivity of DNA in a human embryonic cell line of alveolar epithelial (L-132) cells exposed to DMAA. They found that formation of alkali-labile sites, most likely apurinic/apyrimidinic (AP) sites, occurred prior to the induction of SSB and protein crosslinks, and the AP sites were formed by cleavage

of the N-glycosyl bond between the deoxyribose moiety and nucleic acid bases [Yamanaka et al., 1995].

The finding that human fibroblasts were 10-fold more susceptible to sodium arsenite than Chinese hamster ovary (CHO-K1) cells [Lee and Ho, 1994a] may account, at least in part, for the difference in arsenic carcinogenicity between humans and animals. Comparison of cellular antioxidant enzyme activity showed that CHO-K1 cells contained 3- to 8-fold more glutathione-peroxidase and catalase activities, respectively, than human fibroblasts [Lee and Ho, 1994a]. In an arsenite resistant CHO cell line, SA7, there was an elevated level of glutathione-S-transferase activity, whereas in an arsenite resistant human lung adenocarcinoma cell line, CL3R, the level of heme oxygenase was found to be increased [Lee and Ho, 1994a, b]. These results suggest that the differential toxicity of arsenic to human and animal cells could be due to differential levels of antioxidant enzymatic activities. This may result in more efficient protection against arsenic-induced oxidative damage in animal cells than in human cells.

Other mechanisms of arsenic genotoxicity have also been suggested. As mentioned earlier, arsenite is highly reactive with sulfhydryls, especially the closely spaced (vicinal) protein-bound dithiol groups [Joshi and Hughes, 1981; Knowles and Benson, 1983], which are common structural features among DNA binding proteins, transcription factors, and DNA repair proteins [Berg, 1990]. Based on these facts, it is reasonable to postulate that arsenite may interact with the dithiol groups of DNA repair proteins, thereby interfering with their normal repair functions and potentiating induced chromosomal damage. The observation that inorganic arsenicals inhibit the removal of UV-induced thymine dimers from the DNA of human SF34 cells and potentiate the lethal effects of UV in excision-proficient normal and xeroderma pigmentosum (XP) variant cells but not in excision-defective XP group A cells [Okui and Fujiwara, 1986], indicates that, in this case, the toxic effects of arsenic are excision/repair-dependent. Sodium arsenite was also found to inhibit the repair of N-methyl-N-nitrosourea (MNU)-induced lesions by affecting the incorporation of dNMPs into damaged DNA templates or by interfering with the ligation step [Li and Rossman, 1989a, b]. In addition, arsenite was shown to retard the

disappearance of UV- or alkylation-induced DNA strand breaks in Chinese hamster ovary cells [Lee-Chen et al., 1994], suggesting an inhibition in the rejoining of the broken ends. In contrast, Dong and Luo (1993) found that 1-5 μM sodium arsenite produced DNA-protein crosslinks and protein-associated DNA-strand breaks in human fetal lung fibroblasts, as well as increased unscheduled DNA synthesis (UDS) values of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)-treated human fetal lung fibroblasts [Dong and Luo, 1994]. This suggests that arsenic damages DNA directly via a protein-associated mechanism rather than via inhibiting DNA repair.

1.7.3 Co-mutagenicity

As most carcinogens are also mutagens, the mutagenicity of inorganic arsenic compounds has been investigated. Interestingly, little evidence was found to support its mutagenicity in bacteria, animals, and humans [Lõfroth and Ames, 1978; Amacher and Pailler, 1980; Rossman et al., 1980; Jacobson-Kram and Montalbano, 1985]. Arsenic is not able to induce mutations at single gene loci. Rather, co-mutagenic effects of arsenic compounds have been reported. For example, post-treatment of UV light irradiated CHO cells with sodium arsenite enhanced the mutation frequency at the hypoxanthine (guanine) phosphoribosyltransferase (hprt) locus, and altered the mutational spectrum by increasing the frequency of transversions from 57% (UV-induced only) to 70% (UV plus sodium arsenite-induced). In addition, sodium arsenite increased mutation frequency at the 5' position of TT and the 3' position of CT, suggesting that arsenite may interfere with the process of mutation fixation of TT and CT dimers during DNA replication [Yang, et al., 1992].

In contrast to the observations in eukaryotic systems, sodium arsenite was found to strongly inhibit mutations induced by UV light, 4-nitroquinoline-1-oxide (4NQO), furylfuramide (AF-2), and methyl methanesulfonate (MMS), as well as to reduce spontaneous mutations in the *E. coli* WP2*uvrA*/pKM101 reversion assay. However, no such effect was observed in mutagenesis induced by N-methyl-N'-nitro-N-nitrosuguanidine (MNNG). The mechanism of antimutagenesis by sodium arsenite was

thought to be two fold: i) as an inhibitor of *umuC* gene expression, and ii) as an enhancer of *uvrA* and *recA*-dependent error-free repairs [Nunoshiba and Nishioka, 1987].

The lack of mutagenicity further supports the notion that arsenic is a tumor promoter and not an initiator. Other genetic effects concerning its tumor-promoting activity have also been observed. Arsenite was found to stimulate expression of transcription factor AP-1 [Cavigelli et al. 1996], a complex of the *c-jun* and *c-fos* gene products, whose activity can also be stimulated by several other tumor promoters. Arsenite-induced *c-fos* and *c-jun* transcription correlates with the activation of Jun kinases (JNKs) and p38Mpk2, which phosphorylate transcription factors that activate immediate early genes, such as *c-jun* and *c-fos*. Stimulation of JNKs by arsenite is mediated by inhibition of a constitutive dual specificity of JNK phosphatase, which acts to maintain low basal JNK activity in non-stimulated cells. Inhibition of the JNK phosphatase by arsenite may result in tumor promotion through induction of proto-oncogenes, such as *c-fos* and *c-jun*, and stimulation of AP-1 activity [Cavigelli et al., 1996].

1.7.4 Embryotoxicity

Arsenic exerts profound detrimental effects on developing embryos [For review, see Domingo, 1994; Shalat et al., 1996]. Both arsenite and arsenate have been reported to have teratogenic effects in mammals [Domingo, 1994; Tabacova et al., 1996]. Exencephaly, micrognathia, open eye, tail defects, renal agenesis, gonadal agenesis and skeletal anomalies of the ribs and vertebrae are the most common defects associated with arsenic treatment on experimental animals [Domingo, 1994]. Different routes of maternal exposure may cause different extents of effects. Oral administration usually has less effect on conceptus than treatment by intraperitoneal (ip) injection [Baxley et al., 1981; Hood and Harrison, 1982]. This suggests that fetal arsenic uptake is more rapid and extensive following ip than oral maternal exposure [Hood et al., 1978, 1987]. *In vitro* studies revealed higher teratogenicity for arsenite than for arsenate. In mouse embryos, arsenite was found to be teratogenic between 3 and 4 μM and embryolethal at higher concentrations, whereas arsenate only produced similar effects when concentrations were

10 times higher [Chaineau et al., 1990]. Another study showed that, with respect to malformations and lethality in mouse whole embryos, arsenate and arsenite induce similar malformations, but arsenite is approximately three times more potent than arsenate [Tabacova et al., 1996]. Rasco and Hood (1994) investigated the effects of arsenate on the mouse conceptus when combined with maternal restraint stress, and found that arsenate, when combined with maternal restraint stress, may have a greater effect on the conceptus than exposure to either agent alone.

To understand the molecular mechanism of arsenic-induced teratogenicity, Wlodarczyk et al. (1996) examined the effects of arsenic on the expression of several cell cycle controlling genes, and found that arsenate treatment on pregnant LM/Bc mice upregulated the expression of the *bcl*-2 and P53 genes at gestational day 9:0, compared to their control values. The increased expression of both of these genes suggests that arsenic inhibits cell proliferation, which delays neural tube closure and ultimately leads to the neural tube defects, a characteristic of arsenic-induced embryo malformation observed in exposed embryos [Wlodarczyk et al. 1996]. The teratogenic effects of arsenate was also observed in the topminnow, *Fundulus heteroclitus* [Craig et al., 1996], at a level similar to that observed in murine embryos. The most common response to arsenate was a delay in growth and development, which affected approximately 90% of the surviving embryos. To delineate the specific molecular alterations that are responsible for both developmental delay and congenital defects, Craig and colleagues (1996) examined the effect of arsenate on the expression of 47 genes. They found that arsenate treatment induced alteration of expression of 11 genes, including glucocorticoid receptor (GCR) and cellular retinal binding proteins 1 and 2 (CRBP-1 and CRBP-2), which are known to play a pivotal role in embryogenesis [Craig et al., 1996].

Gallium arsenide (GaAs) was shown to cause testicular spermatid retention and epididymal sperm reduction in hamsters and rats [Omura et al., 1996a, b]. However, the same effects were not observed when the animals were treated with indium arsenide (InAs) or arsenic trioxide (As_2O_3), indicating that gallium may play an important role in the testicular toxicity of GaAs [Omura et al., 1996a, b].

1.7.5 Other effects

In addition to the above mentioned detrimental effects to plants, animals, and humans, other miscellaneous effects have also been reported. It has been observed that arsenic in the sera of GaAs-exposed mice inhibited bacterial growth and increased host resistance to *Streptococcus pneumoniae* and *Listeria monocytogenes* infections [Burns et al., 1993]. GaAs treatment of male rats has also been shown to cause a decrease in blood GSH levels, inhibition of δ-aminolevulinic acid dehydratase (ALAD) activity, an increase in urinary δ-aminolevulinic acid (ALA) excretion and blood zinc protoporphyrin levels [Flora and Kumar, 1996]. Moreover, multiple GaAs exposure produced adverse effects on the hematopoietic, renal, and immune systems [Flora and Kumar., 1996]. Disodium arsenate strongly inhibits heme biosynthesis in mouse spleen by depressing the activities of ALA synthase (ALAS), ALAD, and porphobilinogen deaminase (PBGD) [Kondo et al., 1996]. Treatment of male rats with sodium arsenate (oral dose of 10 mg As/kg/day) for two days caused an increase in hepatic GSH levels, ascorbic acid levels in both liver and plasma, and triglyceride content, but a decrease in the plasma level of uric acid [Schinella et al., 1996]. The lipoprotein levels in arsenate-treated animals demonstrated a greater tendency for *in vitro* oxidation than in control animals [Schinella et al., 1996].

Gluconeogenesis is one of the metabolic pathways severely affected by acute arsenic poisoning [Muckter et al., 1993; Liebl, et al., 1995a]. Among the arsenicals tested, trivalent oxoarsines, such as oxophenylarsine (PhAsO), were the most potent arsenicals in inhibiting glucose uptake in both rat kidney tubules (RKT) and Madin-Darby canine kidney (MDCK) cells, followed by arsenite, arsenate, and phenylarsonate in order of decreasing potency. Conversely, methylarsonate had virtually no effect on gluconeogenic activity [Liebl et al., 1995a]. The toxicity of PhAsO is thought to be due to its inhibitory effects on pyruvate dehydrogenase, which leads to a decrease in acytyl-CoA formation and a slow-down of the citric acid cycle. Glucose has protective effects on the toxicity of PhAsO in MDCK cells, probably due to an increase in glycolysis that prevents the lethal shortage of cellular ATP [Liebl et al., 1995b]. Moreover, the inhibitory effects of PhAsO on glucose uptake in MDCK cells can be reversed by addition of dithiol molecules,

suggesting an As-thiol interaction involved in the mechanism of PhAsO toxicity [Liebl et al., 1995c].

When studying arsenic toxicity, the interactive action of arsenic with other metals or surrounding materials should be considered. Evidence has shown that when *Ceriodaphnia dubia*, an aquatic invertebrate, was exposed to arsenic, molybdenum (Mo), and selenium (Se), separately or in combination, selenium exhibited the strongest effects on survival and fecundity. Molybdenum and arsenic in binary combinations appear to be strongly antagonistic in their effects on *C. dubia*, even at their respective IC_{50} concentrations. Moreover, addition of Se to the As-Mo mixtures significantly reduced survival and reproduction. All tertiary metal mixtures, except the combination of the $IC_{12.5}$ concentrations of each metal, significantly reduced *C. dubia* fecundity [Naddy et al., 1995]. Selenium and iron have also been shown to antagonize the effect of arsenic on progression of blackfoot disease [Wang et al., 1994a].

In spite of these detrimental effects, arsenic has been considered to be an essential human micronutrient, with a predicted requirement of 12 μg per day. The physiological role of arsenic as a trace element is thought to be its involvement in methyl group transport and metabolism [Nielsen, 1991].

1.8 CELLULAR DEFENSE MECHANISMS

Living cells are constantly exposed to their ever-changing environment. To survive environmental stress, various mechanisms have evolved to protect cells from environmental perturbations. Several mechanisms involved in arsenic detoxification have been described in the scientific literature, including energy-dependent efflux systems, stress responses and adaptation, and sequestration. Moreover, biotransformation processes, such as oxidation-reduction and methylation-demethylation, are also implicated in arsenic metabolism and detoxification, as have been described in previous sections. This section will focus on the major mechanisms involved in arsenic detoxification.

1.8.1 Efflux

The most extensively studied mechanism of arsenic detoxification is the energy-dependent efflux systems [For review, see Silver et al., 1989; Kaur and Rosen, 1992a; Silver and Ji, 1994; Rosen, 1995; Silver, 1996]. The best characterized bacterial arsenic efflux system is the *Escherichia coli* plasmid R773 encoded *ars* operon, which consists of five ORFs, in the order *arsRDABC*, and organized as a single transcription unit [Owolabi and Rosen, 1990]. The operon confers resistance to arsenite, arsenate, and antimonite oxyanions by energy-dependent efflux of the oxyanions, preventing their concentration from reaching toxic levels [Silver and Keach, 1982]. The resistance phenotype can be induced by all three oxyanions, as well as bismuthate, another member of the nitrogen group in periodic table. The structures and functions of the *ars* gene products have been elucidated. The three structural gene products include ArsA, an arsenite/antimonite-stimulated ATPase [Rosen et al., 1988], ArsB, an inner membrane channel, also serving as a membrane anchor for the ArsA protein [Tisa and Rosen, 1990; Wu et al., 1992], and ArsC, an arsenate reductase [Gladysheva et al., 1994]. The ArsA and ArsB proteins are sufficient for arsenite resistance [Chen et al., 1985b], whereas the ArsC protein mediates the reduction of arsenate to arsenite, and thus is required only for arsenate resistance [Rosen and Borbolla, 1984; Chen et al., 1985b]. The operon is negatively regulated by two repressors, ArsR and ArsD [Wu and Rosen, 1991; 1993a]. Two unique features are found to be associated with this arsenic efflux system. First, it is evolutionarily unrelated to other classes of transport ATPases [Endicott and Ling, 1989; Silver et al., 1993a]. Secondly, the substrates for this efflux pump are anions rather than cations.

The catalytic subunit of the R773 *ars* operon is the ArsA protein, which is an arsenite/antimonite-stimulated ATPase with two nucleotide binding sites, one in the N-terminal half and one in the C-terminal half of the protein [Chen et al., 1986b]. Both nucleotide-binding sites interact with each other [Li et al., 1996] to form a catalytic unit that is required for resistance and ATP binding [Karkaria et al., 1990; Kaur and Rosen, 1992b; 1994a, b]. The binding of purified ArsA protein to ATP is specific and requires the presence of Mg(II) ions. AMP, ADP, GTP, UTP, and CTP are not able to compete with

ATP for binding [Rosen et al., 1988; Karkaria and Rosen, 1991; Zhou et al., 1995], nor can these nucleotides substitute ATP in providing energy for oxyanion transport [Dey et al., 1994a]. The ArsA protein is allosterically activated by tricoordinate binding of As(III) or Sb(III) to three cysteine thiolates [Bhattacharjee et al., 1995], and metalloactivation of the protein is associated with the formation of ArsA homodimers [Mei-Hsu et al., 1991].

The *arsB* gene product is an integral membrane protein with a predicted molecular mass of 45 kDa [San Francisco et al., 1989]. The protein contains 12 membrane-spanning regions, which are thought to compose an arsenite/antimonite-specific channel for exporting the oxyanions [Wu et al., 1992]. The ArsB protein serves as an anchor for the ArsA protein to localize the Ars pump to the inner membrane of *E. coli* cells [Tisa and Rosen, 1990]. Synthesis of the ArsB protein is the rate-limiting factor for assembly of the pump [Owolabi and Rosen, 1990; Dey et al., 1994c].

The ArsC protein, encoded by the arsenical resistance operon of plasmid R773, catalyzes the reduction of arsenate to arsenite in *E. coli* [Gladysheva et al., 1994; Oden et al., 1994]. The enzymatic activity of the ArsC protein has been shown to require reduced glutathione and glutathione reductase [Gladysheva et al., 1994; Oden et al., 1994], suggesting that thiol chemistry might be involved in the reaction mechanism.

The ArsR and ArsD proteins are inducer-dependent *trans*-acting repressors that control the basal and upper levels of the operon expression, respectively [Wu and Rosen, 1993a, b; Shi et al., 1994; Chen and Rosen, 1997]. Both repressors function by binding to the same sequence in the *ars* operator/promoter region, but with different temporal control [Chen and Rosen, 1997]. The ArsR repressor was found to be dissociated from the operator sequence in the presence of low levels of arsenic/antimony oxyanions. Whereas the ArsD repressor requires higher levels of inducers to relief the repression [Chen and Rosen, 1997]. Arsenite and antimonite, but not arsenate are effective inducers for bot repressors [Wu and Rosen, 1993b; Shi et al., 1994; Chen and Rosen, 1997].

Similar arsenic resistance operons have been identified and characterized in plasmids of Gram-positive bacteria *Staphylococcus aureus* (pI258) [Ji and Silver, 1992a] and *Staphylococcus xylosus* (pSX267) [Rosenstein et al., 1992]. The staphylococcal *ars*

operons contain only the *arsR*, *B*, and *C* genes. Although the *arsA* and *D* coding sequences are absent, the resistant cells still actively extrude arsenite [Ji and Silver, 1992a; Rosenstein et al., 1992]. This suggests that the ArsB protein can transport arsenite in the absence of the *arsA* gene product. Interestingly, when the R773 ArsA was provided in *trans* to the *S. aureus* ArsB, increased resistance to arsenite was observed [Brõer et al., 1993]. This suggests that the ArsB protein may function in a dual mode mechanism. Indeed, Dey and Rosen (1995) found that cells expressing the R773 *arsB* gene alone exhibited intermediate level of energy-dependent arsenite resistance compared to cells expressing both the *arsA* and *arsB* genes. Arsenite extrusion in the cells expressing *arsB* alone was coupled to electrochemical energy, while in cells expressing both *arsA* and *arsB* genes, extrusion was coupled to chemical energy, most likely ATP. The ArsC protein of the *S. aureus* pI258 *ars* operon was purified from the cytoplasmic fraction of the cells [Ji and Silver, 1992b; Ji et al., 1994]. *In vitro*, reduction of arsenate to arsenite by the plasmid pI258 ArsC protein is coupled with thioredoxin and dithiothreitol. Reduced GSH and glutaredoxin are not required for its activity *in vitro* [Ji and Silver, 1992b; Ji et al., 1994]. Between the staphylococcal *ars* and the R773 *ars* operons, the amino acid sequence homology of ArsR, B, and C is 30, 58, and 18%, respectively [Silver et al., 1993a]. The ArsR proteins of the staphylococcal *ars* operons, like the R773 ArsR, are *trans*-acting repressors [Rosenstein et al., 1994; Ji and Silver, 1992a].

Studies indicate that arsenic resistance mediated by the R773-encoded arsenic efflux system requires ATP [Mobley and Rosen, 1982; Rosen et al., 1988; Karkaria and Rosen, 1991; Zhou et al., 1995]. However, the extrusion of arsenic and antimony oxyanions by the staphylococcal plasmid *ars* operons is coupled to membrane potential rather than ATP [Ji and Silver, 1992; Rosenstein, et al., 1992; Brõer et al., 1993; Silver et al., 1993].

Following the discovery of the aforementioned plasmid-encoded *ars* operons, many more arsenic resistance plasmids have been found [Dabbs et al., 1990; Cervantes and Chávez, 1992; Bruhn et al., 1996; Neyt et al., 1997]. The arsenic resistance plasmid, pUM310, from a *Pseudomonas aeruginosa* clinical isolate is similar to the *ars* operons in

E. coli and staphylococcal plasmids, as it confers resistance to arsenite, arsenate, and antimonite, and is inducible by these oxyanions. Phosphate was found to protect cells containing pUM310 from arsenate toxicity, but the protective effect on arsenite was not observed [Cervantes and Chávez, 1992]. More recently, an arsenical resistance operon of the IncN plasmid R46, consisting of 4696 bp coding for five ORFs, *arsRDABC*, was identified and characterized [Bruhn, et al., 1996]. This operon shares high homology to the R773 *ars* operon at both the DNA and protein levels, and is the second version found to contain the *arsD* and *arsA* coding sequences after that of the R773 plasmid [Bruhn et al., 1996]. In addition, an *ars* operon has been found in transposon Tn2502 of the low-virulence strain of *Yersinia enterocolitica* [Neyt et al., 1997]. Four genes are involved in arsenic resistance by this *ars* operon. Three of them are homologous to *arsRBC*. The fourth gene, *arsH*, has not been found in any other known *ars* operons. Although the function of the gene is currently unknown, ArsH is required for arsenic resistance encoded by the Tn2502 *ars* operon [Neyt et al., 1997]. Genetic determinants for arsenite and arsenate resistance were also found in *Rhodococcus* spp. [Dabbs and Sole, 1988], in which the resistance phenotype has been employed to develop shuttle vectors for cloning purposes [Dabbs et al., 1990; Quan and Dabbs, 1993].

Interestingly, in addition to the well-recognized plasmid-located arsenic resistance systems in various bacterial isolates, an *E. coli* chromosomal *ars* homolog, containing *arsRBC*, was discovered by two research groups using different strategies [Sofia et al., 1994; Diorio et al., 1995, see Chapter 2 of this thesis]. Moreover, the *E. coli* chromosomal *ars* homolog was found to be conserved in the chromosomes of many other Gram-negative bacterial species [Diorio et al., 1995, see Chapter 2 of this thesis], suggesting a functional importance of the *ars* operon during evolution. The sequence homology between the *E. coli* chromosomal *ars* and the R773 and staphylococcal plasmid *ars* operons may implicate a common ancestor of the bacterial *ars* operons [Diorio et al., 1995].

The functional importance of the arsenic efflux system appears to extend beyond prokaryotic systems, since the energy-dependent efflux mediated resistance to arsenic has

also been observed in eukaryotic systems [Ouellette and Borst, 1991; Wang and Rossman, 1993; Dey et al., 1994b]. Wang and Rossman (1993) isolated several arsenite resistant and hypersensitive Chinese hamster V79 cell line variants. Some of the arsenite resistant subcell lines are cross resistant to arsenate and potassium antimonyl tartrate, a trait similar to that observed in bacterial *ars* operons. The level of resistance can be increased by pretreatment of the cells with nontoxic concentrations of the oxyanions [Wang and Rossman, 1993; Wang et al., 1994b]. In the resistant variants, arsenic resistance occurs as a result of decreased accumulation of the oxyanions, and is mediated by energy-dependent efflux [Wang et al., 1996]. Moreover, it appears that an arsenite-glutathione interaction is involved in this arsenite resistance mechanism, since addition of two inhibitors of glutathione S-transferase (GST) to the system decreased arsenite efflux [Wang et al., 1996]. A similar effect of GST inhibitors on the resistance phenotype was also observed in SA7, an arsenite resistant Chinese Hamster Ovary cell line, which expresses GST at high levels [Lo, et al., 1992; Wang and Lee, 1993]. This suggests that GST is involved in arsenic detoxification in Chinese hamster cells.

A functionally related arsenic/antimony resistance mechanism has been found in unicellular eukaryotes, including important parasites of man, such as leishmanial and trypanosomal species. The resistance in *Leishmania tarentolae* and *Trypanosome brucei* is determined by a P-glycoprotein (PgpA) associated function [Ouellette and Borst, 1991; Jackson et al., 1990; Grõgl et al., 1992]. It is specific to arsenicals and antimonials [Callahan and Beverley, 1991; Papadopoulou et al., 1994; 1996], and resembles that of the *E. coli ars* operon [Kaur and Rosen, 1992a]. Transfection experiments showed that the *pgpA* genes of *L. tarentolae* and *L. major* were implicated in the low level oxyanion resistance [Callahan and Beverley, 1991], whereas the high level resistance is found to be associated with amplification of the *pgpA* gene [Grondin et al., 1993]. A null mutant of the *pgpA* gene of *L. tarentolae* exhibited increased sensitivity to arsenite and antimonite, and decreased intracellular survival inside murine macrophages [Papadopoulou et al., 1996]. Transfection of an intact *pgpA* gene into a *L. tarentolae pgpA* null mutant reversed the resistance levels to those of the wild-type cells [Papadopoulou et al., 1996]. Experiments

with disrupted chromosomal *pgpA* locus in the arsenic resistant mutant indicate that PgpA is not essential for resistance to oxyanions, although it might be required in the establishment of resistance in the early stages [Papadopoulou et al., 1996]. Recent studies on the mechanism of arsenic resistance of *L. tarentolae* showed that the arsenic resistant cells overproduce intracellular thiols, mainly trypanothione [Mukhopadhyay et al., 1996], which forms an $As(SG)_3$ or $Sb(SG)_3$ complex in an ATP-dependent manner [Dey et al., 1996]. The direct substrates for the arsenic/antimony pump are As(III)/Sb(III)-thiol complexes, which accumulate in the plasma membrane vesicles prepared from either wild type or *pgpA* null mutant strains at similar rates, indicating that the *pgpA* gene does not encode the As-thiol efflux pump [Dey et al., 1996; Mukhopadhyay et al., 1996]. However, the As(V)/Sb(V)-containing compounds, including the antileishmania drug, Pentostam, are proposed to be reduced intracellularly to their trivalent equivalents, followed by the formation of metalloid-thiol complexes in order to be extruded [Dey et al., 1996; Mukhopadhyay et al., 1996].

Although the leishmanial P-glycoprotein A does not seem to function as an efflux pump, a strong analogy does exist between the overall structure of the bacterial arsenic translocating ATPase (encoded by the R773 *ars* operon) and the mammalian multidrug resistance system (MDR) [Silver et al., 1989]. The mammalian MDR system is composed of a P-glycoprotein (P-gp) with a duplicated ATPase domain anchored to the cell membrane through the integral membrane regions [Gros et al., 1986]. The MDR phenotype results from amplification and overexpression of the MDR gene when cells are exposed to a single drug, thus increasing the efflux of multiple unrelated chemotherapeutic agents [Endicott and Ling, 1989]. The common features in the overall structure and function of these energy-dependent efflux systems may imply a fundamental cellular defense mechanism conserved in many organisms during evolution.

1.8.2 Stress responses and adaptation

A sudden change in growth conditions may elicit a series of responses within a cell. These responses may involve gene activation and synthesis of stress proteins, which allow

the cell (or organism) to adapt to its new environment. One commonly observed stress response in all living organisms is the heat shock response, in which an abrupt shift to a higher temperature leads to the synthesis of a set of heat shock proteins (HSPs). Many HSPs function as chaperones and chaperonins, and assist in protein folding and renaturing under elevated growth temperature conditions [Morimoto et al., 1990]. The heat shock response appears to be a universal stress response, as it is observed in all living organisms. Although the classical stimulator for the heat shock response is a rapid up-shift in growth temperature, many external stimuli are able to elicit synthesis of HSPs. These stimuli include changes in oxidative growth conditions, viral infection, and exposure to a variety of chemicals, such as ethanol, 2,4-dinitrophenol, sodium azide, hydrogen peroxide, heavy metals, and amino acid analogues [Welch, 1990]. Kato et al. (1993) found that exposure of human glioma cells to arsenite co-induced the synthesis of two low molecular weight stress proteins, αB crystallin and HSP28, which belong to the low-molecular-weight HSP family and can also be induced by heat shock treatment. Increased synthesis of HSP70 and HSP90 was found in precision-cut rat liver slices exposed to sodium arsenite or heat shock [Wijeweera et al., 1995]. A stress protein response was found in arsenite-exposed Fathead minnows (*Pimephales promelas*) [Dyer et al. 1993], which showed significant increases in the synthesis and accumulation of 20-, 40-, 70-, 72-, and 74-kD proteins in gill, and 20-, 30-, 68-, 70-, and 90-kD proteins in muscles. Although the identity and physiological function of these proteins are not yet clear, synthesis and accumulation rates of arsenite-induced proteins 20-, 70-, 72-, and 74-kD in gill and the 70-kD protein in the muscle were significantly correlated with arsenic-induced mortality [Dyer et al, 1993]. Falkner and colleagues (1993) found that arsenite can induce NAD(P)H:Quinone acceptor oxidoreductase (QOR) activity in liver, lung, kidney, and heart, and glutathione S-transferase activity in kidney of the rat. QOR is primarily a cytosolic protein that catalyzes the two electron reduction of quinones to hydroquinone, an important detoxification pathway [Cadenas et al., 1992]. Albores et al. (1995) found that subcutaneous administration of sodium arsenite to Sprague-Dawley male rats selectively increased lung

cytochrome P450 isozyme 1A1-dependent monooxygenase activity, indicating the activation of this cellular defense mechanism against arsenite-induced oxidative stress.

In addition to stress responses and physiological adaptation, living organisms can also undergo genetic adaptation, in which a mutant is selected by a new environment to become the predominant type in the population. Genetic adaptation to arsenic has been recently reported in some organisms [Teixeira et al., 1995; Kondrayeva et al., 1995; 1996]. For example, two arsenate resistance mechanisms have been observed in *Thiobacillus ferrooxidans* strain VKM-458 [Teixeira et al., 1995], an important bacterium employed for metal-leaching in mining processes. The low level arsenate resistance observed in this strain is thought to be mediated by a constitutive mechanism that allows the bacterial cells to sustain levels of arsenic ranging from 0 to 4 g/l. The high level of arsenate resistance was acquired by an adaptation process resulting from the growth of cells in a semi-continuous culture with relatively low concentrations of arsenate (4 g As/l) in a fermentor. The bacterial cells so adapted showed an improved growth profile in the presence of arsenic concentrations as high as 8 g/l, and an increased rate of ferrous iron oxidation, a characteristic of the metabolic activity of the species [Teixeira et al., 1995]. Comparison of the protein profiles of the cell-free extracts from the adapted cells grown in the absence and presence of 8 g/l arsenate by SDS-PAGE revealed the appearance of four extra protein bands in the later case [Teixeira et al., 1995]. This suggests that the arsenate resistance resulted from adaptation involves synthesis of novel proteins. In addition, pulse-field gel electrophoresis of the chromosomal DNA from an experimentally enhanced arsenate resistant *T. ferrooxidans* strain (VKM-458As2) and its parental strain (VKM-458) was performed. The strain VKM-458As2, which developed arsenic resistance after 10 passages on growth medium containing low levels of arsenic as arsenopyrite, showed an *Xba*I restriction fragment of 28 kb in size, which was not present in the parental strain VKM-458 [Kondrayeva et al., 1995; 1996]. This observation indicated an involvement of gene amplification during this arsenic adaptation process. The arsenic resistance gene might be localized within the amplified DNA fragment.

Arsenic tolerance resulting from adaptation has also been observed in Yorkshire Fog, *Holcus lanatus* L. (poaceae) collected from arsenate contaminated mine soils [Meharg et al., 1993]. The levels of arsenic in the contaminated soils range from 2.2 to 18.6 μmol As/g soil dry weight, 10 to 100 times higher than those found in uncontaminated soils. Root tests showed that 92-100% of the individuals from arsenate contaminated soils were tolerant, compared to the 50% from the uncontaminated soils [Meharg et al., 1993]. It is known that arsenate is taken up by angiosperms through both the high and low affinity phosphate transport systems [Meharg and Macnair, 1990]. In arsenate contaminated soils, arsenate competes for phosphate transport, resulting in a condition equivalent to phosphate starvation. To reduce the cost of living under such nutrient-poor conditions, the high affinity phosphate transport system in *H. lanatus* is suppressed. As a consequence, the arsenate uptake is also reduced in the plants adapted to phosphate-poor conditions [Meharg and Macnair, 1990; 1992], leading to the observed arsenate tolerance.

1.8.3 Sequestration

One common mechanism for detoxifying heavy metals is sequestration, a process in which metals bind to cellular proteins. Metallothioneins (MTs), a group of low-molecular-weight, sulfhydryl-rich, metal-binding proteins, play an important role in the homeostasis of essential metals, in heavy metal detoxification, and in the scavenging of free radicals [Dunn et al., 1987]. The production of MTs can be induced by glucocorticoid hormones, acute stress, various organic chemicals [Dunn et al, 1987; Andrews, 1990], and a variety of metals [Maitani and Suzuki, 1982; Waalkes and Klaassen, 1985].

It has been reported that arsenicals are able to induce the *in vivo* production of hepatic metallothionein (MT) in mouse and rat [Albores et al., 1992; Kreppel et al. 1993]. Among arsenite, arsenate, MMAA, and DMAA, arsenite is the most potent inducer of MT, and at a dose of 80 μmol/kg induces a 30-fold increase in MT. Moreover, 3-, 50-, and 120-fold molar amounts of arsenate, MMAA, and DMAA, respectively, are needed to cause a similar level of MT induction. However, the level of hepatic MT is highest with

MMAA (80-fold), followed by arsenite (30-fold), arsenate (25-fold), and DMAA (10-fold) [Kreppel et al., 1993]. Induction of MT by arsenite has also been observed in other mammalian species including cultured human (HeLa) cells [Guzzo et al., 1994]. Zinc was shown to induce the synthesis of hepatic MT as well as arsenite tolerance in mice. However, induction of MT by zinc did not appear to be responsible for the induced arsenite tolerance [Krepple et al., 1994].

Although MT has been implicated in detoxification of a variety of heavy metals, the *in vivo* binding of MT to arsenic appears to be insignificant [Chen and Whanger, 1994]. Therefore, the role of MT in arsenic detoxification remains to be clarified. Other cellular proteins may bind to arsenic and thus reduce the toxic effects of arsenic ions. *In vitro* methylation assays of rat liver cytosol and of liver and kidney cytosol of arsenite-treated mice showed that most inorganic arsenic was protein bound. Appreciable fractions of organoarsenical metabolites were also protein bound [Styblo et al., 1996b]. It is known that arsenite or arsenate administered to rabbits is bound initially to hepatocyte proteins before methylated arsenic appears in urine [Marafante et al., 1981; 1982; Vahter and Marafante, 1983]. This protein binding may decrease the toxicity of inorganic arsenic *in situ* by decreasing its metabolic availability until it is methylated enzymatically. It was found that when cytosolic proteins were incubated with inorganic arsenic, the amount of As(III) bound is 13 times greater than that for As(V). Three arsenite-binding proteins were found in the cytosolic portion of rabbit liver [Bogdan et al., 1994]. However, the identity and function of the three arsenite-binding proteins need to be further studied.

In prokaryotic systems, detoxification via sequestration has been reported for the copper resistance systems. In *E. coli* and *Pseudomonas syringae*, copper resistance is mediated by two periplasmic copper binding proteins and two other membrane components [Silver and Ji, 1994]. The first well-defined bacterial metallothionein was found in the cyanobacterium *Synechococcus*. Bacterial MT is encoded by the *smtA* gene and contains 56 amino acids, including 9 cysteine residues. The synthesis of the bacterial MT is controlled by several mechanisms, including derepression of repressor activity, deletion of the repressor gene at fixed positions (over a long term), and amplification of

the MT gene [Silver and Ji, 1994]. Although arsenic specific binding proteins have not yet been reported, such sequestration mechanisms may also play a role in arsenic detoxification, and remain to be further investigated.

1.8.4 Other mechanisms

Arsenic tolerance can also result from genetic defects that cause decreased uptake of arsenicals. For example, mutations in phosphate transport systems, through which arsenate is transported into cells, are responsible for arsenate resistance in some *E. coli* strains [Willsky and Malamy, 1980b]. Melaminophenyl arsenical drugs, such as melarsen, have been used to treat African sleeping sickness. Recently, trypanosomes resistant to melarsen have been found. The resistance is due to the absence of an unusual adenosine transporter that is also responsible for the uptake of melaminophenyl arsenical drugs [Carter and Fairlamb, 1993].

1.9 CHEMICAL AND BIOLOGICAL MONITORING OF ARSENIC

1.9.1 Analytical methods

Development in analytical chemistry has made it possible to identify arsenic compounds in environmental and biological samples. Numerous sophisticated analytical techniques, such as atomic absorption spectrometry (AAS), atomic emission, atomic fluorescence, high performance liquid chromatography (HPLC), and gas chromatography (GC), and combinations of spectrometry and chromatography, have been developed for quantitative analysis of arsenic and other metal pollutants [Harriot et al., 1988]. These methods are highly sensitive and selective in detecting different species of metallic and metalloid compounds. A very popular analytical method for arsenic speciation is the hydride generation atomic absorption spectrometry (HG-AAS), which selectively determines arsenite ion, arsenate ion, MMAA, and DMAA concentrations in aqueous solutions with high specificity and low detection limits [Braman and Foreback, 1973; Braman et al., 1977; Norin and Vahter, 1981; Masscheleyn et al., 1991b]. MMAA and DMAA are the major urinary metabolites detected after exposure to inorganic arsenic.

Organic arsenic compounds of marine origin are not biotransformed into inorganic arsenic or methylated arsenic acids to any significant degree in human body, and thus will not be detected by this method. This analytical procedure involves reduction of arsenicals to their corresponding arsines, which are collected in a liquid N_2 trap. After sequential volatilization, the arsines are detected by an emission detection system [Braman and Foreback, 1973]. In this assay system, As(III) ion is the only form that can be reduced to arsine by sodium borohydrate between pH 4 and 9. As(V) ions must first be reduced to As(III) ions by sodium cyanoborohydride before they can be reduced to arsine by sodium borohydride. MMAA and DMAA are reduced to methylarsine and dimethylarsine, respectively, by sodium borohydrade between pH 1 and 2. The detection limits of this procedure are 0.05 ng for As(III) and As(V), and 0.5 ng for methylarsinic acid [Braman and Foreback, 1973]. Recently, various improved analytical methods have been developed for full speciation of organic and inorganic arsenic compounds in biological and environmental samples [Lamble and Hill, 1996; Lopezgonzalvez et al., 1996; Le et al., 1994; 1996]. An effective urine clean-up method has been developed by Lopezgonzalvez et al. (1996) for the determination of arsenite, arsenate, MMAA, DMAA, AB, and AC in urine samples. This method involves precipitation of most high molecular mass inorganic salts and organic compounds in the urine sample in ethanol at -15°C prior to injection into an HPLC column. The six arsenic species of the cleaned-up urine extract are then separated on an anionic HPLC column coupled to a microwave-assisted oxidation-hydride generation-AAS (HPLC-MO-HG-AAS) [Lopezgonzalvez et al., 1996]. Because urine is the main pathway of arsenic excretion and the toxic species, As(III), As(V), MMAA, and DMAA are not transformed to the non-toxic species (AB or AC), separate quantification of these species may reveal the source of arsenic, i.e. from occupational exposure (toxic species) or from food ingestion (non-toxic species), and thus enable an adequate toxicological evaluation of arsenic exposure. The HPLC at elevated temperature and selective hydride generation atomic fluorescence detection method [Lamble and Hill, 1996; Le et al., 1996] reduced chromatography retention times for AB, AC, tetramethyl-arsonium, and arsenosugars, and enabled differentiation of more toxic from less toxic

arsenic species [Le et al., 1996]. In addition to the most common and widely applicable HG-AAS and HPLC assays, other improved analytical techniques, including HPLC coupled to an inductively-coupled plasma-mass spectrometer (ICP-MS), and ion-exchange chromatography with detection of arsenic by instrumental neutron activation, are now in use, although of limited availability.

1.9.2 Biological assay systems

The well-developed analytical chemistry systems, as described above, can provide accurate data on the concentration and speciation of arsenic compounds. However, the procedures are usually time-consuming and expensive, and the results cannot distinguish between compounds that are available to biological systems from those that exist in inert, unavailable forms.

In an effort to overcome the drawbacks of these analytical methods, various *in vivo* and *in vitro* biological assay systems, including biosensors, have been developed. The biosensor system that is gaining increasing popularity in environmental monitoring is the bioluminescence system due to its high sensitivity, rapid response, large dynamic range, high accuracy, ease of measurement, and economy [Danilov and Ismailov, 1989; Van Dyk et al., 1994]. Bioluminescence is an inherent trait of relatively few organisms, ranging from bacteria and fungi to insects and fish. The chemical basis of bioluminescence varies from species to species. The most popular and best studied bioluminescence system to date is bacterial bioluminescence. There are currently six recognized marine luminous bacterial species, all belonging to the families *Enterobacteriaceae* and *Vibrionaceae*. These luminous marine bacteria are ubiquitous in marine environments. They emit visible blue-green light at a rate of 10^2 to 10^4 quanta per second per cell [Danilov and Ismailov, 1989]. The light emitting reaction of all luminous bacteria is catalyzed by a specific enzyme, luciferase, and involves oxidation of a reduced monoflavin nucleotide ($FMNH_2$) and an aliphatic aldehyde in the presence of oxygen, to form FMN, aliphatic acid, water, and blue-green light, which can be readily detected at 490 nm [Danilov and Ismailov, 1989; Meighen, 1991].

Luminescent biosensors have been used to monitor cell survival under various growth conditions, to identify the presence of specific toxins, heavy metals and other xenobiotic substances, to monitor gene expression, and to study the intracellular levels of the substrates involved in the bioluminescence reaction. For *in vivo* bioluminescence assays, two major types of bioluminescent organisms have been used. One type exploits naturally luminous organisms that constitutively express bioluminescence and microorganisms that contain a constitutively expressed luciferase gene on a plasmid. This type is often used for monitoring environmental changes that affect cell physiology and thus affect bioluminescence at either the substrate or enzyme level. For example, a plasmid-carried constitutively expressed luciferase gene system has been used to monitor the thermal inactivation of *Salmonella typhymurium* in the presence of competitive microflora [Duffy et al., 1995]. The MICROTOX® assay system, a commercially available *in vivo* bioluminescence system, uses the bioluminescent bacterium, *Photobacterium phosphoreum*. The freeze-dried bacteria and all the reaction components are supplied as a kit (Beckman Instruments Inc.). The system has been used for predicting the relative toxicity of metal ions using ion characteristics [McCloskey et al., 1996]. The second type of *in vivo* bioluminescence assay system consists of genetically-engineered luminous microorganisms, in which the expression of bioluminescence is controlled by the regulatory elements of interest, so that the system can be used to evaluate the effects or the level of a given compound. These luminous microorganisms have been used to detect the presence of specific environmental pollutants, such as heavy metal ions [Selifonova et al., 1993; Guzzo and DuBow, 1994] and organic contaminants [King et al., 1990; Heitzer et al., 1992], to monitor pathogenic microbes [Shaw and Kado, 1986], to identify a specific microorganism in a heterogenous mixture of microbial flora [Rattray et al., 1990], and to monitor gene expression [Guzzo and DuBow, 1994; Corbisier et al., 1993; Van Dyk et al., 1994; Kondo et al., 1993; Kondo and Ishiura, 1994]. Although there have been few reports on arsenic specific bioluminescent biosensors, this appears to be a promising strategy for monitoring the bioavailability of arsenic compounds.

In addition to bioluminescent biosensor systems, bioassays for detecting the toxic effects of various environmental pollutants, such as arsenic, have been developed. For example, based on the fact that arsenic induces oxidative stress [Ahmad, 1995], Zaman and colleagues (1995) developed an insect model in which the oxidative stress related to arsenic toxicity can be monitored. In this assay, changes in the activities of several antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), glutathione transferase (GST), the peroxidase activity of glutathione transferase (GSTPX), and glutathione reductase (GR) are monitored. When adult female houseflies, *Musca domestica*, were treated with As(III), an increase in enzymatic activity was found in SOD (1.3-fold), GST (1.6-fold), and GR (1.5-fold), but the activity of CAT and GSTPX were not affected. As(V) had no effect on these parameters. In another insect species, *Tricoplusia ni*, the antioxidant enzyme activities were not affected by As(III), except for SOD, which was suppressed by 29.4% and GST, which was induced 1.4-fold. As(V) had no effect, except the suppression of SOD by 41.2%. Lipid peroxidation and protein oxidation were elevated in both insects by up to 2.9-fold. These data suggest that the effects of arsenic-induced oxidative stress may differ between the two insects [Zaman et al., 1995]. Based on the haemolytic activity of arsine gas, an *in vitro* model for arsine toxicity has been reported using isolated red blood cells [Hatlelid et al., 1995].

1.10 OBJECTIVES AND OUTLINE OF THE THESIS

Owing to the prominent toxic effects of arsenic on ecosystems and the increasing levels of arsenic released into the environment from various sources, the objective of this study was to reveal the molecular basis of genetically-programmed responses to arsenic exposure in bacterial cells. Our first effort was to identify bacterial genes whose expression is derepressed in the presence of arsenic oxyanions. This task was achieved using transposable bacteriophage MudI (*lac*, Ap^r) [Casadaban and Cohen, 1979] to create a random *lacZ* gene fusion library of *E. coli*, and screening for arsenic induction of *lacZ* expression in a *lac*-deleted strain of *E. coli*. One arsenic inducible clone was isolated, and the chromosomal arsenic responsive operon, the *ars* operon, was preliminarily

characterized as described in Chapter 2. Further analysis was performed to elucidate the expression and transcriptional regulation of the *E. coli* chromosomal *ars* operon, as depicted in Chapter 3. Chapter 4 is devoted to exploring of the potential of the transcriptional gene fusion strains, constructed during the course of this study, in biologically monitoring the presence of arsenic in chromated copper arsenate, a commonly used wood preservative. Chapter 5 describes the identification and characterization of a chromosomal *ars* operon homolog in the pathologically and environmentally important species *Pseudomonas aeruginosa*, and the comparison of this operon with its homologs in other bacterial species, in order to shed light on the evolutionary relationships and functional importance of this operon. Lastly, a general conclusion has been made, in Chapter 6, to summarize the importance and significance of the present study.

CHAPTER 2

AN *ESCHERICHIA COLI* CHROMOSOMAL *ARS* OPERON HOMOLOG IS FUNCTIONAL IN ARSENIC DETOXIFICATION AND IS CONSERVED IN GRAM-NEGATIVE BACTERIA

2.1 ABSTRACT

Arsenic is a known toxic metalloid, whose trivalent and pentavalent ions can inhibit many biochemical processes. Operons which encode arsenic resistance have been found in multicopy plasmids from both Gram-positive and Gram-negative bacteria. The resistance mechanism is encoded from a single operon which typically consists of an arsenite ion-inducible repressor that regulates expression of an arsenate reductase and inner membrane-associated arsenite export system. Using a *lacZ* transcriptional gene fusion library, we have identified an *Escherichia coli* operon whose expression is induced by cellular exposure to sodium arsenite at concentrations as low as 5 μg/liter. This chromosomal operon was cloned, sequenced, and found to consist of three cistrons which we named *arsR*, *arsB*, and *arsC* because of their strong homology to the plasmid-borne *ars* operons. Mutants in the chromosomal *ars* operon were found to be approximately 10 to 100-fold more sensitive to sodium arsenate and arsenite exposure than wild type *E. coli*, while wild type *E. coli* that contained the operon cloned on a ColE1-based plasmid was found to be at least 2 to 10-fold more resistant to sodium arsenate and arsenite. Moreover, Southern blotting and high-stringency hybridization of this operon with chromosomal DNAs from a number of bacterial species showed homologous sequences among members of the family *Enterobacteriaceae*, and hybridization was detectable even in *Pseudomonas aeruginosa*. These results suggest that the chromosomal *ars* operon may be the evolutionary precursor of the plasmid-borne operon, as a multicopy plasmid location would allow the operon to be amplified and its products to confer increased resistance to this toxic metalloid.

2.2 INTRODUCTION

Arsenic is a metalloid found in the environment and exists commonly in the trivalent or pentavalent ionic forms [Lindsay and Sanders, 1990]. Its toxic properties are well known, and have been exploited in the production of antimicrobial agents, such as the first specific antibiotic (Salvarsan 606) and the African sleeping sickness drug Melarsen, in addition to the commonly-used wood preservative chromated copper arsenate [Carter and

Fairlamb, 1993; Weis and Weis, 1992a]. Because of the increasing environmental concentrations as a result of industrialization, perhaps, it is not surprising that plasmid-located genes which confer resistance to arsenic have been isolated from bacteria [Kaur and Rosen, 1992a; Silver et al., 1993a]. These arsenic resistance determinants (*ars*), isolated from both Gram-positive and Gram-negative bacterial species, have been found to be very homologous and generally consist of either three or five genes that are organized into a single transcriptional unit [Silver and Walderhaug, 1992]. In the well-studied *ars*-containing plasmid R773, isolated from *Escherichia coli* [Chen et al., 1986b; Silver et al., 1981], the operon consists of five genes that are controlled from a single promotor located upstream of the first cistron (*arsR*). These cistrons, *arsRDABC* (in that order), encode an arsenic inducible repressor (*arsR*) [Wu and Rosen, 1991], a negative regulatory protein that controls the upper level of transcription (*arsD*) [Wu and Rosen, 1993a], an ATPase plus a membrane-located arsenite efflux pump (*arsA* and *arsB*, respectively) [Karkaria et al., 1991; Rosen et al., 1988; Silver et al., 1993a; Tisa and Rosen, 1990], and an arsenate reductase (*arsC*) [Gladysheva et al., 1994]. In the well-studied *ars*-containing plasmids isolated from *Staphylococcus* species, (plasmids pI258 and pSX267), the *arsR*, *arsB*, and *arsC* cistrons are conserved, while the *arsD* and *arsA* cistrons are absent [Ji and Silver, 1992a; Rosenstein, et al., 1992]. In this case, the ArsB protein is believed to use the cell's membrane potential to drive the efflux of intracellular arsenite ions [Ji and Silver, 1992b]. The origin of these homologous plasmid-borne arsenic resistance determinants has not yet been defined.

Given the ubiquitous presence of arsenic, we sought to determine if bacteria contain chromosomally located genes whose expression is induced at elevated arsenic ion concentrations and which aid cells in detoxification of episodic increases in extracellular arsenic [Smith et al., 1992]. We have previously reported the presence of aluminum [Guzzo et al., 1991] and nickel [Guzzo and DuBow, 1994]- inducible genes in *E. coli* by screening a library of 3000 single-copy, *Vibrio harveyi* luciferase gene fusion chromosomal insertion clones [Guzzo and DuBow, 1991] for changes in light emission upon addition of these metals. Using a collection of *lacZ* chromosomal gene fusions prepared with MudI [Casadaban and Cohen, 1979], we report here the identification of an

arsenic-inducible operon in the chromosome of *E. coli* located at 77.5 min. The cloning and sequencing of this operon revealed that it can encode proteins that are highly homologous to plasmid-encoded *ars* determinants and that its expression is inducible at arsenic ion concentrations just above the environmental background [Dudka and Markert, 1992; Smith et al., 1992]. We also show that this operon is present in the chromosomes of a wide variety of Gram-negative bacterial species and that it is a functionally important determinant in detoxification of arsenic ions in *E. coli*.

2.3 MATERIALS AND METHODS

2.3.1 Bacterial strains and phages

The following bacterial strains used were all derivatives of *E. coli* K12: *E. coli* 40 (Δ*pro-lac*, *rpsL*, *trp*), *E. coli* BU5029 (a *recA* mutant derivative of strain 40), and those described (including sources) by Autexier and DuBow (1992). Phages MudI and P1*vir* were kind gifts of M. Casadaban (University of Chicago) and R. Stewart (McGill University), respectively.

2.3.2 DNA manipulations

All restriction endonuclease hydrolyses and DNA ligations were performed as described by Tolias and DuBow (1985). DNA sequencing of both strands (Fig. 2A) was performed by the dideoxy DNA sequencing method with single-stranded DNAs from cloned fragments in plasmids pUC118 or pUC119 [Vieira and Messing, 1987] by using the Sequenase kit Version 2.0 from United States Biochemicals. Southern blotting and hybridization, as well as isolation of total cellular DNA, were performed according to the method of Autexier and DuBow (1992), while P1 transduction was done according to the method of Miller (1972). DNA was isolated from stationary-phase cells grown in Luria-Bertani (LB) [Miller, 1972] broth (*E. coli* strain 40 and *P. aeruginosa* strain PA01) or nutrient broth (Difco Laboratories; *Shigella sonneii*, *Citrobacter freundii*, *Enterobacter cloacae*, *Salmonella arizonae*, *Erwinia carotovora*, and *Klebsiella pneumoniae*) at 37°C except for *E. coli* strain 40, *P. aeruginosa* PA01, and *S. arizonae*, which were grown at

32°C. For Southern blotting, 10 μg of total cellular DNA was digested with the appropriate restriction enzyme and blotted onto nylon (Hybond-N; Amersham) membranes following electrophoresis through 0.75% agarose gels [Sambrook et al., 1989]. Membranes were probed with 2×10^8 to 4×10^8 cpm/ml of an α-^{32}P-labelled 1188-bp *Eco*RV (bp 1664 to 2852 [see Fig. 2A]), or a 587-bp *Nde*I- *Eco*RV (bp 1077 to 1664 [see Fig. 2A]) DNA fragment (prepared by the random priming method [Sambrook et al., 1989]) under high stringency conditions [Autexier and DuBow, 1992]. After being washed, membranes were exposed to Agfa Curix RPI X-ray film.

2.3.3 Construction of strains LF20001 and LF20018

lacZ fusions to chromosomal genes were constructed by infecting *E. coli* 40 with MudI (*lac* Ap^r) bacteriophages as described by Casadaban and Cohen (1979). The resultant clones were picked to a master plate and replicated on LB agar plates that contained ampicillin and 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-Gal; Research Organics Inc.) plus increasing concentrations of sodium arsenate (0.1-10 μg/ml). One clone, which became blue when it was grown in the presence of sodium arsenate and remained white in its absence, was named LF20001 and isolated for further study. *E. coli* LF20018 was constructed by P1*vir* transduction [Miller, 1972] of *E. coli* LF20001 into *E. coli* 40 and selection on LB plates that contained ampicillin. The resultant Ap^r clone (*E. coli* LF20018) was tested for arsenic induction of ß-galactosidase, and the location of the MudI prophage was determined by Southern blotting and hybridization, with the *lacZ* gene as the probe.

2.3.4 Isolation of the arsenic-inducible operon

To isolate the proximal portion of the arsenate-inducible operon, a *lac* operon-Mu *attR*- *E. coli* DNA fragment was cloned from strain LF20001 via isolation of total cellular DNA [Guzzo and DuBow, 1991], cleavage with *Bgl*II, ligation into *Bam*HI-cleaved pBR322 DNA, and transformation into *E. coli* BU5029. One colony, which developed a blue color on LB agar with ampicillin and X-Gal (because of amplification of the *lac*

operon), was selected and its plasmid designated pJS29 (Fig. 1). The cloned chromosomal DNA adjacent to the right end of the MudI insertion was isolated and used as a probe to identify a 15-kb *Pst*I fragment from the chromosome of *E. coli* 40. The 15-kb fragment was cloned into pBR322 (digested with *Pst*I) to yield plasmid pJC076 (Fig. 1) by standard procedures [Sambrook et al., 1989]. A 3-kb *Nsi*I-*Bgl*I fragment that encompassed the site of MudI insertion in strain LF20001 was subcloned (plasmid pJC103) and completely sequenced.

2.3.5 ß-Galactosidase assays

ß-Galactosidase assays were performed as described by Miller (1972) by the chloroform- sodium dodecyl sulfate cell lysis procedure. Cells were grown to an A_{550} of 0.4 in LB broth at 32°C and exposed to various arsenic and antimony compounds, and aliquots were removed for ß-galactosidase assays after 30 min.

2.3.6 Arsenic sensitivity tests

The sensivities of *E. coli* strains to trivalent and pentavalent arsenic ions were determined by preparing petri plates that contained LB agar and various concentrations of sodium arsenate and sodium arsenite. Overnight cultures of *E. coli* strains grown in LB broth were diluted in fresh LB broth and grown at 32°C to an A_{550} of approximately 0.4. Then, cells were diluted 10^5-fold in LB broth, and 0.1ml of these dilutions were spread (in triplicate) on different agar plates. Petri dishes were incubated at 32°C overnight and then colonies were counted.

2.3.7 Nucleotide sequence accession number

The nucleotide sequence reported here has been submitted to the EMBL database, and assigned accession number X80057.

2.4 RESULTS

2.4.1 Discovery of a chromosomal *ars* operon homolog

A collection of *lacZ* transcriptional gene fusions was prepared using *E. coli* strain 40 and the MudI bacteriophage [Casadaban and Cohen, 1979]. In order to identify any gene whose transcription is specifically affected by arsenic salts, this collection of clones was replicated on petri dishes in the absence and presence of various concentrations of sodium arsenate and the ß-galactosidase indicator substrate X-Gal. A single clone which formed blue colonies on petri plates that contained sodium arsenate and white colonies in its absence was identified. This clone, designated strain LF20001, was isolated for further study. A P1*vir* transductant of the MudI prophage region was also prepared in *E. coli* 40 and designated *E. coli* LF20018. The DNA adjacent to the MudI prophage was mapped by Southern blotting (with the *lac* operon as the probe), cloned, and used as a probe to map and isolate the DNA sequences that flank the MudI insertion site from the chromosome of *E. coli* 40 (Fig. 1). By hybridization of the chromosomal DNA [Guzzo et al., 1991] in plasmid pJC103 to the Kohara phage set [Kohara et al., 1987], it was determined that the arsenate-inducible gene was located at 77.5 min on the *E. coli* genetic map. A total of 2.973 kb of DNA was sequenced (EMBL accession number X80057) (Fig. 2A) from plasmid pJC103 and used to scan databases with the University of Wisconsin Genetic Computer Group sequence analysis software package. It was found that this region of the chromosome is highly homologous to the arsenic-inducible *ars* operons of plasmid isolates from *E. coli* [Chen et al., 1986b; San Francisco et al., 1990; Wu and Rosen, 1993a], *Staphylococcus aureus* [Ji and Silver, 1992a] and *Staphylococcus xylosus* [Rosenstein et al., 1992] (Fig. 2B); thus because of its homology and arsenate inducibility, it was designated *ars*. This chromosomal *ars* operon was found to consist of three cistrons, which we have named *arsR*, *arsB*, and *arsC* because of the strong homology to the plasmid-borne *ars* operons. The *arsR* cistron is 77.0% homologous (at the protein level) to the same cistron in the *ars* operon isolated from plasmid R773 of *E. coli*, while the *arsB* and *arsC* cistrons are 90.7 and 94.3% homologous, respectively, with this operon. Weaker, though still significantly homologous, are the plasmid-encoded ArsR,

ArsB, and ArsC proteins of both *S. aureus* and *S. xylosus* (Fig. 2B). The location of the MudI prophage in *E. coli* LF20001 was found to be in the *arsB* gene, 799 bp downstream from the ATG start codon. Transcription of the inserted promoterless *lac* operon would occur from an upstream promoter (Fig. 2A), presumably located in a position similar to that of the *ars* operon in plasmid R773 [Wu and Rosen, 1993b].

2.4.2 Effects of various arsenic and antimony compounds on *ars* gene expression

The plasmid-borne *ars* operons are inducible by various toxic arsenic and antimony compounds [Silver and Walderhaug, 1992]. In order to measure induction of expression of the chromosomal *ars* operon by these compounds, *E. coli* LF20001 was grown in LB broth and exposed to various compounds, and ß-galactosidase activity was quantified. Maximal levels of ß-galactosidase activity were reached at approximately 60 min after exposure of strain LF20001 to sodium arsenate at final concentrations that ranged between 1-10 µg/ml (not shown). When strain LF20001 was exposed to increasing concentrations of sodium arsenite, induction of gene expression was detectable 30 min postexposure at 5 µg/l, with maximal induction observed at 1000 µg/l (Fig. 3). Sodium arsenate, the pentavalent (and less toxic) form of arsenic, did not induce *ars* operon expression at 30 min postexposure and 5 µg/l. However, higher concentrations (100 and 1000 µg/l) were able to induce expression of the *arsB*::*lacZ* fusion. Antimony (as antimony oxide), located just below arsenic in the periodic table, was also found to induce *ars* operon expression, as it does for the plasmid-borne *ars* operons [Silver et al., 1981]. However, cacodylic acid, a relatively nontoxic pesticide which contains arsenic in an organic formulation [Brannon and Patrick, 1987], was unable to induce expression of the *arsB*::*lacZ* fusion, even when added at concentrations as high as 1000 µg/l arsenic (Fig. 3). The observed threshold and concentration-dependent induction of the chromosomal *arsB*::*lacZ* fusion are very similar to those observed with the plasmid-borne *ars* operons [Corbisier et al., 1993]. No induction of the *arsB*::*lacZ* fusion was observed with other (e.g. Pb, Zn, Cu) metal ions at concentrations that ranged from 10-10000 µg/l, nor was ß-galactosidase activity from wild type *E. coli* induced or affected by arsenic and antimony

Figure 1: Strategy used to clone the chromosomal arsenic-inducible gene. See Materials and Methods for details. Displayed at the top of the figure is a schematic drawing (not drawn to scale) of the MudI insertion in the chromosome. Mu DNA (), the *bla* gene (), the *lac* operon (), *trp*-derived sequences (), and *E. coli* chromosomal DNA (wavy line) are indicated. The direction of transcription from the putative arsenic-inducible promoter-operator region (P/O) is also shown. The construction of pJS29 and pJC103 is outlined. Lane M in the Southern blot, the positions of λ DNA fragments generated after cleavage with *Hin*dIII. The 2973-bp *Nsi*I - *Bgl*I fragment from pJC103 was subcloned and sequenced.

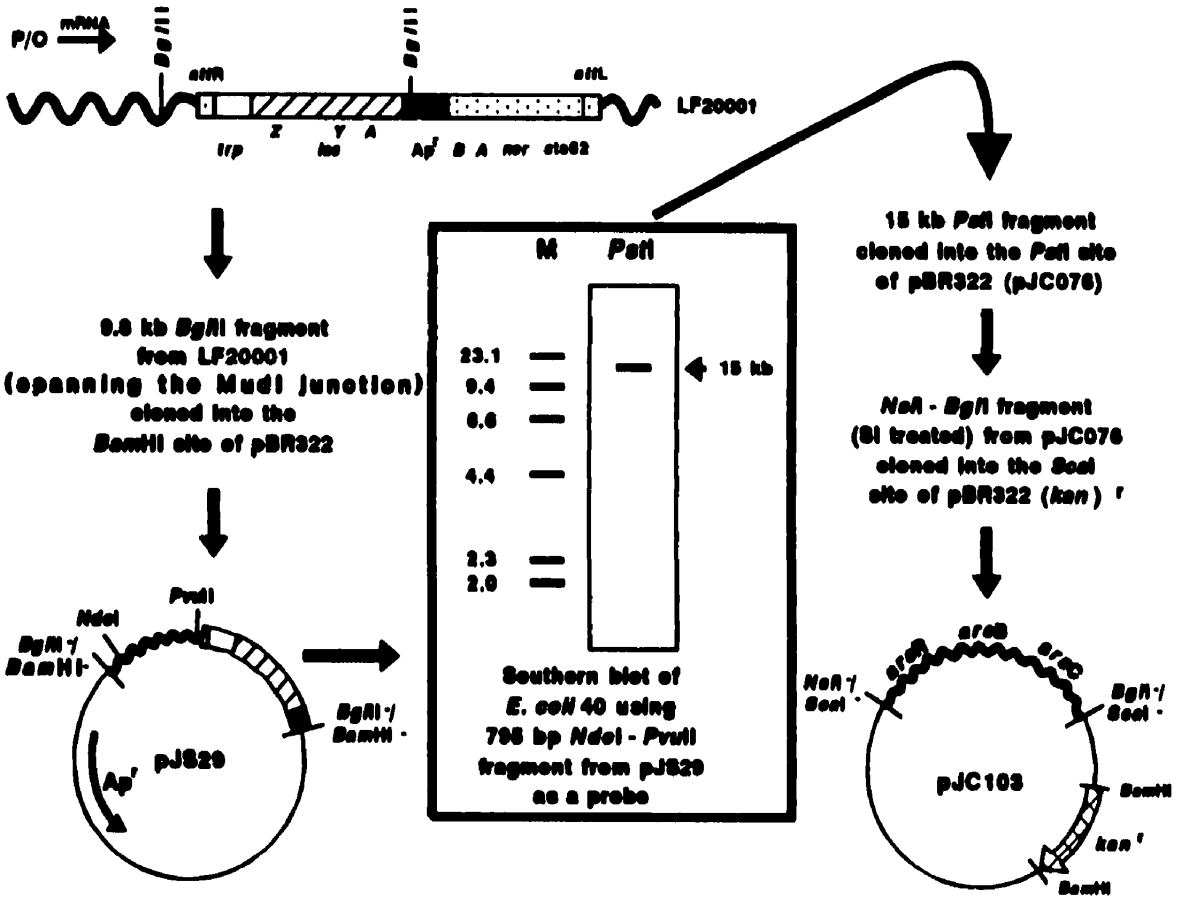
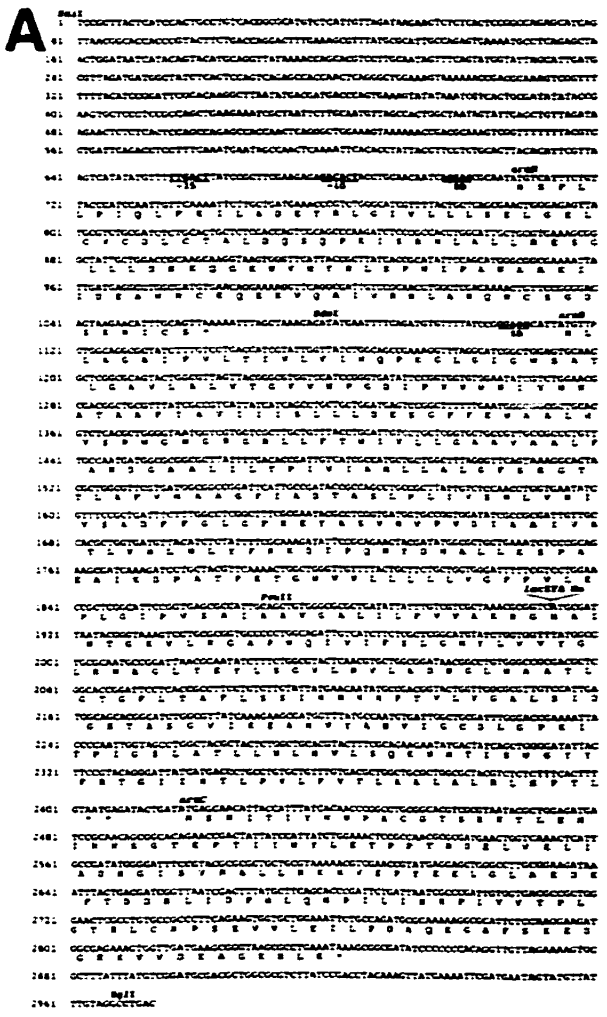
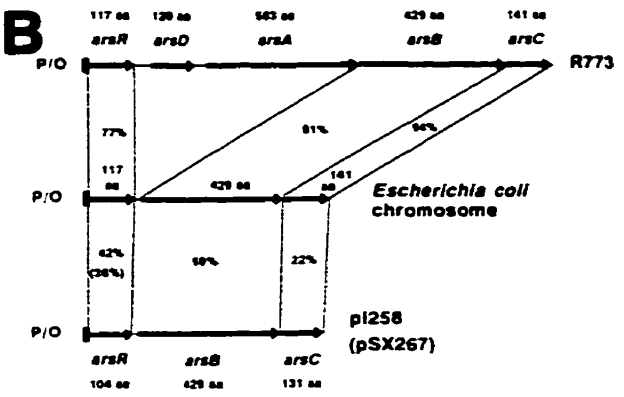

P/O
mRNA
BglII
attR
attL
LF20001
Z
Y
A
trp
lac
Ap^r
B
A
rev
ste82
9.8 kb BglII fragment
from LF20001
(spanning the MudI junction)
cloned into the
BamHI site of pBR322
NdeI
PvuII
BglII /
BamHI -
BglII /
BamHI -
pJS29
Ap^r
M
PstI
23.1
9.4
6.6
4.4
2.3
2.0
15 kb
Southern blot of
E. coli 40 using
795 bp NdeI - PvuII
fragment from pJS29
as a probe
15 kb PstI fragment
cloned into the PstI site
of pBR322 (pJC076)
NaeI - BglI fragment
(S1 treated) from pJC076
cloned into the SacI
site of pBR322 (kan) r
araB
araC
araD
NaeI /
SacI -
BglI /
SacI -
pJC103
BamHI
kan r
BamHI

Figure 2: (A) Nucleotide sequence of DNA cloned in pJC103 that contained the *arsRBC* genes. The predicted amino acid sequences of gene products are shown below the DNA sequence. Stop codons are marked by asterisks. Shine-Dalgarno (SD) sequences are printed in bold and underlined. The putative promoter sequences for σ^{70} RNA polymerase are printed in bold, underlined, and identified as -10 and -35. The location and orientation of the MudI insertion, as determined by DNA sequencing from LF20001, as well as several restriction enzyme sites, are also shown.

(B) Homologies among the arsenic resistance determinants of *E. coli* plasmid R773, *Staphylococcus* plasmids pI258 and pSX267, and the chromosomal *ars* operon of *E. coli*. The promoter-operator (P/O) regions are indicated. The sizes of putative gene products (in amino acid residues [aa]), are shown above or below the genes. The numbers between lines are percentages of similarity among the ArsR, ArsB, and ArsC proteins.


A


B
117 aa
139 aa
563 aa
429 aa
141 aa
arsR
arsD
arsA
arsB
arsC
P/O
R773
77%
81%
94%
117 aa
429 aa
141 aa
Escherichia coli chromosome
42% (38%)
69%
22%
pI258 (pSX267)
arsR
arsB
arsC
104 aa
429 aa
131 aa

compounds at the concentrations used here (data not shown).

2.4.3 A functional role for the *ars* operon in protection from arsenic toxicity

In order to determine if the chromosomal *ars* operon plays a functional role in protecting cells from arsenic toxicity, clones with (*E. coli* 40) and without (*E. coli* LF20001 and LF20018) a functional *ars* operon were tested in identical genetic background for their growth in arsenic-containing LB media. Increasing concentrations of sodium arsenite or sodium arsenate were added to LB agar on petri dishes, and the colony-forming capacities of the various strains were examined after overnight growth. The 50% lethal concentrations of sodium arsenate and sodium arsenite of *E. coli* 40 were found to be between 200 and 2000 μg/ml (Fig. 4), and similar results were obtained for *E. coli* strain MG1655 (data not shown). Disruption of the chromosomal *ars* operon by MudI insertion was found to increase the sensitivity of *E. coli* 40 to sodium arsenite (Fig. 4A) and arsenate (Fig. 4B) by approximately 10- to 100-fold (Fig. 4A). However, when the complete *ars* operon, cloned on a multicopy plasmid (pJC103), was introduced into wild-type *E.* coli 40, resistance to sodium arsenite (Fig. 4A) and arsenate (Fig. 4B) increased by at least 3 to 10-fold, though the absolute levels of resistance were somewhat lower than those observed for *E. coli* that contained the *arsRDABC* operon of plasmid R773 [Silver et al., 1981]. The ability of *E. coli arsB* mutant strains (LF20001 and LF20018) to survive at arsenic levels which induce *ars* operon expression (0.01-1 μg/ml) may be due to other cellular detoxification mechanisms, such as those provided by glutathione and thioredoxin [Greer and Perham, 1986; Huang et al., 1993; Huckle et al., 1993].

2.4.4 Sequences homologous to the chromosomal *ars* operon are highly conserved

Because of the high degree of homology between the protein products of the *E. coli* chromosomal *ars* operon and those found on plasmids from both Gram-negative and Gram-positive bacteria, we sought to determine if the chromosomal operon was conserved at the DNA level (and thus possibly the progenitor of the plasmid-based arsenic resistance determinants). DNA was isolated from a number of plasmid-free, Gram-negative bacterial

species [Autexier and DuBow, 1992], hydrolysed with restriction enzymes, and Southern blotted after agarose gel electrophoresis. After hybridization with an *E. coli ars*-specific probe, sequences that were homologous to the *E. coli* chromosomal *ars* operon were found in all of the enterobacterial species examined. Moreover, homologous sequences to the *ars* operon were detected in the non-enterobacterial species *P. aeruginosa* (Fig. 5). This large degree of evolutionary conservation at the DNA level strongly reinforces notions that the chromosomal *ars* operon is functionally important and that its chromosomal presence is not of recent origin.

2.5 DISCUSSION

We have discovered a functional, arsenic-inducible operon present in the chromosome of *E. coli*, with homologous sequences detectable in many other Gram-negative bacterial species. This operon displays strong homology, both in protein sequence and genetic organization, with plasmid-borne arsenic detoxification operons. During the latter stages of this work, continued sequencing of the *E. coli* genome also uncovered this chromosomal *ars* operon homologue, though no functional studies were performed [Sofia et al., 1994]. The names *arsE*, *arsF*, and *arsG* were given to these three homologous cistrons, but *arsR*, *arsB*. and *arsC* more accurately reflect their evolutionary relatedness and probable function(s). Thus, it is likely that the chromosomal *ars* operon is organized as a single transcription unit that is regulated by the arsenic- and antimony-inducible ArsR repressor. Moreover, the structural genes of the chromosomal *ars* operon appear to encode an arsenate reductase (*arsC*) and an arsenite-specific efflux system (*arsB*). The apparent strong evolutionary conservation of chromosomal *ars* determinants also suggests that this operon may be the progenitor of plasmid-borne *ars* operons. The origins of many plasmid-borne resistance determinants has not yet been elucidated. However, it is known that ß-lactamases are also highly conserved, whether their location is chromosomal or on plasmids [Nordmann and Naas, 1994; Seoane and Garcia Lobo, 1991]. In addition, hemolysins show similar strong evolutionary conservation [Baba et al., 1991; Frey et al., 1991]. More recently, however, a chromosomal homolog of a plasmid-borne copper

Figure 3: ß-Galactosidase levels in *E. coli* LF20001 cells at 30 min after exposure to arsenic and antimony compounds. Cells were grown to an A_{550} of 0.4 and exposed to the indicated final concentrations of sodium arsenate (+), sodium arsenite (□), cacodylic acid (■), and antimony oxide (*), and the amounts of ß-galactosidase produced after 30 min were measured as described in Materials and Methods.

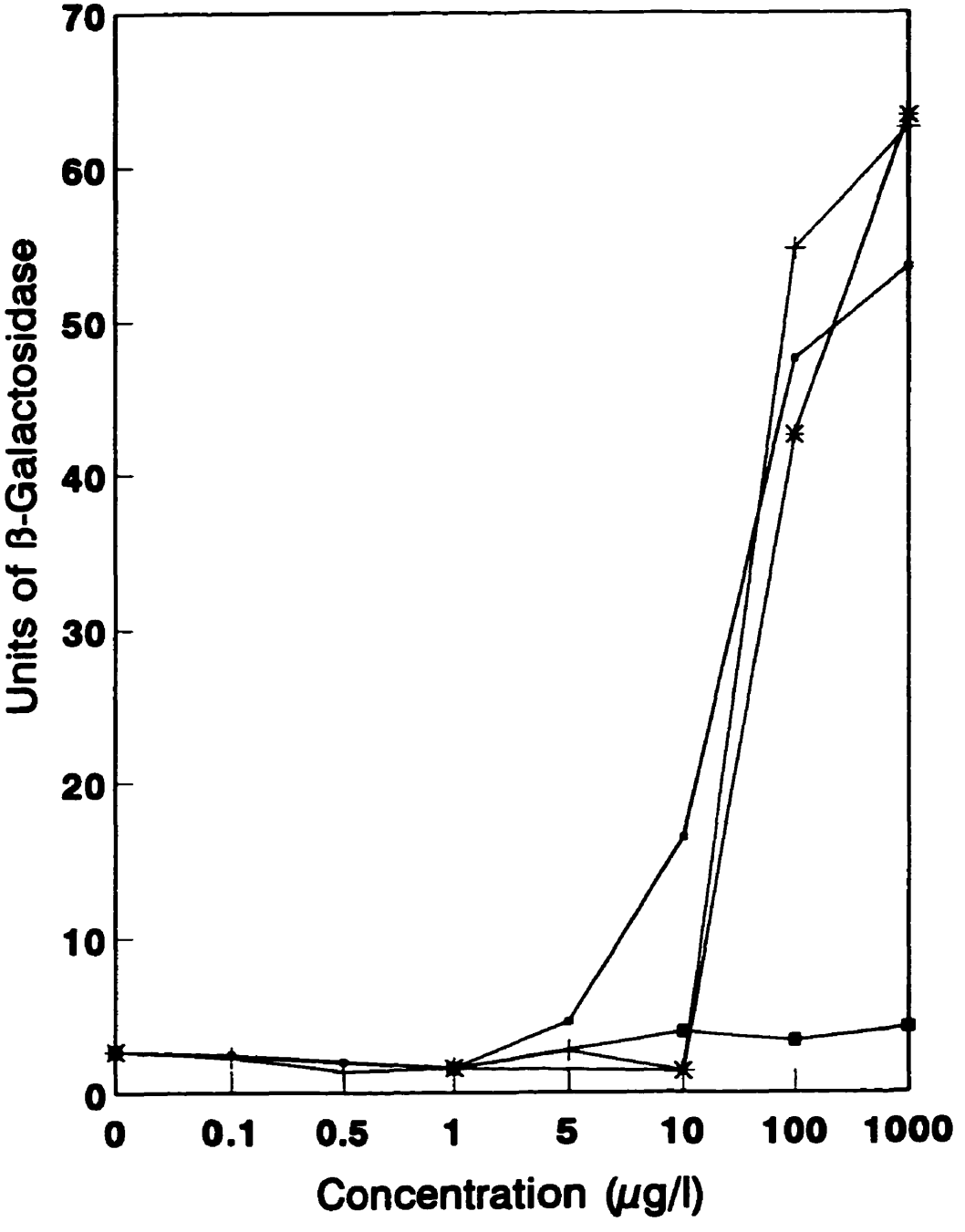
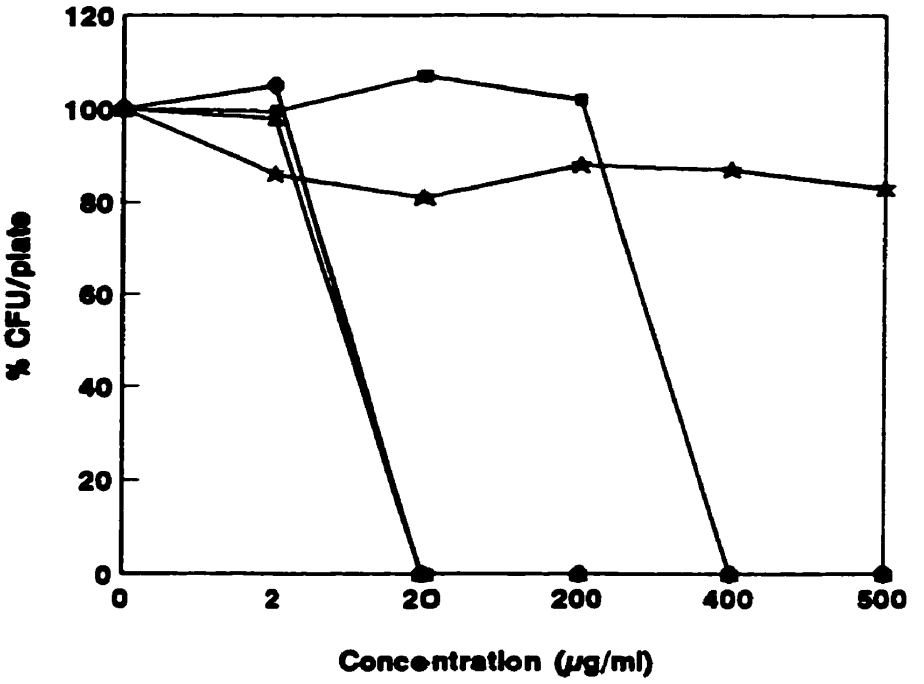

70
60
50
40
30
20
10
0
Units of β-Galactosidase
0
0.1
0.5
1
5
10
100
1000
Concentration (μg/l)

Figure 4: Arsenic sensitivity tests on solid media. Log-phase cultures of *E. coli* 40 (□), LF20001 (▲), LF20018 (●), and LF20020 [*E. coli* 40 (pJC103)] (★) were diluted and spread on petri dishes that contained the indicated concentrations of sodium arsenite (A) and sodium arsenate (B), as described in Materials and Methods. Colonies were counted after 18 h and expressed as the average percentage of CFU per plate, compared with those obtained in the absence of added arsenic compounds.

A


120
100
80
60
40
20
0
% CFU/plate
0
2
20
200
400
500
Concentration (μg/ml)

B

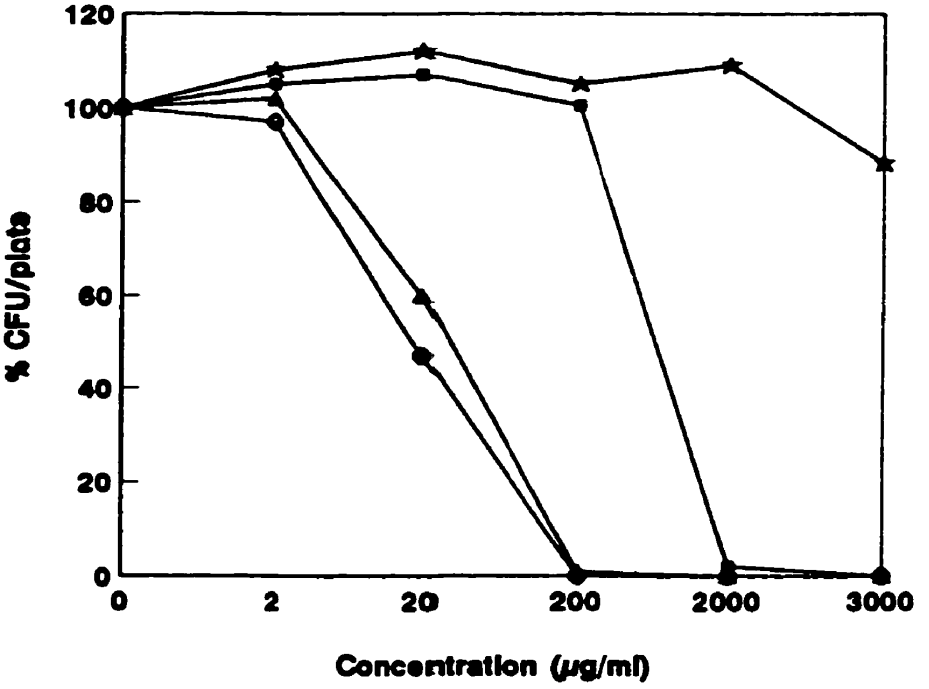
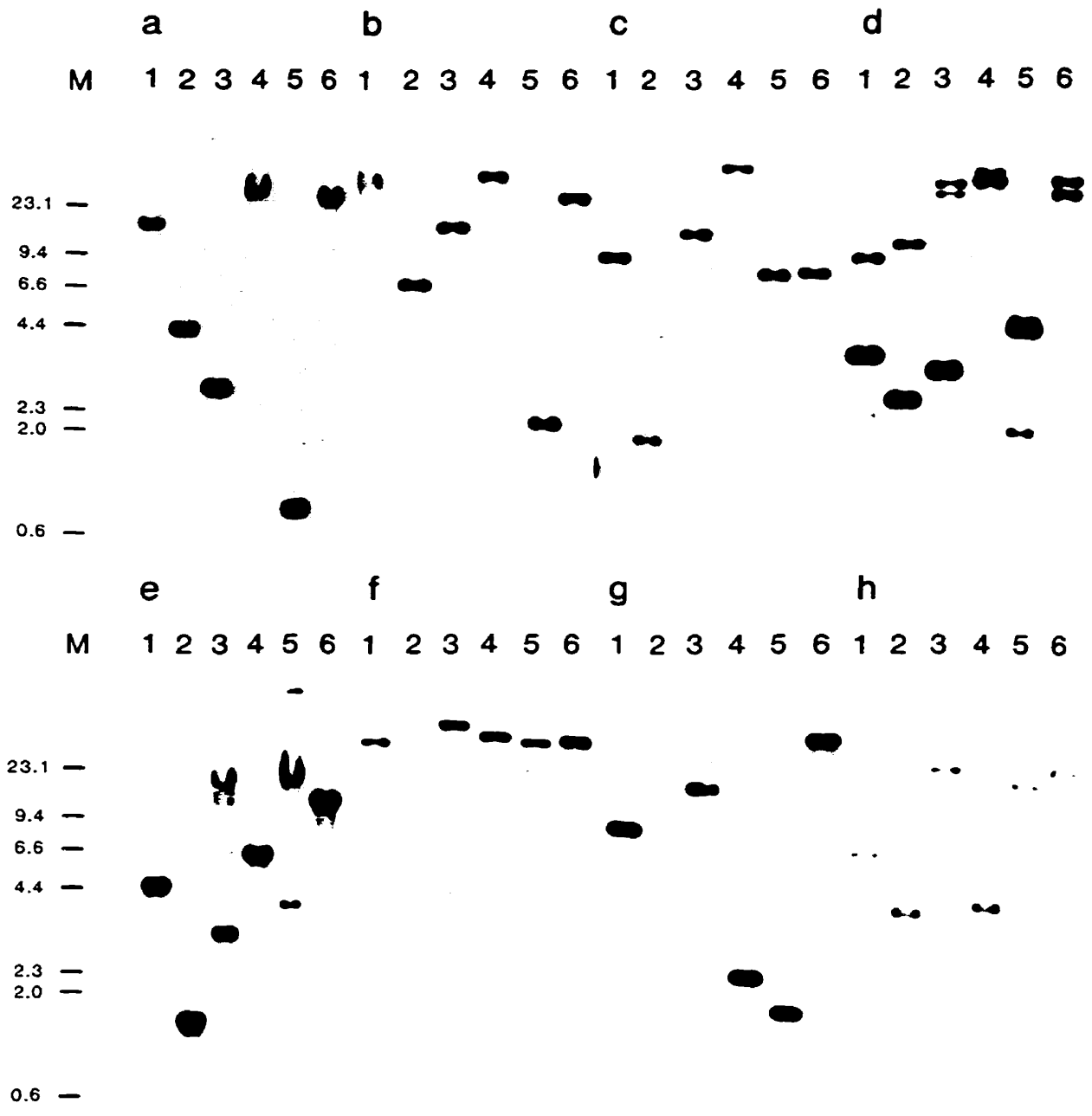

120
100
80
60
40
20
0
% CFU/plate
0
2
20
200
2000
3000
Concentration (μg/ml)

Figure 5: Southern hybridization analyses of chromosomal DNAs from various bacterial species to detect sequences homologous to those of the *E. coli* chromosomal *arsRBC* genes. DNA was digested with *Pst*I (lanes 1), *Eco*RV (lanes 2), *Bgl*II (lanes 3), *Bam*HI (lanes 4), *Pvu*II (lanes 5), and *Hin*dIII (lanes 6). (a) *Escherichia coli* 40; (b) *Shigella sonneii*; (c) *Citrobacter freundii*; (d) *Enterobacter cloacae*; (e) *Salmonella arizonae*; (f) *Erwinia carotovora*; (g) *Klebsiella pneumoniae*; (h) *Pseudomonas aeruginosa*; Lanes M, markers (in kilobases) are λ DNA digested with *Hin*dIII. (a and b) Hybridized with an *Nde*I-*Eco*RV *arsB* probe; (c to h) probed with an *Eco*RV fragment that contained the *arsB* and *arsC* genes. See Materials and Methods for details.


a
b
c
d
M 1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6
23.1
9.4
6.6
4.4
2.3
2.0
0.6
e
f
g
h
M 1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6
23.1
9.4
6.6
4.4
2.3
2.0
0.6

resistance operon has been found in *Pseudomonas syringae* [Lim and Cooksey, 1993]. To our knowledge, its evolutionary conservation has not been determined.

It has been proposed that the structure of the plasmid-borne, ATP-driven arsenic efflux pump, made up of the ArsA and ArsB proteins, may be structurally related to the multiple drug resistance ATP-driven efflux pump found amplified in mammalian cancer cells [Silver et al., 1989; Silver et al., 1993b; Wu et al., 1992]. During chemotherapy of cancer patients, cells become resistant to anti-cancer chemotherapeutic agents by amplification of the number of copies of the multidrug resistance gene and thus overexpression of the multidrug resistance pump [Roninson, 1992]. In analogous fashion, amplification of the chromosomal *ars* operon, by its presence on multicopy plasmids, should allow increased resistance to cellular exposure to toxic arsenic salts. In this regard, we found that the presence of this operon in pBR322 (pJC103), under its own regulation, conferred at least a 2 to 10-fold increase in resistance of *E. coli* to arsenate or arsenite exposure.

A *lacZ* fusion to the chromosomal *ars* operon was found to be induced by arsenic compounds at concentrations that reflect their relative toxicity (arsenate < arsenite). Moreover, antimony oxide could also induce expression of the *arsB*::*lacZ* fusion. These results are consistent with those observed for the R773 *ars* operon of *E. coli* [Wu and Rosen, 1993b]. We also observed a threshold of *ars* operon expression; induction was not observable at a concentration less than 1 μg arsenite per liter. A similar threshold effect of induction has been shown for the *mer* operon by its inducer, mercury [Ralston and O'Halloran, 1990]. In addition, cacodylate did not induce the *arsB*::*lacZ* fusion, even at elevated concentrations. Thus, the induction of *ars* gene expression appears to reflect the relative toxicities of the arsenic and antimony compounds. The LF20001 (*lacZYA* inserted in *arsB*) clone may therefore prove useful in determining the potential cytotoxicity of arsenic compounds (to enteric bacteria), as the assay for ß-galactosidase is both rapid and quantitative [Miller, 1972].

In contrast to the *E. coli* plasmid-borne *ars* operon, the chromosomal *ars* operon contains neither the *arsD* nor *arsA* cistron. Low-stringency Southern blotting [Sol et al., 1986] and hybridization with cloned *arsR*, *arsB*, and *arsC* cistrons from plasmid R773

enabled these cistrons to be detected in the chromosome of *E. coli*, and the pattern of fragments was consistent with restriction enzyme mapping of the chromosomal *ars* region (data not shown). However, no signal was detected with the *arsD* and *arsA* cistrons as probes, even after long exposure times. These results imply that the *arsD* and *arsA* cistrons are not present at another location on the *E. coli* chromosome. Whether (and from where) plasmid-borne *ars* operons have gained the *arsD* and *arsA* cistrons or the *E. coli ars* operon has lost them is not clear at present. In this regard, it is interesting that the chromosomal *ars* operon organization more closely resembles that of Gram-positive bacteria in lacking *arsD* and *arsA*, even though it is more homologous to that of Gram-negative bacterial species.

The lack of an *arsA*-encoded ATPase subunit in the chromosomal *ars* operon is striking. Nonetheless, the ArsB subunit may function as an arsenite-specific efflux system which uses membrane potential instead of ATP hydrolysis provided by the *arsA* cistron. This mechanism has been observed in the plasmid-borne ArsB protein from *Staphylococcus* [Bröer et al., 1993] and in tetracycline efflux for the Tn*10*-derived tetracycline resistance determinant [Kaneko et al., 1985]. Determination of the structures and functions of other chromosomal *ars* operons and the origin(s) of the *arsD* and *arsA* genes, is currently in progress.

CHAPTER 3

EXPRESSION OF THE *ESCHERICHIA COLI* CHROMOSOMAL *ARS* OPERON

3.1 PREFACE

In Chapter 2, an *Escherichia coli* chromosmal *ars* operon has been identified using a random *lacZ* gene fusion strategy. The operon was found to play a role in protecting cells from arsenic toxicity and be evolutionarily conserved in the chromosomes of a number of bacterial species. The *lacZ* reporter gene in the arsenic-responsive clone was found to be induced by inorganic arsenic and antimony compounds, and was inserted within the *arsB* coding sequence. A further investigation of transcriptional regulation and expression of the *E. coli* chromosomal *ars* operon is described in Chapter 3.

3.2 ABSTRACT

A chromosomally-located operon (*ars*) of *Escherichia coli* has been previously shown to be protective against arsenic toxicity. DNA sequencing revealed three open reading frames homologous to the *arsR*, *arsB*, and *arsC* open reading frames of plasmid-based arsenic resistance operons isolated from both *E. coli* and Staphylococcal species. To examine the outline of transcriptional regulation of the chromosomal *ars* operon, several transcriptional fusions, using the luciferase-encoding *luxAB* genes of *Vibrio harveyi*, were constructed. Measurement of the expression of these gene fusions demonstrated that the operon was rapidly induced by sodium arsenite and negatively regulated by the *trans*-acting *arsR* gene product. Northern blotting and primer extension analyses revealed that the chromosomal *ars* operon is most likely transcribed as a single mRNA of approximately 2100 nucleotides in length and processed into two smaller mRNA products in a manner similar to that found in the *E. coli* R773 plasmid-borne *ars* operon. However, transcription was found to initiate at a position that is relatively further upstream of the initiation codon of the *arsR* coding sequence than that determined for the *E. coli* R773 plasmid-encoded *ars* operon.

3.3 INTRODUCTION

Resistance to elevated levels of arsenic and antimony in both Gram-negative and Gram-positive bacteria was originally found to be encoded by plasmid-located determinants, called *ars* operons [Novick and Roth, 1968; Hedges and Baumberg, 1973]. The well-studied *E. coli* conjugative plasmid R773 *ars* operon encodes an ATP-driven, arsenite- and antimonite-specific membrane efflux pump, conferring resistance to arsenic and antimony salts by extrusion of intracellular toxic oxyanions from the cell [Kaur and Rosen, 1992a]. This operon consists of five ORFs, in the order *arsRDABC*, organized as a single transcription unit initiated at a site just upstream of *arsR*. It encodes an arsenic-inducible *trans*-acting repressor (ArsR), controlling the basal level of expression of the operon [San Francisco et al., 1990; Wu and Rosen, 1991; 1993a]; a negative regulator (ArsD) that independently controls the upper level of expression of the operon [Wu and Rosen, 1993b]; an arsenite- and antimonite-stimulated ATPase (ArsA) [Rosen et al., 1988; Mei-Hsu et al., 1991]; a membrane pump for efflux of

arsenite and antimonite ions (ArsB) [Wu et al., 1992; Dey and Rosen, 1995]; and an arsenate reductase (ArsC) [Rosen et al., 1991; Gladysheva et al., 1994]. The *arsR*, *arsB*, and *arsC* genes are highly conserved in plasmid-based *ars* operons (plasmids pI258 and pSX267) isolated from Gram-positive Staphylococcal species [Broër et al., 1993; Rosenstein et al., 1992]. However, the *arsD* and *arsA* genes are absent. The efflux of arsenic and antimony oxyanions by Staphylococcal *ars* operons has been shown to be powered by membrane potential rather than ATP [Ji and Silver, 1992a; Rosenstein et al., 1992; Bröer et al., 1993; Silver et al., 1993].

The recent discovery of a chromosomally-located *ars* operon homolog in *E. coli* and other Gram-negative bacterial species [Carlin et al., 1995; Diorio et al., 1995; Sofia et al., 1994] revealed a potential evolutionary relatedness between the plasmid-encoded and the chromosomal *ars* operons in bacteria. The *E. coli* chromosomal *ars* operon is composed of three ORFs which could potentially encode polypeptides which would share strong homology with the ArsR, ArsB, and ArsC proteins of the plasmid R773 *ars* operon, and weaker, though significant, homology with those encoded by the Staphylococcal plasmid *ars* operons [Diorio et al., 1995]. Disruption of the chromosomal *arsB* coding sequence by a MudI insertion, or deletion of the operon, resulted in increased sensitivity of the cells to arsenic salts [Carlin et al., 1995; Diorio et al., 1995], implying a protective role of this operon against arsenic toxicity in cells that do not harbour any arsenic resistance plasmids. Conversely, the presence of this operon cloned on a multicopy plasmid was found to confer increased resistance to elevated levels of sodium arsenate or sodium arsenite [Diorio et al., 1995]. Expression of this operon has been shown to be induced by arsenic and antimony oxyanions, and the inducibility of arsenic compounds tested appears to reflect their relative toxicity [Diorio et al., 1995].

To outline the parameters underlying transcriptional regulation of the *E. coli* chromosomal *ars* operon, several *E. coli* strains containing *arsB*::*luxAB* or *arsR*::*luxAB* gene fusions were constructed. Expression of the luciferase reporter gene in these fusion strains confirmed the role of the *arsR* gene product as a *trans*-acting repressor. Northern blotting and primer extension analyses were performed to determine the transcript size and transcriptional initiation site. These results, in turn, were compared and contrasted with those of the plasmid-based *ars* operons.

3.4 MATERIALS AND METHODS

3.4.1 Bacterial strains and plasmids

The *E. coli* strains and plasmids used in this study are described in Table 1. Cells were grown in Luria-Bertani (LB) broth [Miller, 1972] at 37°C for all strains. Ampicillin (40 μg/ml), tetracycline (10 μg/ml), and kanamycin (50 μg/ml) were added to the growth medium at the indicated final concentrations when required. Phage P1*vir* was a kind gift from Dr. R. Stewart (University of Maryland, College Park, Md.).

3.4.2 Materials

All restriction endonucleases and nucleic acid-modifying enzymes were purchased from Gibco BRL (Burlington, Ont.), New England Biolabs (Missisauga, Ont.), and Pharmacia Biotech (Baie d'Urfe, Qué.). Radioisotopes were purchased from Amersham (Oakville, Ont.). All other chemicals were obtained from commercial sources.

3.4.3 DNA manipulations

The conditions for DNA restriction endonuclease hydrolyses and DNA ligation reactions were as described by Tolias and DuBow (1985). Plasmid isolation and transformation (using the calcium chloride method) were performed as described [Sambrook et al., 1989]. P1 transduction was performed (using P1*vir*) following the procedure described by Miller (1972).

3.4.4 Construction of chromosomal *arsB*::*luxAB* and *arsR*::*luxAB* gene fusions

A 7.4 kb DNA fragment containing a promoterless *Vibrio harveyi luxAB* reporter gene, plus a tetracycline resistance (Tc^r) gene from Tn10, was isolated from *Bam*HI-*Hin*dIII digested plasmid pAG3 [Autexier and DuBow, 1992], made blunt-ended by backfilling the 5' extensions of the restriction enzyme sites with Klenow enzyme, and ligated with plasmid pJC103 [Diorio et al., 1995] digested with either *Sca*I for the *arsB* fusion, or *Bgl*II (plus Klenow enzyme blunt-ended) for the *arsR* fusion. The ligation mixtures were used to transform *E. coli* strain NM522, followed by selection on kanamycin- and tetracycline-containing LB agar plates. The recombinant plasmids, containing the *arsB*::*luxAB* (pJC202) or *arsR*::*luxAB*

Table 1. Bacterial strains and plasmids.

Table 1. Bacterial strains and plasmid

Strain or plasmid	Characteristics	Reference
E. coli		
40	F', Δ *pro-lac*, *rpsL*, *trp*	Bukhari and Metlay 1973
LF20012	*arsB*::*lacAB* in *E. coli* 40	This work
LF20013	*arsR*::*lacAB* in *E.coli* 40	This work
LF20012R	LF20012 containing pDK1 and pJC612	This work
LF20013R	LF20013 containing pDK1 and pJC612	This work
LF20013R'	LF20013 containing pJC613	This work
D308	*recD*1009, *lacY*, *eda*	Russell et al. 1989
LF20010	*arsB*::*lacAB* in *E. coli* D308	This work
LF20011	*arsR*::*lacAB* in *E. coli* D308	This work
NM522	Δ *pro-lac*, *rpsL*, *thi*1, *hsdR*4, *supE44*/F' *traD36*, *proA*$^+$*B*$^+$, *lacI*^{q}ZΔM15	Gough and Murray 1983
LF20014	*arsB*::*lacAB* fusion in *E.coli* NM522	This work
LF20015	*arsR*::*lacAB* fusion in *E. coli* NM522	This work
Plasmids		
pJC103	a 2970 bp DNA fragment containing the entire *E. coli* chromosomal *ars* operon cloned into the *Sca*I site of a pBR322 derivative (Km^r)	Diorio et al. 1995
pAG3	ColEI containing a Tn*5*::*LacAB* derivative, Tc^r	Autexier and DuBow 1992
pJC202	a *lacAB* Tc^r insertion in *arsB* of pJC103 in the *Sca*I site	This work
pJC301	a *lacAB* Tc^r insertion in *arsR* of pJC103 in the *Bgl*II site	This work
pKK223-3	Ap^r, *trp-lac* (P_{tac}) P/O containing vector	Brosius and Holy 1984
pJC612	a 411 bp *Nde*I-*Bst*BI DNA fragment containing the *arsR* gene cloned into the *Sca*I site of pKK223-3	This work
pJC701	a DNA fragment containing the intact *arsBC* genes, followed by a 515 bp pBR322 sequence (from the *Sca*I to *Eco*RI sites) cloned into the *Eco*RI site of pKK223-3	This work
pUC119	Ap^r *lac* P/O containing vector	Vieira and Messing 1987
pJC502	a 411 bp *Nde*I-*Bst*BI DNA fragment containing *arsR* gene cloned into the *Sma*I site of pUC119	This work
pMC9	a pBR322 derivative with a *lacI*^{q} gene (1.7 kb) cloned into the *Eco*RI site.	Miller et al. 1984
pDO1	an 1.7 kb *Eco*RI fragment, carrying a *lacI*^{q} gene from pMC9, cloned into the *Eco*RI site of pACYC184, Tc^r.	Fortin et al. in preparation
pDK1	an 1.1 kb *Bam*HI fragment containing a kanamycin resistance gene from pJC103 cloned into the *Bam*HI site of pDO1, Km^r.	This work
pAB100	a pBR322 derivative containing the R773 *arsRAB* genes under the P_{tac} control, Ap^r	Turner et al. 1992
pJC613	a pBR322 derivative containing the R773 *arsR* gene under the P_{tac} control, constructed by deleting a 3 kb *Hin*dIII fragment from pAB100, Ap^r	This work

(pJC301) fusions, were identified by a luminescence assay as previously described [Guzzo and DuBow, 1991], and confirmed by restriction enzyme mapping analyses.

To replace the intact chromosomal *ars* operon with the gene fusions, plasmids pJC202 and pJC301 were linearized with the restriction enzyme *Hin*dIII, which cleaves within the vector sequence only. The linearized plasmids were then transformed into the *recD*⁻ *E. coli* strain D308 [Russell et al., 1989] and plated on LB agar containing tetracycline. The recombinant clones were initially identified by a luminescence assay on LB agar plates [Guzzo and DuBow, 1991] and mapped [Autexier and DuBow, 1992], and the gene fusion replacements for the *arsB* and *arsR* fusions were designated LF20010 and LF20011, respectively. These luciferase fusions were subsequently placed into the chromosome of strain *E. coli* 40 by P1 transduction, resulting in strains LF20012 (*arsB*::*luxAB*) and LF20013 (*arsR*::*luxAB*), respectively, which were further confirmed by Southern blotting analyses using the *luxAB* and *ars* genes as probes. To examine the *trans*-acting effects of the *arsR* gene product, these strains were transformed with pDK1 (Table 1), a pACYC184 derivative that contains a $lacI^q$ gene to control the activity of the P_{tac} promoter, followed by plasmid pJC612 containing the *arsR* gene under the control of the P_{tac} promoter (see below), yielding strains LF20012R and LF20013R, respectively.

To examine whether any strain-specific effects occurred with the *luxAB* gene fusions, the *arsB*::*luxAB* and *arsR*::*luxAB* chromosomal fusions were introduced into strain NM522 by P1 transduction from LF20012 and LF20013, resulting in *E. coli* strains LF20014 and LF20015, respectively.

3.4.5 Cloning and expression of *ars* gene products

Plasmid pJC103 was digested with *Nde*I and *Bst*BI. Three fragments were generated: a 411-bp *Bst*BI-*Nde*I fragment containing the *arsR* coding sequence, a 5.8-kb *Nde*I-*Nde*I fragment containing the *arsBC* sequence followed by the flanking pJC103 DNA, and a third, 2.2-kb *Nde*I-*Bst*BI fragment containing the rest of the pJC103 DNA. The DNA fragments in the digestion mixture were backfilled at the 5' extensions of the restriction enzyme sites with Klenow enzyme and ligated with *Sma*I-digested plasmid pKK223-3 [Brosius and Holy, 1984].

The ligation mixture was used to transform *E. coli* strain NM522 using the calcium chloride method [Sambrook et al., 1989], followed by selection for ampicillin-resistant transformants. The recombinant plasmids were mapped by restriction enzyme analyses, and two recombinant plasmids were isolated. One, designated pJC612, contains the entire *arsR* gene (without the P_{ars} promoter) under the control of the P_{tac} promoter. The other, designated pJC604, contains the 5.8-kb *Nde*I-*Nde*I fragment. Plasmid pJC701 was subsequently created by cloning a 2.4-kb *Eco*RI fragment from pJC604 into the *Eco*RI site of pKK223-3. In this construct (pJC701), expression of the *arsBC* genes is under the control of the P_{tac} promoter. The *in vivo* expression assay was carried out using a modified [Tolias and DuBow, 1986] chloramphenicol release procedure [Neidhardt et al., 1980].

3.4.6 Luciferase assays

Overnight cultures of *E. coli* strains grown in LB medium, containing the required antibiotics, were diluted 100 fold in the same medium and allowed to grow to early log phase (A_{600}= 0.2 ~ 0.3). Cells were exposed to increasing concentrations of sodium arsenite and sampled every 30 min for a period of 2 h (not shown). However, maximum induction of gene expression under these conditions was observed at 60 min post-exposure. Samples were diluted in LB broth to a final A_{600} of 0.05 and then introduced into a Tropix Optocomp I luminometer (MGM Instruments, Hamden, Conn.). Total relative light units were determined for a 10-s interval, after injection of 100 µl of dodecyl aldehyde (diluted 1:100 in LB broth). Luciferase activity is expressed as the number of photons emitted per second per A_{600} unit.

3.4.7 Northern blotting analysis

Total cellular RNA was isolated from arsenite-induced (for 60 min with 0.8 µM sodium arsenite) and uninduced *E. coli* strain 40 using the diethylpyrocarbonate (DEPC)-saturated NaCl method as described by Ausubel et al. (1989).

Total cellular RNA (10 µg) from arsenite-induced and uninduced cells were denatured by heating at 65°C for 15 minutes in formamide loading buffer, and then subjected to electrophoresis through a 1% formaldehyde agarose gel as described by Sambrook et al.

(1989), except that the RNA was prestained by addition of 9.5 μg ethidium bromide/ml (final concentration) to the loading buffer. After electrophoresis, the gel was soaked in 20 × SSC [3M sodium chloride, 0.3M sodium citrate (pH 7.0)] for 20 min and transferred overnight by capillary blotting onto a nylon membrane filter (Hybond™-N, 0.45 micron, Amersham). The RNA was fixed to the filter by baking at 80°C for 1 h, and the filter was prehybridized [in a solution containing 0.5M sodium phosphate (pH 7.2), 1mM EDTA, 1% (w/v) bovine serum albumin (BSA), and 7% (w/v) sodium dodecyl sulfate (SDS)] at 65°C for 1 h and then hybridized to an α-^{32}P-labelled DNA fragment containing the *E. coli* chromosomal *arsRBC* genes, isolated as a 2 kb fragment from *Stu*I-digested pJC103, at 65°C for 24 h. The DNA probe was labelled with [α-^{32}P]dATP (3000 Ci/mmol, 1 Ci = 37 GBq) using the random priming method [Sambrook et al. 1989] and random hexanucleotide primers (Regional DNA Synthesis Laboratory, University of Calgary, Calgary, Alta.). The 0.4- to 9.5-kb RNA ladder (Gibco BRL) was subjected to electrophoresis in parallel with the above samples and hybridized to α-^{32}P-labelled Lambda DNA cleaved with *Hin*dIII. Unhybridized probes were removed by washing the filters four times at 65°C in a solution containing 40mM sodium phosphate (pH 7.2), 1mM EDTA, and 1% (w/v) SDS. The blots were air dried for 30 min and exposed to Kodak XAR-5 film (Eastman Kodak, Rochester, N.Y.) under Dupont Cronex intensifying screens at -70°C.

3.4.8 Primer extension analysis

A synthetic 24-mer oligonucleotide (5'-TAACTCTCCCAGTTCGCTGAGCAG-3'), complementary to the putative transcribed strand of *arsR*, was used as a primer. The primer was end-labelled with [γ-^{32}P]ATP (6000 Ci/mmol), using T4 polynucleotide kinase [Sambrook et al., 1989].

For the primer extension reaction, 10 μg of total cellular RNA, isolated as described above, were mixed with 2 pmol of radiolabelled primer. The reaction was performed using Gibco BRL reagents (i.e. 1 × RT buffer, 10 μM dithiothreitol (DDT), 1.25 μl RNA Guard, 0.5 mM dNTPs, and 200 U of Moloney murine leukemia virus reverse transcriptase) following the manufacturer's instructions and terminated by addition of 1 μl 0.5 M EDTA (pH 8.0) plus 1 μl

of DNase-free pancreatic RNase (5 μg/ml) to a reaction volume of 20 μl at 37°C for 30 min. The reaction mixture was extracted with phenol:chloroform (1:1, v/v) and precipitated with ethanol following the procedure described by Sambrook et al. (1989), dissolved in formamide loading buffer, heated at 95°C for 5 min, and immediately chilled on ice. A sample (2.5 μl) was then loaded onto a 5% polyacrylamide-7M urea sequencing gel. The length of the reverse transcriptase product was measured against a size standard produced by dideoxy sequencing of the pUC119 vector [Vieira and Messing, 1987] containing the *arsR* gene (pJC502) using the above primer.

3.5 RESULTS

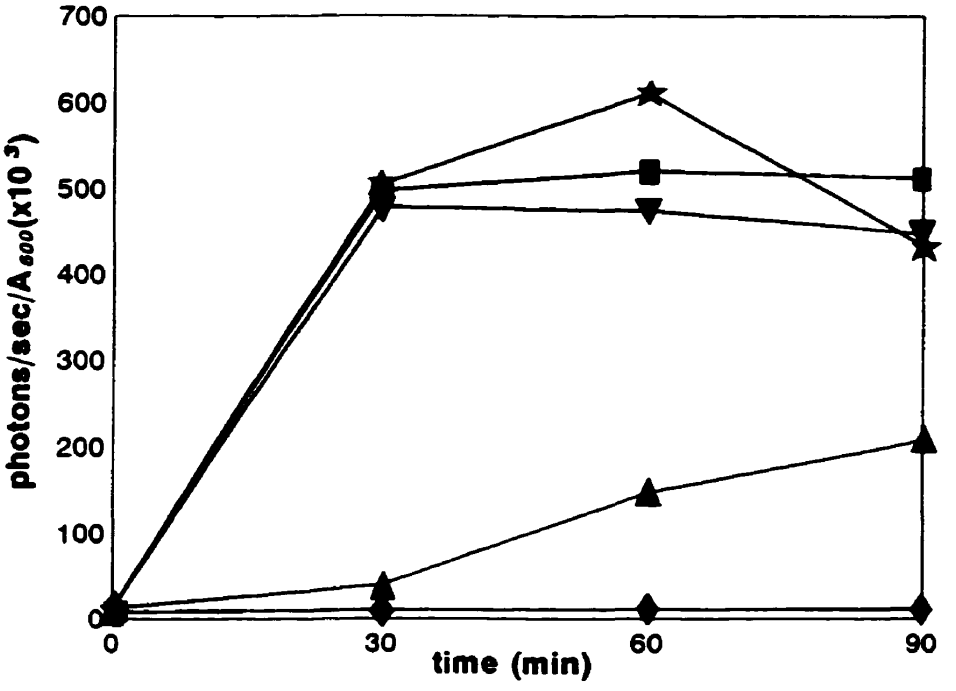
3.5.1 Construction and expression of the *ars::luxAB* gene fusions

To measure the expression of the *arsR* and *arsB* genes, a 7.4 kb *Bam*HI-*Hin*dIII fragment (from pAG3) containing a promoterless *Vibrio harveyi luxAB* gene and a Tc^r gene, was introduced into the chromosomal *arsR* and *arsB* genes, respectively, of *E. coli* strain 40, yielding strains LF20013 (*arsR::luxAB*) and LF20012 (*arsB::luxAB*). Subsequently, plasmids pJC612 (containing the *E. coli* chromosomal *arsR* gene under the control of P_{tac} promoter) and pDK1 (containing the $lacI^q$ gene) were transformed into these strains, yielding strains LF20013R and LF20012R, respectively. The same chromosomal gene fusion constructs were also introduced into *E. coli* strain NM522 by P1 transduction, resulting in strains LF20015 and LF20014. The ability of the plasmid R773 *arsR* gene product to control the expression of the chromosomal *ars* operon was examined by transforming plasmid pJC613 (Table 1), containing the R773 *arsR* gene under the control of the plasmid-based arsenic-inducible promoter, into strain LF20013 and then measuring the luminescence of the resulting transformant (LF20013R') with and without added arsenic oxyanions.

The above gene fusion strains were grown in the absence and presence of sodium arsenite and luciferase activity was analysed as described in Materials and Methods. Expression of the *arsB::luxAB* fusion in strain LF20012 was found to be induced by arsenite in a concentration-dependent manner (Fig. 1A). Maximum induction of luciferase expression was observed at 60 min after addition of sodium arsenite at concentrations ranging from 0.8 to 2

Figure 1. Expression of the *arsB*::*luxAB* fusion (A) and *arsR*::*luxAB* fusion (B) in *E. coli* strain 40. Overnight cultures of strains LF20012 and LF20013 were diluted (1:100) in tetracycline-containing LB media, grown to early log phase, and then exposed to increasing concentrations of sodium arsenite: ◆, 0 μM; ▲, 0.08 μM; ★, 0.8 μM; ■, 1.2 μM; ▼, 2 μM. Aliquots were removed every 30 min after the addition of sodium arsenite and luciferase activity was determined as described in Materials and Methods.

A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600
500
400
300
200
100
0
photons/sec/A600(x10 3)
0
30
60
90
time (min)

B

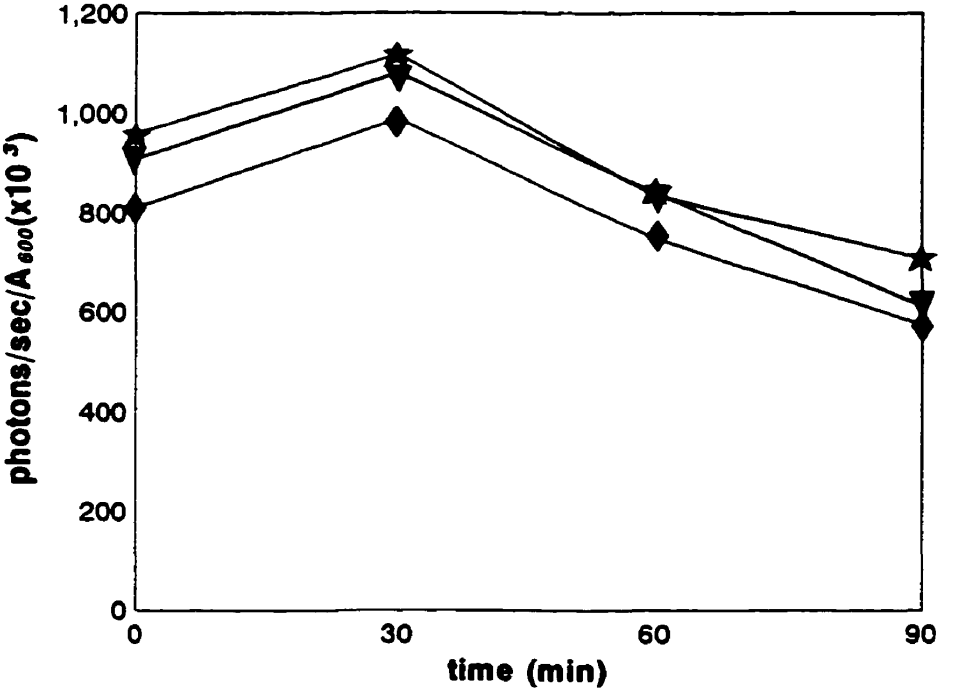
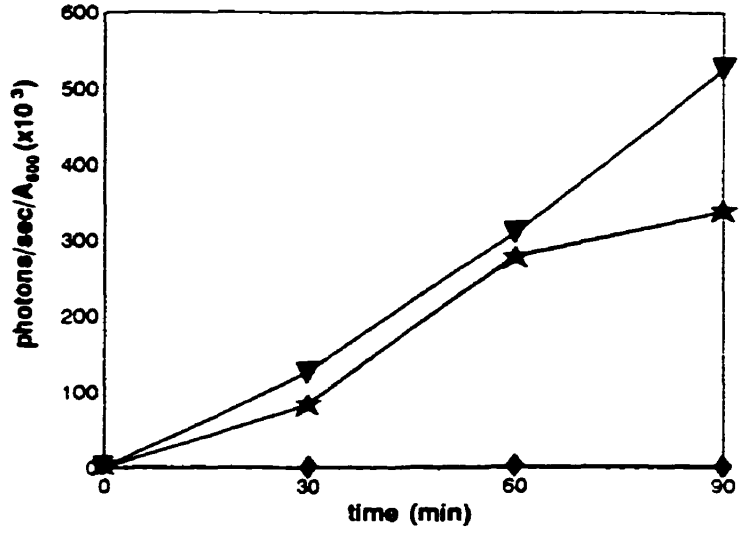
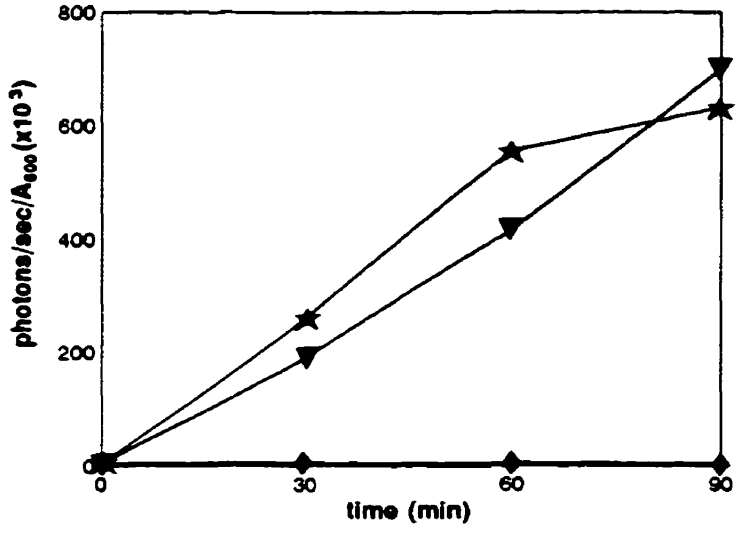

1,200
1,000
800
600
400
200
0
photons/sec/A600(x10 3)
0
30
60
90
time (min)

Figure 2. Expression of the *arsB::luxAB* fusion (A) and *arsR::luxAB* fusion (B) in *E. coli* strain 40 with pJC612 (*arsR*), and the *arsR::luxAB* fusion in *E. coli* strain 40 with pJC613 (C). Overnight cultures of LF20012R, LF20013R and LF20013R' were diluted in LB media containing the required antibiotic(s), grown to early log phase, and then exposed to increasing concentration of sodium arsenite: ◆, 0 μM; ★, 0.8 μM; ▼, 2 μM. Samples were taken every 30 min after addition of sodium arsenite and luciferase activity was determined as described in Materials and Methods.

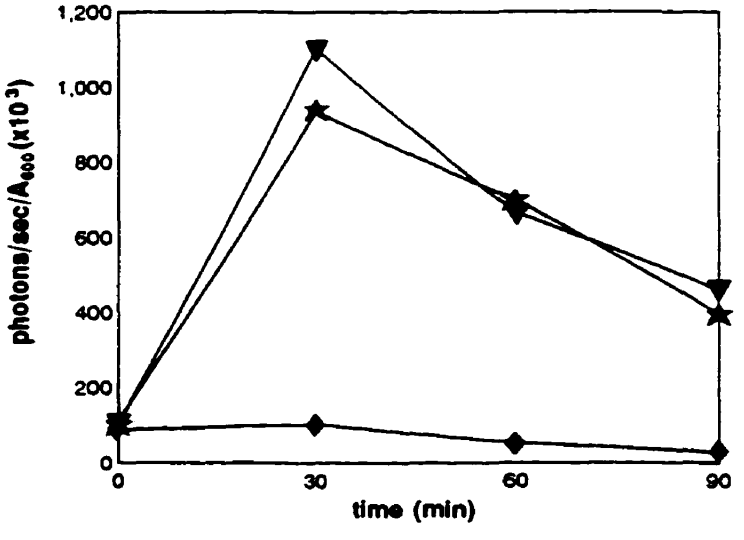
A


600
500
400
300
200
100
0
photons/sec/A600 (x10 3)
0
30
60
90
time (min)

B


800
600
400
200
0
photons/sec/A600 (x10 3)
0
30
60
90
time (min)

C

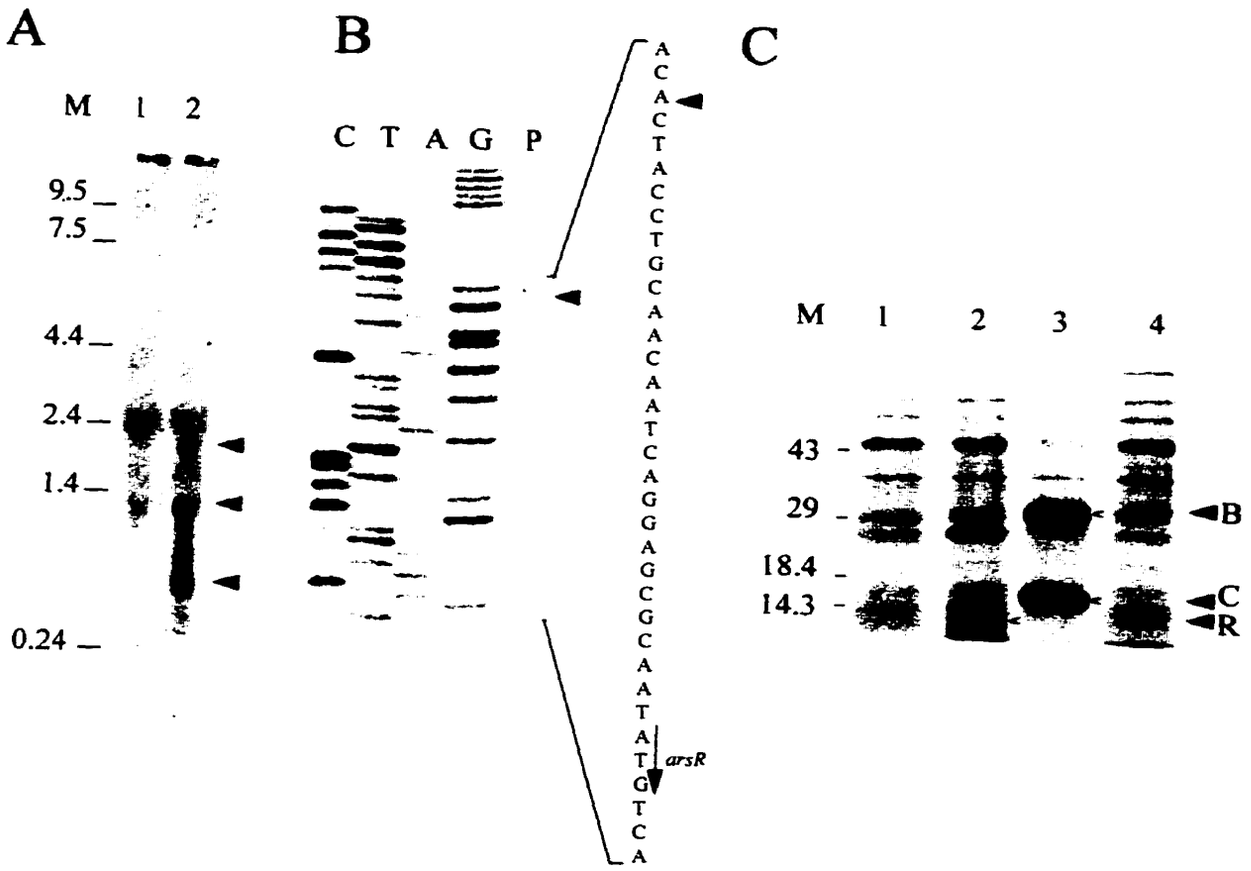

1,200
1,000
800
600
400
200
0
photons/sec/A600 (x10 3)
0
30
60
90
time (min)

μM (Fig. 1A). In strain LF20013, however, the *lacAB* reporter gene, inserted within *arsR*, was found to be constitutively expressed (Fig. 1B). When the wild type chromosomal *arsR* gene was provided in *trans* to these two strains, not only was a reduced basal level of expression of the operon observed in the *arsB*::*lacAB* fusion strain LF20012R (Fig. 2A), but inducible expression of the *lacAB* reporter gene was restored in the *arsR*::*lacAB* fusion strain LF20013R (Fig. 2B), suggesting that the *arsR* gene product functions as a *trans*-acting repressor. Interestingly, the constitutive expression of the chromosomal *arsR*::*lacAB* fusion was found to be repressed by the plasmid R773 *arsR* gene product provided *in trans* (Fig. 2C), indicating that the *arsR* gene products of both plasmid and chromosomal *ars* operons are functionally interchangeable. Similar patterns of reporter gene expression were also observed in *arsB*::*lacAB* and *arsR*::*lacAB* gene fusion strains derived from *E. coli* strains D308 (LF20010 and LF20011) and NM522 (LF20014 and LF20015) (data not shown).

3.5.2 Determination of the transcript size and transcription initiation site of the chromosomal *ars* operon

To measure the size of the transcript produced from the *ars* operon, formamide-denatured total cellular RNA from arsenite-induced and uninduced cells was subjected to formaldehyde agarose gel electrophoresis, transferred to a nylon membrane filter, and hybridized to a radiolabelled DNA fragment containing the *arsRBC* genes. The arsenite-induced sample was shown to hybridize to the probe as three bands over the background of non-specific hybridization with rRNA (Fig. 3A, lane 2). The top *ars*-specific band corresponds to the size of a full-length *arsRBC* transcript, predicted to be approximately 2.1-2.2 kb. The lower two bands of *ars*-specific RNA are most likely RNA processing products of the full-length transcript. A similar mRNA processing pattern was observed in the plasmid R773 *ars* operon in which a single, full-length transcript is processed to produce two smaller mRNAs of approximately 2.7 kb and 0.5 kb in size [Owolabi and Rosen, 1990]. Conservation of the putative cleavage region in the *arsB* gene between the plasmid and the chromosomal *ars* operons could account for the processing and the sizes of the observed smaller mRNAs.

Figure 3. (A) Northern hybridization analysis of total cellular RNA of *E. coli* strain 40 using an [α-^{32}P]ATP-labelled DNA fragment containing the *arsRBC* genes as a probe as described in Materials and Methods. Lane 1, unexposed; Lane 2, exposed to 0.8 μM sodium arsenite for 60 min. The arrowheads indicate the RNA bands that specifically hybridized to the probe. The numbers on the left side represent size markers (M, in kb) generated by hybridizing the RNA ladder (0.24-9.5 kb, Gibco BRL) with α-^{32}P-labelled Lambda DNA cleaved with *Hin*dIII. (B) Determination of the initiation site of the *ars* transcript by primer extension analysis. The primer and reaction were as described in Materials and Methods. Lane P: primer extended with reverse transcriptase using RNA from (0.8 μM) arsenite-induced (for 1 h) *E. coli* strain 40. Lanes C, T, A, G are the sequencing reactions using the same α-^{32}P-labelled oligonucleotide as a primer. The template was single-stranded DNA from pUC119 containing the 411 basepair *Nde*I-*Bst*BI fragment (*arsR*). The nucleotide sequence of the coding strand (complementary to the observed sequence) is shown on the right side. The arrowhead indicates the putative *ars* transcript initiation site. (C) *In vivo* expression of cloned *ars* genes using a modified chloramphenicol release assay, as described in Materials and Methods. Lane 1, host strain NM522 only; Lane 2, NM522 (pJC612); Lane 3, NM522 (pJC701); Lane 4, NM522 (pKK223-3). The numbers on the left indicate size markers (M, kD). The ArsR, B, C protein products are indicated by the arrowheads on the right side of the figure.


A
M 1 2
9.5
7.5
4.4
2.4
1.4
0.24
B
C T A G P
A C A C T A C C T G C A A C A A T C A G G A G C G C A A T A T G T C A
arsR
C
M 1 2 3 4
43
29
18.4
14.3
B
C
R

The above results suggest that the chromosomal *ars* operon is likely transcribed as a single mRNA from a unique promoter upstream of the *arsR* gene. To determine the initiation site of the *ars* transcript, a primer extension assay was performed on RNA isolated from arsenite (0.8 μM) exposed and unexposed *E. coli* strain 40. A 24-mer oligonucleotide, complementary to the *arsR* transcribed strand was used as a primer. The *ars* transcript was found to begin from an A-residue, 27 nucleotides upstream from the *arsR* initiation codon (Fig. 3B).

3.5.3 Identification of *ars* gene products

To determine if the putative *ars* ORFs can express polypeptides *in vivo*, the *arsR* and *arsBC* genes were cloned separately into the plasmid expression vector pKK223-3, resulting in plasmids pJC612 and pJC701, respectively, and expressed *in vivo* under the control of the IPTG-inducible P_{tac} promoter. The *arsR* gene in pJC612 was expressed *in vivo* (Fig. 3C, lane 2), producing a protein product with an observed molecular mass of 13.2 kD, corresponding to the predicted size of the ArsR protein. The *arsB* and *arsC* genes, cloned in pJC701, were found to produce polypeptides with a molecular mass of 15.8 kD for ArsC, and 36 kD for ArsB (Fig. 3C, lane 3). The observed molecular mass of the ArsB protein on SDS-PAGE is similar to that observed with the plasmid-encoded ArsB protein, which is also smaller than its predicted size of 45 kDa [San Francisco et al., 1989].

3.6 DISCUSSION

A chromosomally-located *ars* operon in *E. coli* was found to contain three ORFs that are homologous to plasmid-encoded *arsR*, *arsB*, and *arsC* genes and confer resistance to arsenate, arsenite, and antimonite oxyanions [Carlin et al., 1995; Diorio et al., 1995]. Moreover, this operon was also found to be conserved in the chromosomes of many Gram-negative bacterial species [Diorio et al., 1995], implying a functional importance of this operon during evolution. The plasmid-borne *E. coli* R773 *ars* operon is negatively regulated by two *trans*-acting repressors. The ArsR protein is a metalloregulated repressor, acting by binding to an operator sequence in the *ars* regulatory region [Wu and Rosen, 1993a; Shi et al., 1994]. The

second negative regulator of the R773 *ars* operon, ArsD, is an inducer-independent repressor that controls the upper level of expression of the operon by an unknown mechanism [Wu and Rosen, 1993b]. Since a close relatedness exists between the R773-encoded and the chromosomal *ars* operons, as indicated by their strong homology at both the DNA and protein levels [Diorio et al., 1995], it is reasonable to postulate that the chromosomal *ars* operon may be regulated by the *arsR* gene product in a similar manner to that of the R773-encoded ArsR protein.

To address the question of the regulation of expression of the chromosomal *ars* operon, *arsR*::*luxAB* and *arsB*::*luxAB* transcriptional fusions were constructed. The *arsB*::*luxAB* fusion strains showed arsenic-inducible expression in a concentration-dependent manner in all *E. coli* strains tested, consistent with that observed in an *arsB*::*lacZ* fusion strain [Diorio et al., 1995]. These results also suggest that neither a reporter gene-specific nor a strain-specific effect is responsible for the observed patterns of *ars* gene fusion expression. In addition, expression of the chromosomal *ars* operon has been previously shown to be induced by arsenate and antimonite ions [Diorio et al., 1995]. However, unlike the R773 *ars* operon, which confers moderate levels of resistance to tellurite [Turner et al., 1992], the chromosomal *ars* operon was not found to confer increased resistance to tellurite when cloned on a high copy number plasmid, nor was its expression induced by this oxyanion (data not shown). While expression of the *arsB* gene is induced by sublethal doses of arsenite in the presence of a functional *arsR* gene product, the role of the *arsR* gene product can be deduced from its strong homology with the ArsR protein encoded by the R773 *ars* operon, and examined using the *arsR*::*luxAB* fusion strains. That arsenic-inducible expression of the chromosomal *ars* operon is controlled by the product of the first gene (*arsR*) in the operon is supported by three lines of evidence. First, constitutive expression of the operon was observed when the *arsR* gene was disrupted by a reporter gene insertion (Fig. 1B), although a slight decrease inf luminescence was observed with time. This effect may be due to the fact that as cells grow older, their cellular metabolic rate decreases, thus decreasing the cellular uptake of oxygen required for the luminescence reaction [Meighen, 1991]. This observation also reinforces the notion that a strict quantitative concordance between luciferase activity and gene expression is not always observed [Guzzo et al., 1992; Forsberg et al., 1994]. Second, in the presence of a functional

arsR gene provided in *trans* (pJC612), inducible expression of the *arsR::lacAB* fusion was restored (Fig. 2B). Introduction of the vector plasmid under the same conditions did not affect the constitutive expression of the *arsR::lacAB* gene fusion (data not shown). Third, the dosage of the *arsR* gene affects the level of expression of the operon. Thus, when the *arsR* gene is cloned in a high copy number plasmid (pJC612), both the basal and the induced levels of expression of the *arsB::lacAB* fusion are lower (Fig. 2A) compared with those observed when *arsR* is present in single copy on the chromosome (Fig. 1A). However, overproduction of the *arsR* gene product, by addition of high levels of IPTG, with or without the addition of sublethal concentrations of sodium arsenite, markedly inhibited cell growth in both LF20012R and LF20013R (data not shown). A similar inhibitory effect on cellular growth was also observed when cells containing pJC701 (containing *arsBC* under the control of the P_{tac} promoter) were induced by IPTG (data not shown). Thus, overproduction of the chromosomal *ars* gene products appears to be toxic to the cells, though the reason for this effect is not known at the current time. In addition, expression of the chromosomal *ars* operon was shown to be repressed by the R773 *arsR* gene product (Fig. 2C), suggesting that the insertional mutation of the chromosomal *arsR* gene can be functionally complemented by the plasmid R773 *arsR* gene product. Therefore, the chromosomal *arsR* gene product most likely encodes an arsenic-inducible repressor, in agreement with the function of the homologous plasmid-encoded *arsR* gene product [Wu and Rosen, 1991].

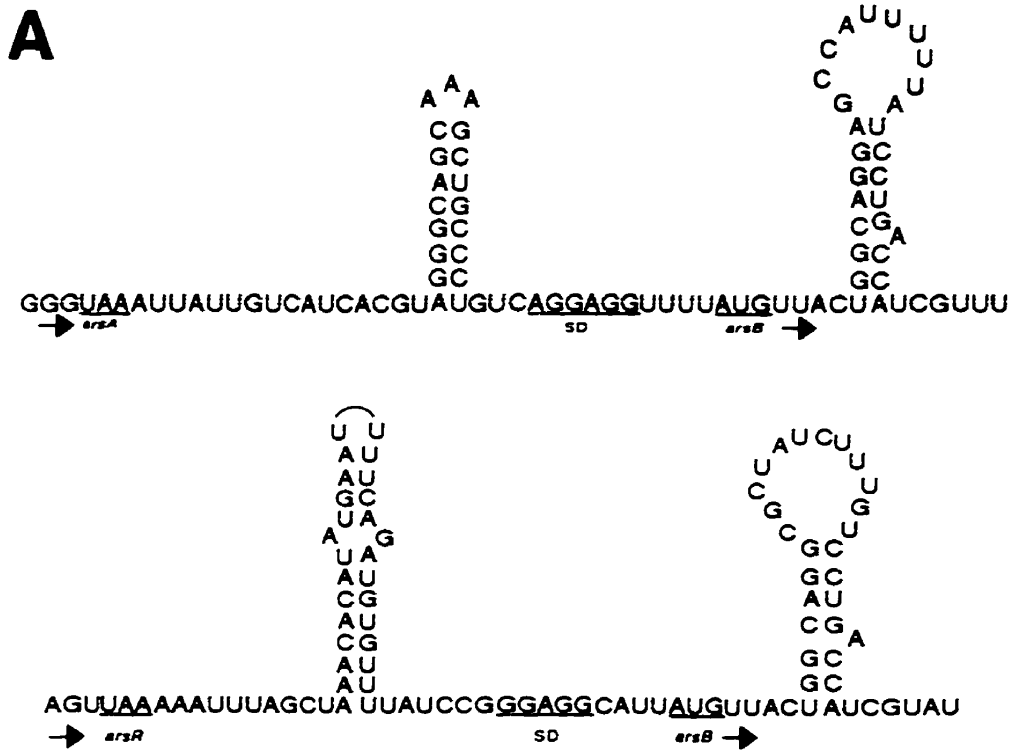
Although the third gene, *arsC*, of the *E. coli* chromosomal *ars* operon was not directly assayed in this study, it is likely that it is cotranscribed with the *arsR* and *arsB* genes as a single transcription unit, as evidenced by the Northern blotting analysis. Moreover, Western blotting analysis, using antiserum against the plasmid-borne R773 ArsC protein [Carlin et al., 1995], showed an increase in chromosomal ArsC protein production in *E. coli* exposed to arsenite ions.

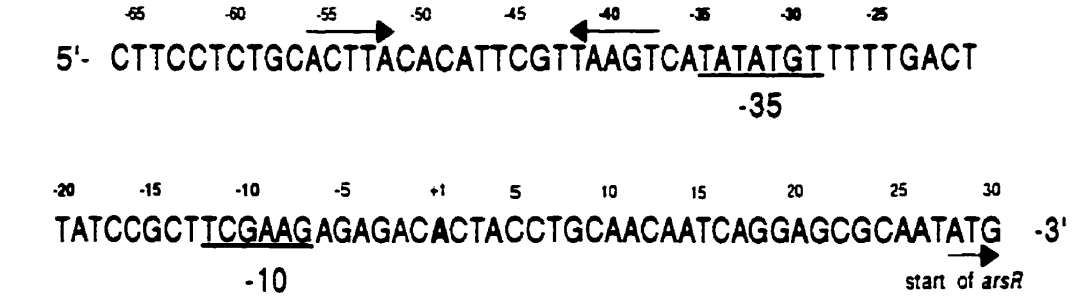
Arsenic-induced expression from a unique *ars* promoter was demonstrated by Northern blotting analysis in which a full-length transcript of the *ars* operon, as well as processed mRNA products, were observed only in the arsenite-induced sample (Fig. 3A, lane 2). The observed increase in *ars*-specific transcripts suggests that regulation of expression of the *ars* operon by arsenic occurs mainly at the transcriptional level, and that transcription of the

operon, plus processing, is similar to that observed for the plasmid R773 *ars* operon, in which processing of the mRNA was believed to occur in a region within the *arsB* coding sequence preceeded by two potentially stable hairpin structures separated by 20 nt. One of the hairpin structures resides in the intergenic region between *arsA* and *arsB*, with an 8-bp perfectly-matched stem and a 3 nt loop. The other resides in the begining of the *arsB* coding sequence, containing an 8 bp stem with 1 nt unmatched and a 10-nt loop [Owolabi and Rosen, 1990]. It is interesting to note that two similar putative hairpin structures, separated by 22 nt, are observed in the chromosomal *ars* operon. The upstream hairpin, containing a 12-bp stem with one pair unmatched nucleotides and a 2 nt loop, is located in the intergenic region between *arsR* and *arsB*. The downstream one is at precisely the same location as that in the R773 *arsB* gene, with 7 bp and 1 nt unmatched in the stem and 12 nt in the loop (Fig. 4A). Albeit the presence of these slight diferences, a similar mechanism, which is perhaps structure-dependent, may occur in the processing of the chromosomal *ars* transcript.

The transcription initiation site of the chromosomal *ars* operon, as determined by primer extension analysis, was found to be located 27 nt upstream of the *arsR* initiation codon (Fig. 3B), 10 to 11 nt further upstream than that observed in the plasmid R773 *ars* operon [San Francisco et al., 1990]. The operator sequence of the plasmid R773 *ars* operon is located from -64 to -40 with respect to the transcriptional initiation site, in a region of imperfect dyad symmetry, and believed to be bound by an ArsR dimer [Wu and Rosen, 1993a]. Close examination of the DNA sequences of the chromosomal *ars* operon reveals a perfect inverted repeat of the sequence 5'-ACTTA-3', separated by 9 bp, immediately upstream of the putative -35 region (Fig. 4B). This potential *arsR* operator sequence is conserved as an inverted repeat of 5'- NCNTA-3' (from bp -59 to -64), separated by 12 bp, in the plasmid R773 *ars* operon, and suggests that the R773 ArsR protein may regulate chromosomal *ars* operon expression through action at this potential operator site. Moreover, the fact that both the putative metal binding motif ELCVCDL and the critical His-50 residue in the putative DNA-binding helix-turn-helix motif identified in the R773-encoded ArsR protein [Shi et al., 1994] are also conserved in the chromosomal *ars* homolog, implies similar DNA- and metalloid-binding properties of the chromosomal *arsR* gene product. However, since the sequences of both operons are not fully identical in the putative operator regions, it is possible that only a few

Figure 4. (A) Comparison of potential secondary structures of the *E. coli* chromosomal (bottom) and plasmid R773 (top) *ars* transcripts in the region spanning the translational termination region of the *arsR* (for chromosomal *ars*) or *arsA* (for R773 *ars*) gene and the initiation region of the *arsB* gene. The orientation of the *ars* genes are indicated by arrows. The UAA translational termination codon of the *arsR* and *arsA* genes, the AUG initiation codon of the *arsB* genes, and the putative ribosome biding site (SD) for the *arsB* genes are underlined. (B) Sequence of the putative operator/promoter region of the *E. coli* chromosomal *ars* operon. The transciption initiation site is indicated in bold face. The numbers on the top of the sequence indicate the position of the nucleotides with respect to the transcription initiation site, which is numbered as +1. The putative -35 and -10 regions are underlined. The inverted repeats which may represent the ArsR binding sites are indicated by arrows on the top of the sequence. The translation initiation site of the *arsR* gene and its orientation are shown by an arrow underneath the sequence.


A
GGGUAAAUUAUUGUCAUCACGUAUGUCAGGAGGUUUUAUGUUACUAUCGUUU
arsA
SD
arsB
AGUUAAAAAUUUAGCUA UUAUCCGGGAGGCAUUAUGUUACU AUCGUAU
arsR
SD
arsB


B
5'- CTTCCTCTGCACTTACACATTCGTTAAGTCATATATGTTTTTGACT
-35
TATCCGCTTCGAAGAGAGACACTACCTGCAACAATCAGGAGCGCAATATG -3'
-10
start of arsR

base pairs in this region are critical for the ArsR protein to bind. The result shown in Fig. 2C demonstrates that the R773 *arsR* gene product can replace the chromosomal ArsR protein in controlling the inducible expression of the chromosomal *ars* operon, reinforcing the similarity of the operators of both *ars* operons. The observed differences in the basal level and the patterns of reporter gene expression between Fig. 2B and Fig. 2C were reproducible and probably caused by a variety of reasons, one of which could be a lower affinity of the plasmid-based ArsR protein for the chromosomal *ars* operator sequence. Secondly, arsenic-inducing conditions not only allow derepression of the chromosomal *arsR*::*luxAB* gene fusion, leading to an immediate increase in the expression of the *luxAB* reporter gene upon cellular exposure to arsenite, but also elevate the level of expression of the R773 *arsR* gene that is present in multicopy in the plasmid pJC613, which, in turn, acts to repress the expression of the single copy chromosomal *arsR*::*luxAB* fusion in strain LF20013R', resulting in a decrease in luminescence with time (Fig. 2C). It is also interesting to note that both the putative -35 region (TATATG) and -10 region (TCGAAG) of the chromosomal *ars* operon (Fig. 4B) are less homologous than those of the R773 *ars* operon (TTGACTT for the -35 and GATACTT for the -10) [San Francisco et al., 1990] to the *E. coli* consensus promoter sequences (TTGACA for -35 and TATAAT for -10) [Hawley and McClure, 1983; Harley and Reynolds, 1987] recognized by the σ^{70} factor of *E. coli* RNA polymerase. It has been proposed that promoter strength is governed by two factors. One is the binding affinity of promoter DNA to RNA polymerase, involving the -35 region, and the other is the rate of open complex formation, involving the -10 region [Gilbert, 1976]. Studies have shown that promoters containing sequences close to the consensus have high values for both parameters [Kajitani and Ishihama, 1983a; 1983b]. Kobayashi et al. (1990) studied the strength of a series of *E.coli lac* UV5 promoters by base substitution within the -35 region. They found that promoters with the consensus sequence (TTGACA) of the -35 region exibited highest values for promoter strength and base substitutions at either nucleotide position -34 (a G residue) or -32 (a C residue) greatly reduced the strength of the promoters. The putative -35 promoter sequence of the chromosomal *ars* operon is poorly homologous to the consensus sequence (2/6 versus 6/6), having neither G_{-34} nor C_{-32} in its -35 region, whereas that of the R773 *ars* operon is much

closer (5/6 versus 6/6). Moreover, the -10 region of the chomsomal *ars* operon is less homologous to the consensus sequence than that of the R773 *ars* operon (3/6 versus 4/6), suggesting that the putative promoter of the chromosomal *ars* operon is weaker than that of the R773 *ars* operon. The plasmid R773 *ars* operon is subjected to both basal and upper levels of transcriptional contol by the *arsR* and *arsD* gene products, while the chromosomal *ars* operon lacks the homolog of the *arsD* gene. Therefore, we can hypothesize that a weaker promoter may have evolved to control the upper level of expression of the chromosomal *ars* operon, since overexpression of the *ars* genes was shown to be toxic to the cells.

CHAPTER 4

USE OF A LUMINESCENT BACTERIAL BIOSENSOR FOR BIOMONITORING AND CHARACTERIZATION OF ARSENIC TOXICITY IN CHROMATED COPPER ARSENATE (CCA)

4.1 PREFACE

In Chapters 2 and 3, we described the identification and characterization of bacterial genes inducible by arsenic compounds. An important strategy, proven to be very useful in such studies, is the construction of transcriptional gene fusions that allow direct monitoring expression of the gene of interest. Gene fusion strains, such as *arsB*::*lacZ*, *arsB*::*luxAB*, and *arsR*::*luxAB* have played an essential role in elucidating the regulation of expression of the *E. coli* chromosomal *ars* operon. Through these studies, we found that sodium arsenite, which is known to be more toxic than sodium arsenate, induced expression of the *arsB*::*lacZ* gene fusion at lower concentrations, while the much less toxic cacodylic acid did not induce expression of the gene fusion even at very high concentrations (Chapter 2, Figure 3), suggesting that the *arsB*::*lacZ* or *arsB*::*luxAB* gene fusion strain may serve as a biological indicator for the toxicity of various arsenic compounds. Chapter 4 describes an investigation of the *arsB*::*luxAB* gene fusion strain as a potential biosensor in monitoring the levels and toxicity of arsenic in a commonly used arsenic-containing wood preservative, chromated copper arsenate (CCA).

4.2 ABSTRACT

An arsenic oxyanion-inducible *Escherichia coli* chromosomal operon (*arsRBC*) has been previously identified. Construction of a luciferase transcriptional gene fusion (*arsB::luxAB*) showed that *ars* operon expression, plus concomitant cell luminescence, was inducible in a concentration-dependent manner by arsenic salts. The present study was conducted to evaluate the potential of the *arsB::luxAB* transcriptional gene fusion for use as a biosensor in monitoring the toxicity of arsenic compounds. Cultures from this gene fusion strain were exposed to increasing concentrations of the wood preservative chromated copper arsenate (CCA), as well as its constituents, sodium arsenate and chromated copper solution (CC). Analysis of luciferase activity revealed that the *arsB::luxAB* gene fusion was expressed in response to CCA and sodium arsenate, but not to the CC solution. The detection limit of arsenic was found to be 0.01 μg As/ml (10 parts per billion, 10 ppb) and therefore well within the range of environmental concerns. A greater induction of luminescence by arsenate was observed when cells were limited for phosphate, as phosphate can act as a competitive inhibitor of arsenate ions. Our results suggest that the *E. coli arsB::luxAB* fusion strain has a promising future as a specific and sensitive biosensor for monitoring bioavailable levels and toxicity of arsenic near sites where CCA-treated wood has been used.

4.3 INTRODUCTION

The increase of arsenic (As) concentration on the earth's surface is due to both natural sources, such as volcanic activity and weathering processes, as well as anthropogenic sources, such as mining activities, agricultural and forestry applications. One example of anthropogenic arsenic contamination in the environment is the use of arsenic-containing wood preservatives. The most extensively used wood preservative is chromated copper arsenate (CCA), which is pressurized into the wood through a process called "Wolmanizing" [Weis and Weis, 1994]. Wood intended for marine uses receives 24 to 40 kg CCA per cubic meter of wood to prevent its destruction by bacteria, fungi, and insects. Each of the three chemicals in CCA is known to be toxic to aquatic biota at

concentrations above trace levels, and found to be leached from the treated wood in both fresh and sea water [Warner and Solomon, 1990; Weis et al., 1991; Weis and Weis, 1992b]. Chemicals leached from CCA-treated wood can affect organisms that grow on the wood itself, those that live adjacent to the CCA-treated bulkheads, and also be adsorbed onto sediments, where they can be slowly released or taken up by benthic organisms [Weis and Weis, 1992a, b; 1995; Weis et al., 1993]. The rate of metal accumulation in sediments and in benthos differs with each chemical in the order: Cu > As > Cr, and decreases with distance and time [Weis and Weis, 1994; 1996]. Benthic organisms living near CCA-treated bulkheads were found to contain elevated levels of Cu and As. The number of individuals, as well as the species diversity, were also decreased at sites adjacent to CCA-treated bulkheads [Weis and Weis, 1994]. Pathologic and genotoxic effects have also been observed in oysters (*Crassostrea virginica*) living on CCA-treated wood [Weis et al., 1995]. In addition, CCA was shown to affect the growth of PCP-degrading bacterial species and their ability to degrade PCP [Wall and Stratton, 1995; 1994]. Thus, monitoring of bioavailable amounts of CCA released by the treated wood is important in order to detect and rectify its toxic effects. Among the three chemical constituents of CCA, arsenic is the most abundant in the environment, and known to have carcinogenic and teratogenic effects on humans upon chronic exposure [Morton and Dunnette, 1994; Hartwig, 1995].

Arsenic belongs to the VA group in the periodic table. It can exist in +5, +3, 0, and -3 oxidation states in nature. The arsenic component in CCA is arsenate, the pentavalent inorganic form that is chemically similar to phosphate. The toxicity of arsenical compounds depends on their bioavailability, oxidation states, and organometalloidal forms [for review, see Tamaki and Frankenberger, 1992]. For the last few decades, numerous analytical methods, including gas chromatography (GC) with flame ionization, reverse phase and high performance liquid chromatography (HPLC), mass spectrometry, X-ray fluorescence, and flame atomic absorption spectrophotometry (AAS), have been developed to detect the concentration of different forms, as well as the total amount, of arsenic in environmental samples. These methods are highly sophisticated and sensitive,

but may require extensive sample pretreatment and high costs. In many cases, the samples have to be converted into other forms (e.g. arsine) in order to be detected by these analytical procedures. Moreover, the bioavailability of the original arsenic species present in a sample, and their potential toxic effects on biota, may not be reflected by these analyses. In this regard, effective means of detecting bioavailable concentrations of arsenic, and its effects on living cells, need to be fully elaborated.

Our laboratory has previously identified, using gene fusion techniques, an *Escherichia coli* chromosomal operon (*ars*), whose expression is induced in the presence of inorganic arsenic and antimony oxyanions [Diorio et al., 1995]. This As/Sb-inducible operon contains three ORFs, *arsR*, *arsB*, and *arsC*, which encode a *trans*-acting repressor (*arsR*) that negatively regulates the expression of the operon [Xu et al., 1996; Cai and DuBow, 1996], a membrane-based pump (*arsB*), which specifically mediates extrusion of arsenite and antimonite anions, and an arsenate reductase (*arsC*). The chromosomal *ars* operon confers moderate levels of resistance to toxic forms of arsenicals and antimonials [Diorio et al., 1995; Carlin et al., 1995]. The *arsRBC* genes of the chromosomal *ars* operon are highly homologous to an *E. coli* plasmid (R773)-encoded *ars* operon, which contains five ORFs in the order *arsRDABC* and encodes an arsenite/antimonite-stimulated ATP-driven efflux pump [Kaur and Rosen, 1992a; Wu and Rosen, 1993a]. Further studies have been conducted to elucidate the regulation of chromosomal *ars* operon expression [Xu et al., 1996; Cai and DuBow, 1996].

The aim of the present study is to examine the effects of CCA and its constituent compounds on bacterial cell growth and expression of the *ars* operon. Moreover, we explore the potential of an *E. coli* chromosomal *arsB::luxAB* gene fusion as a biosensor [Karube and Suzaki, 1990; Danilov and Ismailov, 1989] in detecting biologically important concentrations of arsenic in CCA.

4.4 MATERIALS AND METHODS

4.4.1 Bacterial strains and growth media

The bacterial strains used in this study have been described previously [Cai and DuBow, 1996]. *E. coli* 40 is the strain in which we isolated and sequenced the chromosomal *ars* operon [Diorio et al., 1995]. *E. coli* strain LF20012 is a chromosomal *arsB::luxAB* Tc^r transcriptional fusion strain (and thus *ars*$^-$) derived from *E. coli* 40 [Cai and DuBow, 1996]. All assays were performed in either Luria-Bertani (LB) [Miller, 1972] or LB broth reduced in phosphate, called dephosphorylated LB broth [Bukhari and Ljungquist, 1977], to compare the effects of phosphate on arsenate toxicity. Tetracycline (10 μg/ml, final concentration) was routinely added to cultures of LF20012.

4.4.2 Chemicals

Chromated copper arsenate was purchased from Chemical Specialties Inc. (CSI, Harrisberg, NC), and is a mixture of arsenate (19% as As_2O_5, w/w), chromate (23.5% as CrO_3, w/w), and cupric oxide (9.25% as CuO, w/w). The ratio of As:Cr:Cu in this commercial formula is 13.11 : 14.82 : 9.11. Sodium arsenate ($Na_3AsO_4 \cdot 7H_2O$), chromium oxide (CrO_3) and cupric oxide (CuO) were purchased from American Chemicals Ltd. (A&C, Montréal, QC). A stock solution, containing identical concentrations of chromium oxide (23.5%, w/w) and cupric oxide (9.25%, w/w) as those in CCA, was prepared and designated CC solution. All other chemicals were obtained from commercial sources.

4.4.3 Luciferase assays

Cultures of *E. coli* LF20012 were grown overnight in LB, or dephosphorylated LB, broth containing tetracycline in a 37°C air shaking incubator. The overnight culture was diluted 100-fold in the same medium and growth continued until mid-log phase (A_{600}= 0.3-0.4). The cells were then exposed to increasing concentrations of sodium arsenate, CCA, or CC, respectively, by adding increasing amounts of the stock solutions of each chemical to the culture, and growth was continued for 2 h. Aliquots of these cultures were removed every 30 min for luciferase activity assays. Samples were diluted in LB broth to a

final A_{600} of 0.05. One milliliter (1 ml) of diluted bacterial culture (in triplicate) was placed in a 4 ml cuvette, which was then inserted into a luminometer (Tropix Optocomp I, MGM Instruments, Hamden, Connecticut). Luminescence measurements were initiated by injection of 100 µl of a dodecanal stock solution (1:100 dilution in LB, mixed by vigorous shaking). Total relative light units (RLU) were recorded for a 10 sec interval. The average RLU of each triplicate, as well as their standard deviations, were calculated by the automated program of the luminometer. Luciferase activity was expressed as the number of photons emitted per second per A_{600} unit.

4.4.4 Sensitivity assays

Bacterial cells were grown at 37°C overnight in LB or dephosphorylated LB, broth containing tetracycline when required. The overnight cultures were diluted 100-fold in the same medium containing increasing concentrations of the chemicals of interest. The cultures were incubated in a shaking incubator for 6 h at 37°C. The optical density at 600 nm of each sample (in triplicate) was recorded. The average value of the A_{600} of each culture obtained in the absence of added chemicals after 6 h incubation was used as a reference point, and set at 100%. The percent growth of each culture in the presence of added chemicals at various concentrations was calculated by dividing the average value of the observed A_{600} after 6 h incubation by that of the reference culture. The effects of the chemicals on cell growth was expressed as the percent growth versus arsenic concentration. In the case of CC, the concentration was equivalent to that used for the experiments with CCA. The LD_{50} values of each sample were defined as the elemental concentrations of arsenic in each chemical (except in CC, as designated above) that caused a 50% reduction in cell growth, and obtained according to the above growth curves.

4.5 RESULTS

4.5.1 Expression of the *arsB::luxAB* gene fusion is specific for arsenic in CCA

To evaluate the effects of CCA on the expression of the *ars* operon, and the potential of the *arsB::luxAB* gene fusion for use as a biosensor in detecting bioavailable

levels of arsenic compounds, bacterial cells containing this gene fusion (LF20012) were exposed to sublethal levels of CCA. Luciferase activity was analyzed as described in Materials and Methods. Two other compounds, sodium arsenate, which is the form of arsenic in CCA, and the CC solution, which contains identical concentrations of chromate and cupric oxide as in CCA, but lacks arsenic, were also examined. The results showed that the *arsB::luxAB* gene fusion can be induced by CCA in both LB (Fig. 1D) and dephosphorylated LB broth (Fig. 1A), but not by CC (Fig. 1C and F), indicating that expression of the *arsB::luxAB* gene fusion is specific for the arsenic oxyanion of CCA. These results were supported by the inducible expression of cell luminescence by sodium arsenate (Fig. 1B and E). Expression of luciferase is concentration-dependent within the sublethal concentrations tested, with an assay time of 60 min or less, and can be detected at arsenic concentrations as low as 0.01 µg As/ml (10 parts per billion, 10 ppb). Optimum levels of luciferase activity were achieved at 60 min post-exposure to 0.1 to 1.0 µg/ml arsenic in CCA or sodium arsenate, with a maximum induction of 50- to 90-fold, depending on the experimental conditions used (Fig. 1A, B, D, and E). A decrease in the level of luminescence was observed after 60 min when cells were exposed to CCA in dephosphorylated LB but not in LB (see Discussion). Arsenic concentrations greater than 1.0 µg As/ml caused a reduction of luminescence (data not shown) presumably due to the toxic effects of arsenic on cell physiology and luciferase cofactor levels [Meighen, 1991; Guzzo et al., 1992].

4.5.2 Effects of CCA on bacterial growth

The effect of CCA on bacterial growth was evaluated as described in Materials and Methods. The *arsB::luxAB* fusion strain (*ars*$^-$) was found to be more sensitive to arsenic than the wild type strain (Fig. 2A, B and E), consistent with previous studies of *E. coli* strains in which the *ars* operon is no longer functional [Diorio et al., 1995; Carlin et al., 1995]. The toxicity of CCA or sodium arsenate was higher in dephosphorylated LB than in LB medium, as revealed by the LD_{50} values shown in Table 1. However, when cultures

Figure 1. Luminescence of the *arsB::luxAB* fusion strain LF20012 in the presence of CCA, arsenate, and CC. Cells growing at mid-log phase in dephosphorylated LB (A-C) or regular LB (D-F) were exposed to increasing concentrations of arsenic in CCA (A and D), sodium arsenate (B and E), as well as CC solution (C and F), whose concentration was equivalent to that of CCA. Samples were removed every 30 min. Luciferase activity was measured (in triplicate) as described in Materials and Methods. The standard deviations are shown by error bars. ●, 0 μg/ml; ▲, 0.01 μg/ml; ★, 0.1 μg/ml; ■, 0.5 μg/ml; ▼, 1.0 μg/ml.

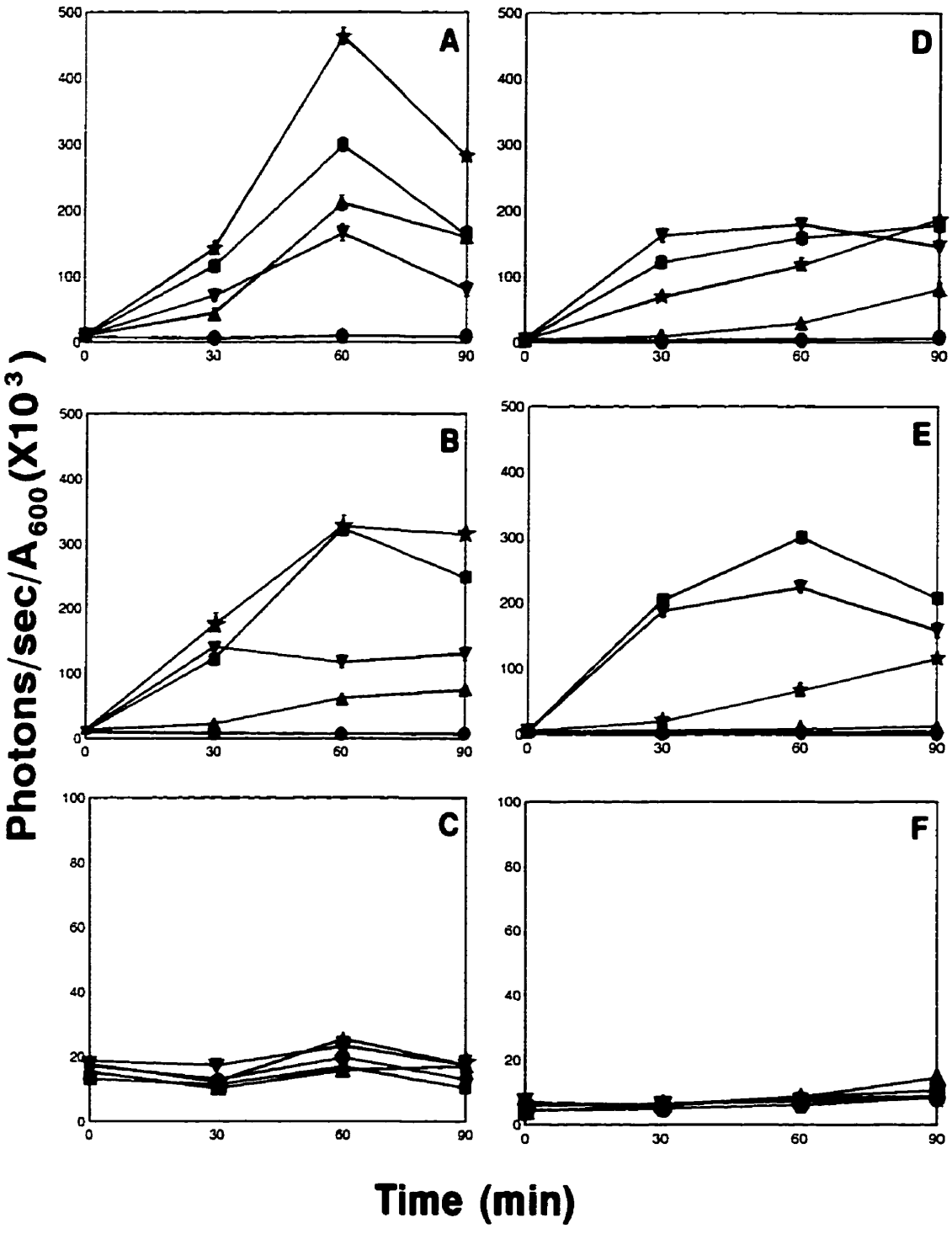

A
B
C
D
E
F
Photons/sec/A600 (X10³)
Time (min)
0
30
60
90
100
200
300
400
500
20
40
60
80

Figure 2. Growth inhibition in the presence of CCA, arsenate, and CC. Cells were grown at 37°C in a shaking air incubator in the presence of increasing concentrations of CCA (A and D), sodium arsenate (B and E), and CC (C and F) in either dephosphorylated LB (A-C) or regular LB (D-F). After 6 h, the absorbance at 600 nm (A_{600}) of each sample (in triplicate) was recorded. The average percent of growth (compared with no added chemical) of each sample was calculated as described in Materials and Methods, and plotted on the Y axes. The standard deviations are shown by error bars. The concentrations indicated on the X axes are the elemental concentrations of arsenic (μg/ml), except in the case of CC, whose concentration is equivalent to that used for the experiments with CCA. ★, LF20012 (*ars*$^-$); ■, 40 (*ars*$^+$).

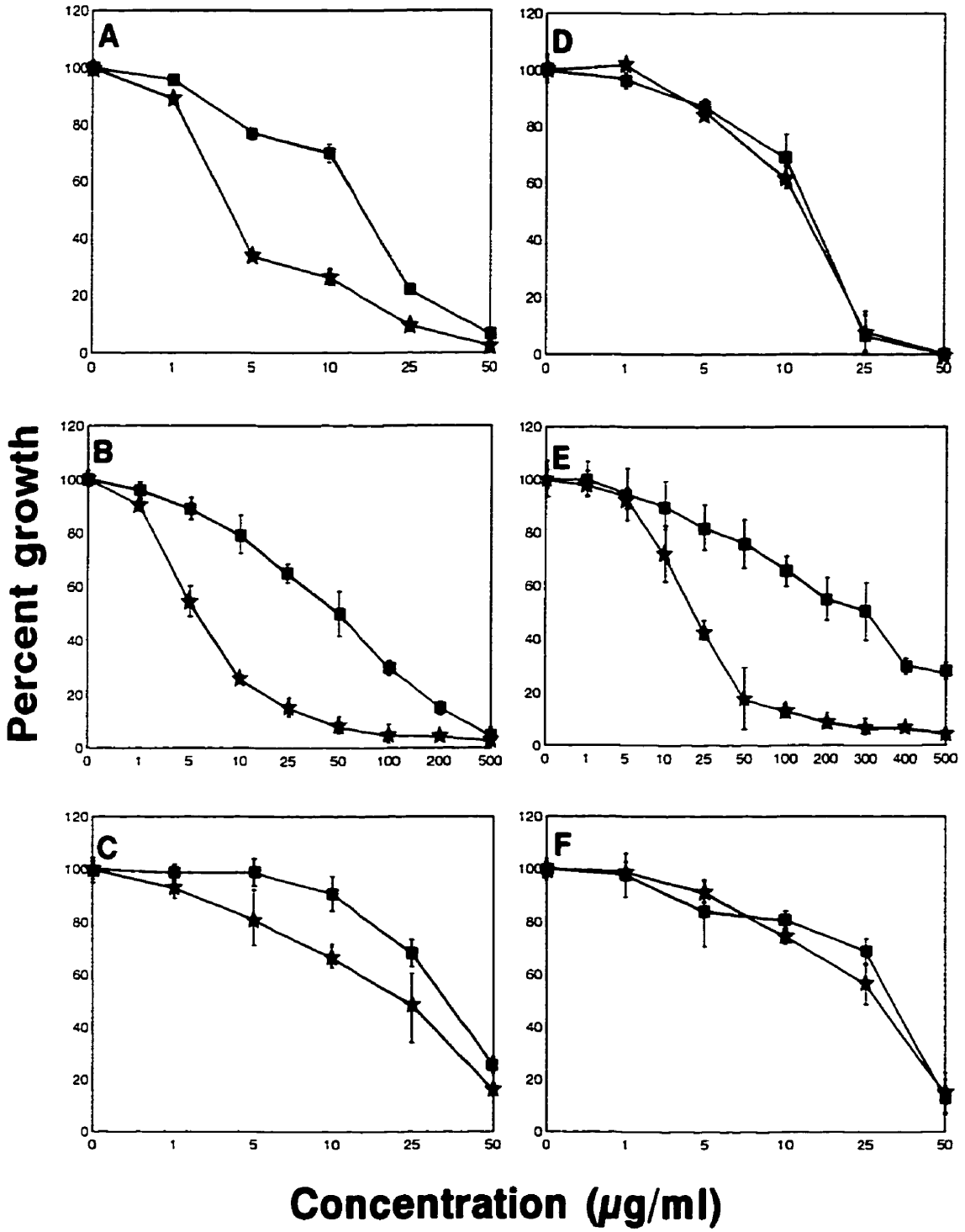

A
B
C
D
E
F
Percent growth
Concentration (µg/ml)

Table 1. LD_{50} (μg As/ml) of chemicals to *E. coli* 40 (*ars*$^+$) and *E. coli* LF20012 (*ars*$^-$).

Table 1.

LD_{50} (μg As/ml)[a] of chemicals to *E. coli* 40 (*ars*$^+$) and *E. coli* LF20012 (*ars*$^-$)

chemical	CCA		Na arsenate		CC[b]	
E. coli strain \ culture medium	de-P LB[c]	LB	de-P LB	LB	de-P LB	LB
40 (*ars*$^+$)	17	14.8	50	300	30	35
LF20012 (*ars*$^-$)	3.9	13.4	5.8	20.8	23.5	30

a. Calculated as described in Materials and methods.

b. The concentrations of chromate and cupric oxide in CC are equivalent to those in CCA.

c. Dephosphorylated LB.

were exposed to CCA in LB broth, there was no significant difference in LD_{50} values between the *arsB::luxAB* fusion strain and the wild type strain at the concentrations tested (Fig. 2D and Table 1). This result may be due to the predominant toxic effects of chromium and copper, over those of arsenic, when phosphate is abundant, and is supported by the observation that wild type cells were more sensitive to CC than to arsenate (Table 1). Nonetheless, greater sensitivity of both strains was observed in media containing CCA than those containing CC only, suggesting a synergistic toxic effect of the three constituents of CCA on the cells.

4.6 DISCUSSION

In the present study, an *E. coli arsB::luxAB* luciferase gene fusion strain (LF20012) was shown to increase luminescence in the presence of increasing concentrations of the commonly used, arsenic-containing wood preservative, CCA, as well as to sodium arsenate, but not to the other two components (chromate and cupric oxide) of CCA. It is interesting to note that the *arsB::luxAB* gene fusion was expressed, in general, to a higher level in dephosphorylated LB medium than in LB medium when the same amount of arsenic was present in the media (Fig. 1). These results may be due, in part, to the fact that arsenate is taken up by most organisms through phosphate transport systems. In *E. coli*, for example, there are two major inorganic phosphate transport systems, the low affinity Pit system and the high affinity Pst system [Willski and Malami, 1980a]. The Pit system has equal affinity for both phosphate and arsenate, while the Pst system has a higher affinity for phosphate than for arsenate [Willski and Malami, 1980b]. Therefore, the depletion of phosphate (in dephosphorylated LB broth) may favor increased arsenate uptake by the cells through both the Pit and Pst systems due to the decreased phosphate competition for arsenate uptake, leading to higher levels of *arsB::luxAB* gene fusion expression in response to arsenate. On the other hand, a general decrease in the level of luminescence was observed after 60 min of cellular exposure to CCA or arsenate. In particular, a greater decrease was seen after 60 min in dephosphorylated LB with CCA than in LB medium. This phenomenon could be

attributed to several factors. First, cells begin to enter stationary phase at the time between 60 to 90 min under these conditions, with concomitantly slower metabolic rates and reduced oxygen uptake, production of luciferase enzyme and its substrate ($FMNH_2$), all of which can lead to a decrease in luminescence in general (see Cai and DuBow, 1996, for example). Secondly, Blouin et al. [1996] suggest that formation of the luciferase-FMNHOOH complex is a primary determining factor for bioluminescence, whereas regeneration of $FMNH_2$, the substrate for the luciferase enzyme, depends on the level of $NAD(P)H+H^+$ and NADH-FMN oxidoreductase [Blouin et al., 1996]. Arsenate is known to uncouple oxidative phosphorylation by arsenolysis [Valee et al., 1960; Summers and Silver, 1978], which may affect the intracellular level of $NAD(P)H+H^+$ generated via the electron transport system, thus decreasing cellular levels of $FMNH_2$. Since the cells presumably contain a higher level of intracellular arsenate in phosphate depleted LB medium than in regular LB medium, this toxic effect may be more pronounced in dephosphorylated LB medium with time, leading to a more rapid decrease in luminescence in dephosphorylated LB.

The advantage of this bacterial gene fusion as an arsenic biosensor lies in its specificity, sensitivity, selectivity, and simplicity of operation. Our results have shown that this biosensor is specific for arsenic, and the detection limit can be as low as 0.01 μg/ml, which is within the federal drinking water standard (i.e. 0.05 μg As/ml). Secondly, bacterial cells are easy to maintain. It has been recently reported that a recombinant *E. coli* biosensor for organophosphorus neurotoxins can be stably maintained at 4°C for over 2 months [Rainina et al., 1996]. Our *arsB*::*luxAB* gene fusion strain has been found to be stable for at least 6 months when stored in 25% (v/v) glycerol at -20°C (data not shown). Moreover, bacterial cells can be easily and inexpensively propagated, luminescence assays are simple to perform, and results can be rapidly obtained after sampling. It is interesting to note that a non-linear dependence of luminescence versus the concentration of arsenic tested was observed in our system, suggesting a complex intracellular biochemistry of arsenic. This is supported by our previous observation that even an *ars*$^-$ mutant can grow in arsenite or arsenate concentrations above those that induce *ars* operon expression

[Diorio et al., 1995], suggesting that the *ars* operon is not the only system acting on toxic arsenic oxyanions. Nonetheless, the value of this gene fusion strain as a living bacterial biosensor cannot be underestimated. With proper control analyses, the use of living biosensor systems can enhance analytical chemistry in quantitatively evaluating not only the levels, but also the bioavailability and toxic effects, of arsenic in the environmental samples. Thus, the use of these luminescent gene fusion biosensors in detecting environmental arsenic contamination may hold promise in environmental assessment.

CHAPTER 5

IDENTIFICATION AND CHARACTERIZATION OF A CHROMOSOMAL *ARS* OPERON HOMOLOG IN *PSEUDOMONAS AERUGINOSA*

5.1 PREFACE

In Chapter 2, we described the identification of the *Escherichia coli* chromosomal *ars* operon and the detection of homologous sequences in the chromosomes of many other Gram-negative bacterial species, including the non-enteric bacterium *Pseudomonas aeruginosa*. This finding was controversial, as other laboratories [Carlin et al., 1995] stated that they could not find one. *P. aeruginosa* is a medically and industrially important bacterial species, and known to be widely present in the environment, thus being frequently exposed to various environmental toxicants. Therefore, it would be of great interest to elucidate potential arsenic detoxification mechanisms in this organism. This chapter describes the cloning and molecular characterization of a chromosomal *ars* operon homolog in *P. aeruginosa*, and characterization of its function in arsenic detoxification.

5.2 ABSTRACT

Operons encoding homologous arsenic resistance determinants (*ars*) have been found in bacterial plasmids isolated from Gram-positive and Gram-negative organisms, as well as in the *Escherichia coli* chromosome. However, there exists conflicting evidence as to the presence of this arsenic detoxification determinant in the medically and industrially important bacterial species *Pseudomonas aeruginosa*. We report here the identification of a *P. aeruginosa* chromosomal *ars* operon homolog via cloning and complementation of an *E. coli ars*⁻ mutant. The *P. aeruginosa* operon contains three potential open reading frames, whose encoded proteins share significant homology to those encoded by the *arsR*, *arsB*, and *arsC* genes of the *E. coli* chromosomal *ars* operon and several plasmid-based *ars* operons. The cloned *P. aeruginosa* chromosomal *ars* operon confers resistance to arsenite, arsenate and antimonite in an *E. coli arsB* mutant strain. Expression of the operon was shown to be induced by arsenite at the mRNA level. Homologous DNA sequences of this operon were detected in some, but not all species of the genus *Pseudomonas*.

5.3 INTRODUCTION

Plasmid-based arsenic resistance in bacteria, encoded by the *ars* operons, has been known for many years [For review, see Kaur and Rosen, 1992a]. The well studied *E. coli* plasmid R773-located *ars* operon contains five open reading frames (ORFs) in the order *arsRDABC*, and is transcribed as a single polycistronic mRNA [Owolabi and Rosen, 1990]. The protein products confer resistance to arsenic and antimony via an ATP-dependent efflux of the oxyanions [Mobley and Rosen, 1982]. Homologous genes, organized in the same fashion, have also been found in the IncN plasmid R46 *ars* operon [Bruhn et al., 1996]. In contrast, the Gram-positive *ars* operons, encoded by staphylococcal plasmids pI258 and pSX267, contain only three ORFs: *arsR*, *arsB*, and *arsC*, and lack the *arsA* and *arsD* coding sequences [Ji and Silver, 1992a; Rosenstein et al., 1992]. In the case of the R773 *ars* operon, the first two ORFs, *arsRD*, encode two *trans*-acting repressors that control the basal and upper levels of operon expression, respectively. ArsR is an arsenic-inducible repressor, whereas ArsD acts in an inducer-

independent manner [San Francisco et al., 1990; Wu and Rosen, 1991; 1993a]. The true inducers for expression of the *ars* operon are trivalent arsenic, antimony, and bismuth oxyanions, while the pentavalent arsenate needs to be reduced to arsenite in order to derepress the operon [Wu and Rosen, 1993b]. The last three genes, *arsABC*, encode the structural components of the arsenic pump [Chen et al., 1986b]. The ArsA protein is an arsenite- and antimonite-stimulated ATPase [Rosen et al., 1988] which forms a complex with the membrane-bound ArsB protein [Tisa and Rosen, 1990; Wu et al., 1992], and functions to actively export arsenite and antimonite ions upon hydrolysis of ATP [Kaur and Rosen, 1994a, b; Li et al., 1996]. The ArsC protein is a small cytosolic protein that acts to reduce arsenate to arsenite [Gladysheva et al., 1994; Oden et al., 1994], which can then be extruded by the ArsAB complex. The staphylococcal *ars* operons, though containing only two structural components, ArsBC, are also able to mediate the efflux of arsenicals and antimonials [Ji and Silver, 1992a; Rosenstein et al., 1992]. However, the energy source coupled to this process is the electrochemical proton gradient rather than ATP [Ji and Silver, 1992a; Rosenstein et al., 1992; Bröer et al., 1993]. The recently discovered *E. coli* chromosomal *ars* operon also contains three ORFs, *arsRBC*, structurally resembling the staphylococcal plasmid *ars* operons [Sofia et al., 1994; Diorio et al., 1995], and has been shown to be functional in arsenic detoxification [Carlin et al., 1995; Diorio et al., 1995]. The significant homology between the *E. coli* chromosomal *ars* operon and the plasmid-encoded *ars* operons [Diorio et al., 1995] suggests that the *ars* operons found in both plasmid and chromosomal locations may have arisen from a common ancestor. Since the *E. coli* chromosomal *ars* operon is the only chromosomally-located *ars* operon identified in bacteria so far, it is important to identify other possible chromosomal *ars* homologs in order to better understand the evolutionary relationship between these *ars* operons and, perhaps, other plasmid and chromosomal-based genes.

We have previously observed (via Southern blotting) that sequences homologous to the *E. coli* chromosomal *ars* operon were present in the chromosomes of many Gram-negative bacterial species, including the non-enteric bacterium, *P. aeruginosa* [Diorio et al., 1995]. Although it has been reported that plasmid pUM310 of *P. aeruginosa* contains a genetic determinant that confers resistance to arsenic and antimony ions [Cervantes and

Chàvez, 1992], the evidence for the presence of a chromosomally-located *ars* operon in the members of the genus *Pseudomonas* has been controversial [Carlin et al., 1995; Diorio et al., 1995]. The aim of this study is to identify and characterize an *ars* operon homolog in the chromosome of *P. aeruginosa*. Here we report the isolation, DNA sequencing, and *E. coli ars* operon complementation of a chromosomal *ars* operon of *P. aeruginosa*. It was found to consist of three ORFs, whose predicted protein products are homologous to those encoded by the *arsR*, *arsB*, and *arsC* genes of other known *ars* operons. The *P. aeruginosa ars* operon is functional in arsenic detoxification, as determined by complementation of an *E. coli arsB* mutant. Moreover, expression of this operon, as measured by RNA dot blots, is induced by sodium arsenite. Sequence analyses revealed that the *P. aeruginosa* chromosomal *ars* operon is evolutionarily related to plasmid-based *ars* operons from both Gram-negative and Gram-positive origins, and is conserved in the chromosomes of some, but not all, *Pseudomonas* species.

5.4 MATERIALS AND METHODS

5.4.1 Bacterial strains and plasmids

E. coli strain 40 (F⁻, Δ*pro-lac*, *rps*L, *trp*) is the strain in which we isolated and sequenced the chromosomal *ars* operon [Diorio et al., 1995]. *E. coli* strain LF20012 is derived from *E. coli* strain 40, but contains an *arsB*::*lacAB* chromosomal transcriptional gene fusion [Cai and DuBow, 1996], and is thus genotypically *arsB*⁻ and hypersensitive to arsenic oxyanions [Cai and DuBow, 1997]. *P. aeruginosa* strains PAO1 and PAK, *P. fluorescens*, *P. stutzeri*, *P. diminuta*, and *Burkholderia cepacia* are all plasmid-free wild type strains obtained from Dr. George Hegeman (Indiana University). *E. coli* cultures were grown in LB medium [Miller, 1972] at 37°C. Cultures from other strains were grown initially in BHI (Brain-Heart Infusion, Difco) broth and then in LB broth at 32°C. Plasmid pJC801 (Ap^r) is a pBR322 derivative that contains the cloned *P. aeruginosa* chromosomal *ars* operon as a 3.4-kb *Eco*RV fragment (described in the following sections). Plasmids pUC119 and pUC118 [Vieira and Messing, 1987; Sambrook et al., 1989] were used as vectors for subcloning and sequencing the *P. aeruginosa* chromosomal *ars* operon. Ampicillin was used at a final concentration of 40 μg/ml when required.

5.4.2 Cloning and sequencing of the *P. aeruginosa* chromosomal *ars* operon

All restriction enzymes and T4 DNA ligase used for cloning purposes were purchased from New England Biolabs (Mississauga, Ont.) and used following the supplier's instructions. Isolation of plasmid and genomic DNA, preparation of competent *E. coli* cells, and DNA transformation into *E. coli* cells were performed as described by Sambrook et al (1989). Alpha-^{32}P-labelling of DNA probes for Southern and RNA dot blotting analyses was performed using the random priming method [Sambrook et al., 1989] with random hexanucleotide primers (Regional DNA Synthesis Laboratory, University of Calgary, Calgary, AB, Canada.). Radioisotopes were purchased from Amersham (Oakville, ON, canada).

To clone the *P. aeruginosa* strain PAO1 chromosomal *ars* operon homolog, restriction fragments of approximately 3.4 kb in size, previously shown to hybridize to the *E. coli* chromosomal *ars* operon [Diorio et al., 1995], were purified from a 0.8% agarose gel of *Eco*RV-cleaved chromosomal DNA of *P. aeruginosa* strain PAO1, and ligated to an *Eco*RV-linearized pBR322 plasmid. The ligation mixture was then transformed into *E. coli* strain JM105 [Yanish-Perron et al., 1985]. Recombinant transformants were selected for ampicillin resistance and screened for tetracycline sensitivity. To identify the plasmids containing the putative *P. aeruginosa* chromosomal *ars* operon homolog, clones from the recombinant transformants were lysed by the "cracking" procedure [Barnes, 1977], subjected to 0.8% agarose gel electrophoresis [Diorio et al., 1995], transfered to a Hybond-N nylon membrane (Amersham, Oakville, ON, Canada) and hybridized [Diorio et al., 1995] to a ^{32}P-labelled *E. coli* chromosomal *arsB* probe, isolated as a 658-bp *Sca*I-*Pvu*II restriction fragment from plasmid pJC701 [Cai and DuBow, 1996]. Following autoradiography, the plasmid DNA from recombinant clones which showed enhanced hybridization signals was isolated [Sambrook et al., 1989] and transformed into *E. coli* LF20012 (*arsB*$^-$). The resulting transformants were then screened for enhanced resistance to sodium arsenite (when compared with *E. coli* LF20012 containing plasmid pBR322) by replica plating them on LB agar plates containing increasing concentrations of sodium arsenite (0, 20, 100, 200, 400, and 800 μg As/ml). One of the clones found to complement the *E. coli arsB*$^-$ mutant for enhanced arsenic resistance was isolated, and the recombinant

plasmid, containing a 3.4-kb *Eco*RV *P. aeruginosa* chromosomal DNA fragment, was named pJC801.

Fragments of the *P. aeruginosa* chromosomal DNA in pJC801 were further subcloned into pUC119 or pUC118 [Sambrook et al., 1989]. Double- or single-stranded DNA templates for sequencing analysis were prepared using QIAprep Spin Mini Prep Kit (QIAGEN Inc., Chatsworth, CA), or following the method of Vieira and Messing (1987). DNA sequencing of both strands of these subclones was performed manually by the dideoxy DNA sequencing method using the ISOTHERM™ sequencing kit (EPICENTRE Technologies, Madison, WI), and by automatic sequencing (Core Facilities for Protein/DNA Chemistry, Biochemistry Department, Queen's University, Kingston, ON, Canada.).

5.4.3 Complementation assays in an *E. coli arsB* mutant strain

The plasmid pJC801 (and the parental plasmid pBR322) was transformed into an *E. coli arsB*⁻ mutant strain LF20012 (*arsB::luxAB*) [Cai and DuBow, 1996] as well as its parental strain, *E. coli* 40. The resistance profile to arsenic and antimony salts was examined for all four transformants by their ability to grow in LB broth containing increasing amounts of arsenic or antimony oxyanions as follows. Overnight cultures of each sample were diluted 50-fold in LB broth containing the appropriate antibiotics and increasing concentrations of sodium arsenite, sodium arsenate, and potassium antimony tartrate hemihydrate. The cultures were incubated for 6 h at 37°C, and the turbidity at 600 nm (A_{600}) of each sample (in triplicate) was determined. The arsenic and antimony oxyanion resistance profile of the clones was expressed as the average value of the percentage of the A_{600} of each strain, calculated as previously described [Cai and DuBow, 1997], as a function of the elemental concentrations of arsenic or antimony in the compounds used.

5.4.4 Southern blotting analysis

Genomic DNAs (10 μg) from the strains of interest were cleaved with *Eco*RV and *Pst*I, respectively, and then subjected to 0.8% agarose gel electrophoresis [Sambrook et

al., 1989]. The subsequent DNA transfer and hybridization with a ^{32}P-radiolabelled probe were performed as previously described [Diorio et al., 1995]. The probe was isolated as a 1.14-kb *Bam*HI-*Sph*I double-cleaved fragment, containing the second half of *arsR* and the first two thirds of *arsB*, from pJC801. After hybridization, the membrane was washed to remove unhybridized probe, and exposed to Kodak XAR-5 film (Eastman Kodak, Rochester, N.Y.), using Dupon Cronex intensifying screens, prior to development.

5.4.5 Preparation of total cellular RNA of *P. aeruginosa* PAO1 and dot blotting analysis

Cultures of *P. aeruginosa* strain PAO1 were grown in LB broth at 32°C in a shaking incubator until mid-log phase (A_{600} = 0.5~0.6), and then sodium arsenite was added to a final concentration of 0.1 μg As/ml. This concentration was chosen based on our previous observations for the *E. coli ars* operon [Cai and DuBow, 1996]. Samples were removed at 0, 15, 30, and 60 min post arsenic addition and kept on ice until all samples were ready. Total cellular RNA was isolated using the RNaid Plus kit (BIO 101, Inc., La Jolla, CA), following the manufacturer's instructions. The RNA concentrations of each sample were spectrophotometrically determined using a UV-1201 spectrophotometer (SHIMADZU Scientific Instruments Inc., Japan). Different amounts (5, 2.5, and 1 μg/slot) of total cellular RNA from arsenite-exposed and unexposed *P. aeruginosa* were loaded onto a Hybond-N nylon membrane (Amersham, Oakville, ON, Canada) in a BioRad (Mississauga, ON, Canada) dot blot apparatus as described [Sambrook et al., 1989]. The RNA was fixed to the membrane by microwave exposure for 2.5 min and hybridized to the same probe as the one used for Southern blotting analysis. Prehybridization and hybridization reactions were performed as described by Cai and DuBow (1996), and the filter was washed and exposed to Kodak XAR-5 film as described in the previous sections.

5.4.6 Nucleotide sequence accession number

The nucleotide sequence reported here has been submitted to the EMBL database, and it has been assigned accession number AF010234.

5.5 RESULTS

5.5.1 Cloning of an *ars* operon homolog of *P. aeruginosa*

Based upon our previous observation that an approximately 3.4-kb *Eco*RV DNA fragment contained sequences homologous to the *E. coli* chromosomal *ars* operon and thus a potential chromosomal *ars* operon homolog of *P. aeruginosa* [Diorio et al., 1995], *Eco*RV fragments of this size were isolated and cloned into plasmid pBR322. Recombinant transformants were screened for the presence of *ars* homologous sequences by hybridization to the *E. coli arsB* probe (see Materials and Methods). Four putative clones were further identified by a complementation assay (Materials and Methods) and isolated due to their ability to confer high levels of resistance to arsenite (i.e. the ability to allow growth on solid media containing 400 μg As/ml as sodium arsenite) when transformed into an *E. coli arsB*⁻ mutant. These four plasmids were designated pJC801 through pJC804, and found to contain an identical DNA insertion based upon subsequent restriction enzyme mapping analysis (data not shown). Therefore, only pJC801 was chosen for further studies.

5.5.2 DNA and predicted amino acid sequence analyses

Subcloning and DNA sequencing analyses showed that the cloned *P. aeruginosa* PAO1 chromosomal DNA fragment, which conferred increased arsenite resistance and was capable of hybridizing with the *E. coli* chromosomal *ars* operon, contains three ORFs (Fig. 1), whose encoded proteins share significant homology to the ArsR, ArsB, and ArsC polypeptides of the *E. coli* chromosomal and plasmid-encoded *ars* operons. Comparison of the amino acid sequences between the *P. aeruginosa ars* operon and other known *ars* operons, using the method of Myers and Miller (1988), revealed striking homology as summarized in Table 1. In the case of ArsR, the amino acid sequence identity between the *P. aeruginosa* chromosomal *ars* operon and other *ars* operons ranges from 28 to 44% (Table 1). It is noted, in particular, that the putative metal binding box, ELCVCDL, and the DNA-binding helix-turn-helix (H-T-H) motif identified in the ArsR proteins of other *ars* operons, and known to be critical in arsenic-dependent regulation of operon expression, are also conserved in the ArsR sequence of the *P. aeruginosa* chromosomal

Figure 1. DNA sequence of the *P.aeruginosa* chromosomal *ars* operon cloned in plasmid pJC801. The predicted amino acid sequences of the gene products are shown below the DNA sequence. The potential *arsR*, *arsB*, and *arsC* ORFs are indicated. Stop codons are indicated by asterisks. Shine-Dalgarno (SD) sequences are marked in bold and underlined.

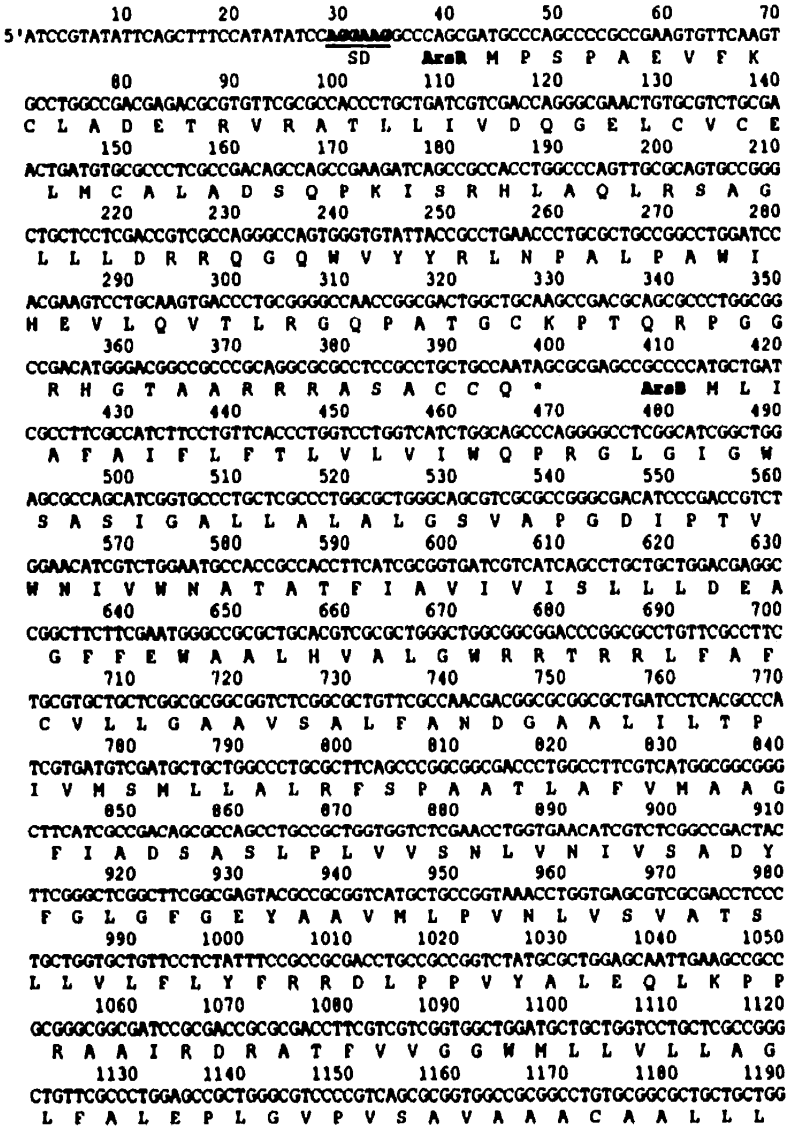
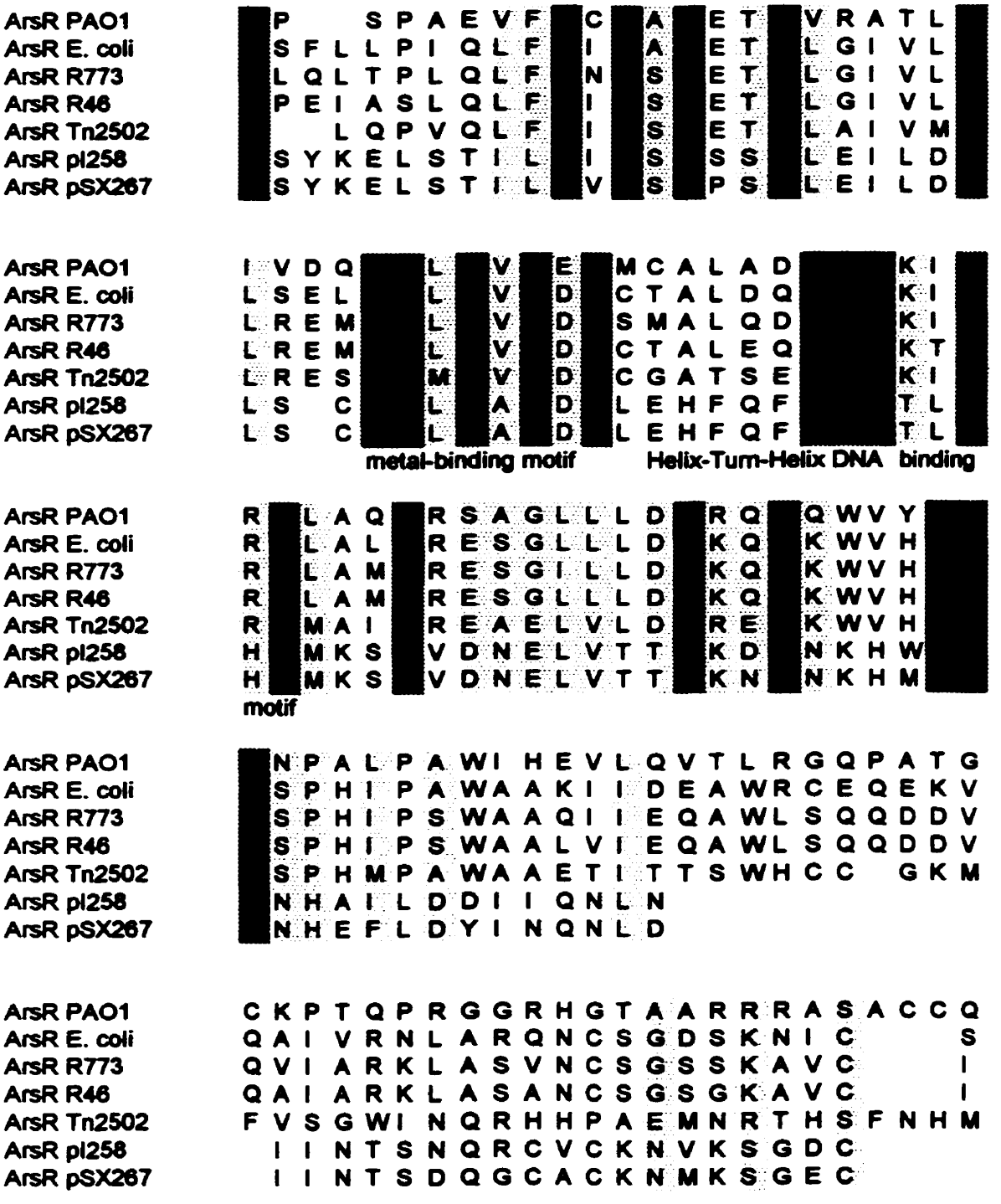

10 20 30 40 50 60 70
5'ATCCGTATATTCAGCTTTCCATATATCCAGGAAGGCCCAGCGATGCCCAGCCCCGCCGAAGTGTTCAAGT
SD AraR M P S P A E V F K
80 90 100 110 120 130 140
GCCTGGCCGACGAGACGCGTGTTCGCGCCACCCTGCTGATCGTCGACCAGGGCGAACTGTGCGTCTGCGA
C L A D E T R V R A T L L I V D Q G E L C V C E
150 160 170 180 190 200 210
ACTGATGTGCGCCCTCGCCGACAGCCAGCCGAAGATCAGCCGCCACCTGGCCCAGTTGCGCAGTGCCGGG
L M C A L A D S Q P K I S R H L A Q L R S A G
220 230 240 250 260 270 280
CTGCTCCTCGACCGTCGCCAGGGCCAGTGGGTGTATTACCGCCTGAACCCTGCGCTGCCGGCCTGGATCC
L L L D R R Q G Q W V Y Y R L N P A L P A W I
290 300 310 320 330 340 350
ACGAAGTCCTGCAAGTGACCCTGCGGGGCCAACCGGCGACTGGCTGCAAGCCGACGCAGCGCCCTGGCGG
H E V L Q V T L R G Q P A T G C K P T Q R P G G
360 370 380 390 400 410 420
CCGACATGGGACGGCCGCCCGCAGGCGCGCCTCCGCCTGCTGCCAATAGCGCGAGCCGCCCCATGCTGAT
R H G T A A R R R A S A C C Q * AraB M L I
430 440 450 460 470 480 490
CGCCTTCGCCATCTTCCTGTTCACCCTGGTCCTGGTCATCTGGCAGCCCAGGGGCCTCGGCATCGGCTGG
A F A I F L F T L V L V I W Q P R G L G I G W
500 510 520 530 540 550 560
AGCGCCAGCATCGGTGCCCTGCTCGCCCTGGCGCTGGGCAGCGTCGCGCCGGGCGACATCCCGACCGTCT
S A S I G A L L A L A L G S V A P G D I P T V
570 580 590 600 610 620 630
GGAACATCGTCTGGAATGCCACCGCCACCTTCATCGCGGTGATCGTCATCAGCCTGCTGCTGGACGAGGC
W N I V W N A T A T F I A V I V I S L L L D E A
640 650 660 670 680 690 700
CGGCTTCTTCGAATGGGCCGCGCTGCACGTCGCGCTGGGCTGGCGGCGGACCCGGCGCCTGTTCGCCTTC
G F F E W A A L H V A L G W R R T R R L F A F
710 720 730 740 750 760 770
TGCGTGCTGCTCGGCGCGGCGGTCTCGGCGCTGTTCGCCAACGACGGCGCGGCGCTGATCCTCACGCCCA
C V L L G A A V S A L F A N D G A A L I L T P
780 790 800 810 820 830 840
TCGTGATGTCGATGCTGCTGGCCCTGCGCTTCAGCCCGGCGGCGACCCTGGCCTTCGTCATGGCGGCGGG
I V M S M L L A L R F S P A A T L A F V M A A G
850 860 870 880 890 900 910
CTTCATCGCCGACAGCGCCAGCCTGCCGCTGGTGGTCTCGAACCTGGTGAACATCGTCTCGGCCGACTAC
F I A D S A S L P L V V S N L V N I V S A D Y
920 930 940 950 960 970 980
TTCGGGCTCGGCTTCGGCGAGTACGCCGCGGTCATGCTGCCGGTAAACCTGGTGAGCGTCGCGACCTCCC
F G L G F G E Y A A V M L P V N L V S V A T S
990 1000 1010 1020 1030 1040 1050
TGCTGGTGCTGTTCCTCTATTTCCGCCGCGACCTGCCGCCGGTCTATGCGCTGGAGCAATTGAAGCCGCC
L L V L F L Y F R R D L P P V Y A L E Q L K P P
1060 1070 1080 1090 1100 1110 1120
GCGGGCGGCGATCCGCGACCGCGCGACCTTCGTCGTCGGTGGCTGGATGCTGCTGGTCCTGCTCGCCGGG
R A A I R D R A T F V V G G W M L L V L L A G
1130 1140 1150 1160 1170 1180 1190
CTGTTCGCCCTGGAGCCGCTGGGCGTCCCCGTCAGCGCGGTGGCCGCGGCCTGTGCGGCGCTGCTGCTGG
L F A L E P L G V P V S A V A A A C A A L L L
1200 1210 1220 1230 1240 1250 1260
CGGTGGCCGGGCGCGGGCGGGTGATCTCGACCCGGCGGGTGCGCGAAGCGCCCTGGCAGATCGTGGTCTT
A V A G R G R V I S T R R V R E A P W Q I V V F
1270 1280 1290 1300 1310 1320 1330
CTCGCTGGGCATGTACCTGGTGGTCTACGGCCTGAAGAACGCCGGTCTAACCGACCTTCTGGCTCAGCTC
S L G M Y L V V Y G L K N A G L T D L L A Q L
1340 1350 1360 1370 1380 1390 1400
CTGAACCGCATGACCGAGCACGGCGTATGGGCGGCCGCGCTGGGTACCGGACTGCTTTCCGCCGCCCTCT
L N R M T E H G V W A A A L G T G L L S A A L
1410 1420 1430 1440 1450 1460 1470
CGTCGGTAATGAACAACATGCCCAGCATGCTGCTCGGCGCCCTGTCGATCCAGGCCAGCGACACCGCCGG
S S V M N N M P S M L L G A L S I Q A S D T A G
1480 1490 1500 1510 1520 1530 1540
GCCGGTGCGCGAAGCGATGATCTACGCCAACGTCATCGGCTGCGACCTGGGTCCGAAGATCACCCCTATC
P V R E A M I Y A N V I G C D L G P K I T P I
1550 1560 1570 1580 1590 1600 1610
GGCAGCCTGGCGACGCTGCTCTGGCTGCACGTGCTGGCGCGCAAAGGCATGCGCATCACCTGGGGCTACT
G S L A T L L W L H V L A R K G M R I T W G Y
1620 1630 1640 1650 1660 1670 1680
ACTTCAGGGTCGGCGCCCTGCTGACCCTGCCGGTCCTGCTGGCGACCCTTTCGGCCCTGGCCCTGCGCCT
Y F R V G A L L T L P V L L A T L S A L A L R L
1690 1700 1710 1720 1730 1740 1750
GGCCATCTGACGGCCGCCGGCGACGGCGGCAGGAGGCATCCCATGCGAGTCCTGTTCATGTGCACGGCCAA
A I * SD AraC M R V L F M C T A N
1760 1770 1780 1790 1800 1810 1820
CAGTTGCCGCAGCATTCTTTCCGAAGCCATGTTCAACCACCTGGCCCCGCCGGGTTTCGAGGCATGCAGC
S C R S I L S E A M F N H L A P P G F E A C S
1830 1840 1850 1860 1870 1880 1890
GCCGGCAGCCAGCCCAGCGGGCGGGTGCATCCGCGCAGCCTGGCGACCCTCGAACAGGCCGGCATCGCCA
A G S Q P S G R V H P R S L A T L E Q A G I A
1900 1910 1920 1930 1940 1950 1960
CCCACGGCCTGTACAGCAAGGGCAGCGAAGCCTTCGAAGGCGCACCACCGGACATCGTCATCACCGTTTG
T H G L Y S K G S E A F E G A P P D I V I T V C
1970 1980 1990 2000 2010 2020 2030
CGACGCCGCCGCGGGGGAAGCCTGCCCGCTGTATCTCGGCGCAGCGCTGAAGGCCCATTGGGGCCTGGCC
D A A A G E A C P L Y L G A A L K A H W G L A
2040 2050 2060 2070 2080 2090 2100
GATCCCTCCGCCCTGGATGGCGACGAAGCCCTGCGGGATGCGGCGTTCCACGCCACCCTGGCACGCATCG
D P S A L D G D E A L R D A A F H A T L A R I
2110 2120 2130 2140 2150 2160 2170
AACAGCGTTGCCTAGCCTTCCTCGGCCTGCCCTTCGCTACCCTGGATCGCGACCAGCTCAAGCGTGAGCT
E Q R C R A F L G L P F A T L D R D Q L K R E L
2180 2190 2200 2210 2220 2230 2240
GGAGCGCATCGGCTCGCTCTGACCGGAGGAAGCATGTCCGAACAACTACCCAACCTCGATCCCGCGCTGC
E R I G S L *
2250 2260 2270 2280 2290 2300 2310
TCGGCGACCCGCCCCCCGTCTCCGGGCACAGGCCGCGCATCCTCCTGCTCTACGGCTCGACCCGCGAGCG
2320 2330 2340 2350 2360 2370 2380
CTCCTTCAGCCGCCTGCTGGTGCTGGAGGCCGCACGCCTGCTCGAACGCTTCGGTGCCGAAACGCGGATT
2390 2400 2410
TTCGACCCTTCCGGGCTGCCATTGCCCGATGCACCGGT 3'

Figure 2. Multiple alignments of amino acid sequences of the ArsR proteins from various *ars* operons using the Clustal computer program (PC-Gene software, Intelligenetics, Inc. Mountain View, CA). Identical amino acid residues are indicated in hatched areas, similar amino acid residues are indicated in dotted areas. The potential metal binding motif and the DNA binding motifs are marked.


ArsR PAO1
ArsR E. coli
ArsR R773
ArsR R46
ArsR Tn2502
ArsR pI258
ArsR pSX267
metal-binding motif
Helix-Turn-Helix DNA binding
motif

ars operon (Figure 2). These results suggest that the *P. aeruginosa* chromosomal *ars* operon is a new member of the arsenic efflux system family, and may be regulated in a manner similar to that used by other known *ars* operons. The highest homology, ranging from 51 to 70%, was found between the ArsB protein of the *P. aeruginosa* chromosomal *ars* operon and that of other known *ars* operons (Table 1). Multiple alignments [Higgins and Sharp, 1988; 1989] of the *P. aeruginosa* ArsB sequence with the ArsB proteins of the *ars* operons listed in Table 1 revealed an overall amino acid identity (similarity) of 45.3% (33.5%) (data not shown). Hydrophobicity analyses, using the method of Eisenberg et al. (1984), indicate that the *arsB* gene product is a hydrophobic protein with 12 putative membrane associated helices, consistent with its potential function as a membrane transporter and the structures of other known ArsB proteins. It is interesting to note that both the ArsR and ArsB proteins of the *P. aeruginosa* chromosomal *ars* operon share higher homology to those of the *ars* operons of Gram-negative bacteria. In contrast, the ArsC protein of the *P. aeruginosa* chromosomal *ars* operon is more homologous to the ArsC proteins of Gram-positive *ars* operons (Table 1).

5.5.3 The cloned *P. aeruginosa* chromosomal *ars* operon confers increased arsenite and antimonite resistance in an *E. coli arsB* mutant

To examine the role of the *P. aeruginosa* PAO1 chromosomal *ars* operon in arsenic resistance, the cloned *ars* operon (in pJC801) was introduced into an *arsB*$^-$ *E. coli* strain, LF20012 [Cai and DuBow, 1996], and the parental strain *E. coli* 40 (*ars*$^+$). The resulting transformants were called LF20012(pJC801) and 40(pJC801), respectively. As a control, the vector plasmid pBR322 was transformed into both *E. coli* 40 and LF20012. Cultures from these four strains were analysed for their ability to grow when exposed to increasing concentrations of arsenite, arsenate, and antimonite ions as described in Materials and Methods, and the results are shown in Fig. 3. Introduction of plasmid pJC801 into *E. coli* conferred an increase in arsenite resistance in both *ars*$^+$ and *ars*$^-$ strains in a manner similar to that observed when the *E. coli* chromosomal *ars* operon is cloned in a multicopy plasmid [Diorio et al., 1995] (Fig. 3A). A similar increase in antimonite resistance

Table 1. Percent identity (similarity) in amino acid sequences with *P. aeruginosa* ArsR, B, and C.

Table 1

Percent identity (similarity) in amino acid sequences with *P. aeruginosa* ArsR, B, and C

	ArsR	ArsB	ArsC
E. coli *ars*	44.4 (11.1)	69.3 (15.6)	10.6 (17)
R773 *ars*	43.6 (16.2)	69.3 (16.3)	12.8 (11.3)
IncN R46 *ars*	43.6 (13.7)	69.9 (15.6)	12.1 (11.5)
Tn2505 *ars*	42.7 (13.7)	68.9 (14.4)	13.5 (15.6)
pI258 *ars*	28.8 (20.2)	51.7 (21)	29 (16.8)
pSX267 *ars*	27.9 (18.3)	51.4 (21.2)	29 (16.8)

was also observed (Fig. 3C). These results suggest that the *P. aeruginosa* chromosomal *ars* operon can complement an *E. coli arsB*⁻ mutant and provide enhanced protection to both the mutant and wild type *E. coli* cells from the toxicity of arsenite and antimonite ions. Interestingly, although the cloned *P. aeruginosa* chromosomal *ars* operon increased arsenate resistance in the *E. coli arsB*⁻ mutant, it did not show any detectable enhancement on arsenate resistance in wild type *E. coli* cells (Fig. 3B) (see Discussion).

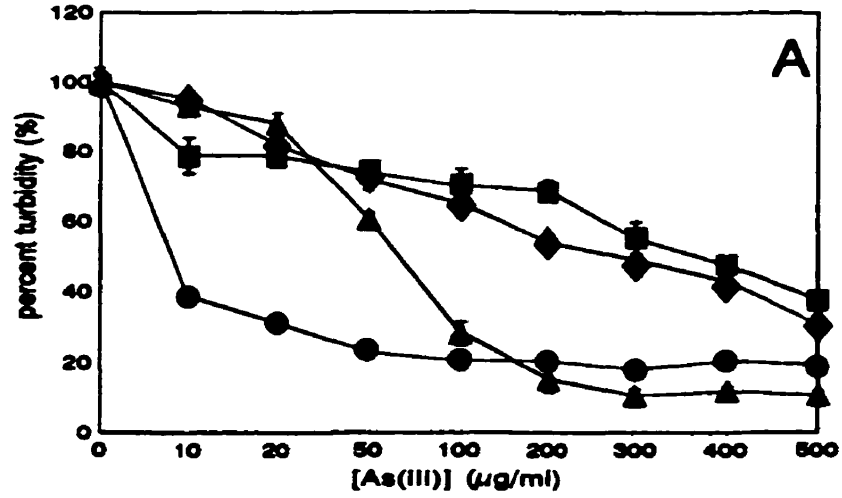
5.5.4 RNA expression of the *P. aeruginosa* chromosomal *ars* operon is inducible by sodium arsenite

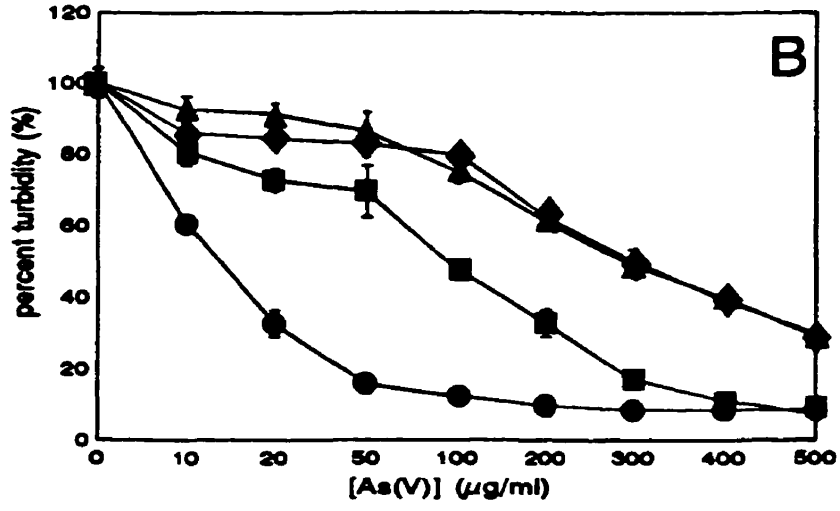
To elucidate arsenic-regulated expression of the *P. aeruginosa* chromosomal *ars* operon, equal amounts of total cellular RNA, isolated from sodium arsenite-exposed (for 15, 30, and 60 min, respectively) and unexposed cells, were loaded onto a nylon membrane, fixed, dried and hybridized to a ^{32}P-labelled DNA fragment containing the *P. aeruginosa* chromosomal *ars* genes as described in Materials and Methods. The autoradiograph (Fig. 4A) showed a dramatic increase in hybridization in the arsenite-exposed RNA samples when compared to the unexposed sample, suggesting that mRNA expression of the *P. aeruginosa* chromosomal *ars* operon is inducible by arsenic at the transcriptional level. Quantitation analysis using the Image Quant™ program (Molecular Dynamics. Synnyrale, CA) revealed at least a 15-fold increase in *ars*-specific RNA upon addition of 0.1 μg As/ml (as sodium arsenite) for 15 min, indicating that *ars* mRNA is rapidly induced by arsenite. Moreover, a decease in the intensity of the *ars*-specific RNA was observed at 60 min post-exposure (Fig. 4A), consistent with previous observations for the *E. coli* chromosomal and the R773 *ars* operons [Cai and DuBow, 1996; Owolabi and Rosen, 1990].

5.5.5 The *P. aeruginosa* chromosomal *ars* operon is conserved in the chromosomes of other *Pseudomonas* species

A Southern blotting analysis with chromosomal DNAs from other *Pseudomonas* species was conducted using a ^{32}P-labeled probe containing part of the *P. aeruginosa* chromosomal *ars* operon (see Materials and Methods). The results, shown in Fig. 4B,

Figure 3. Growth of plasmid-containing *E. coli* strains in the presence of increasing amounts of arsenic and antimony oxyanions. Overnight cultures were diluted 50-fold in LB broth containing appropriate antibiotics and increasing amounts of arsenic or antimony salts. Cellular growth was measured 6 h after addition of chemicals and expressed as percent turbidity versus elemental concentrations of arsenic (antimony) added to the growth media (see Materials and Methods). Standard deviations are represented by error bars. ▲, *E. coli* 40(pBR322); ◆, *E. coli* 40(pJC801); ●, LF20012(pBR322); ■, LF20012(pJC801).


A
percent turbidity (%)
120
100
80
60
40
20
0
0
10
20
50
100
200
300
400
500
[As(III)] (μg/ml)


B
percent turbidity (%)
120
100
80
60
40
20
0
0
10
20
50
100
200
300
400
500
[As(V)] (μg/ml)

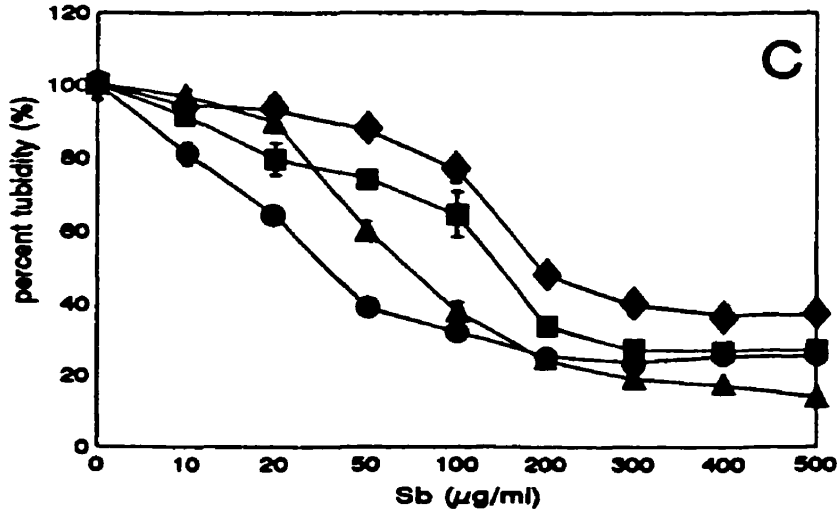
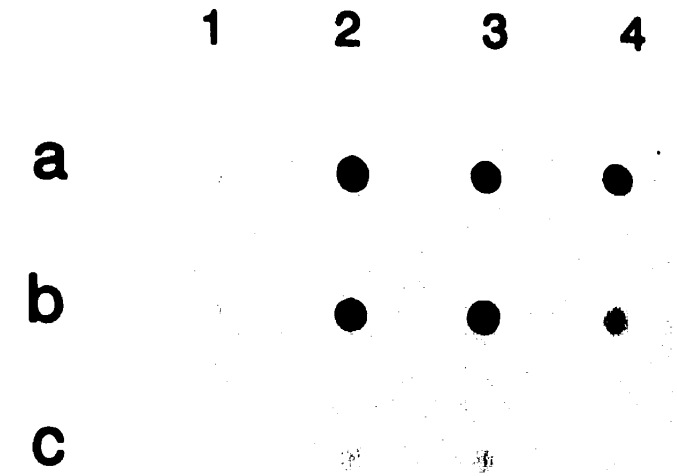
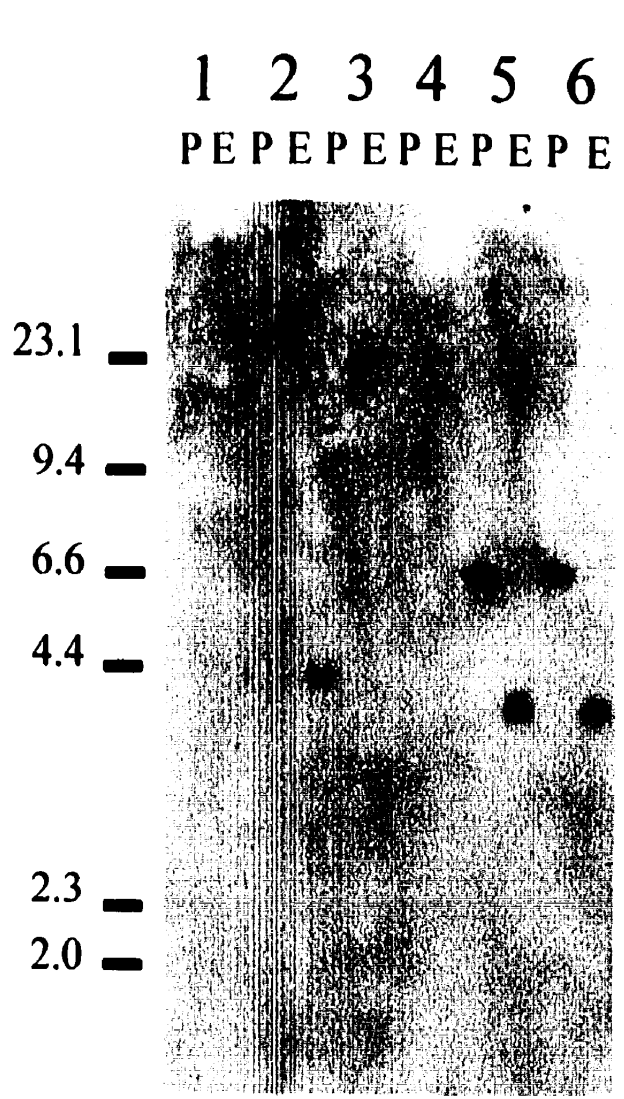

C
percent turbidity (%)
120
100
80
60
40
20
0
0
10
20
50
100
200
300
400
500
Sb (μg/ml)

Figure 4. (A) Dot blot hybridization of total cellular RNA to a ^{32}P-labelled *P. aeruginosa* chromosomal *ars* operon probe (see Materials and Methods). Lane 1 contains RNA from unexposed cultures; lane 2, 3, and 4 are the RNAs from cells incubated with sodium arsenite (at a final concentration of 0.1 μg As/ml) for 15, 30, and 60 min, respectively. Samples in rows *a*, *b*, and *c*, are loaded with 5, 2.5, and 1 μg RNA/slot, respectively.
(B) Southern hybridization of genomic DNAs from various bacterial species to a ^{32}P-labelled *P. aeruginosa* chromosomal *ars* operon probe (see Materials and Methods). The numbers on top indicate different bacterial strains. 1, *B. cepacia*; 2, *P. diminuta*; 3, *P. fluorescens*; 4, *P. stutzeri*; 5, *P. aeruginosa* PAK; 6, *P. aeruginosa* PAO1. The letters on top indicate the restriction enzymes used to digest the genomic DNAs. P, *Pst*I; E, *Eco*RV. The numbers on the right side are DNA size markers (in kb).

A



B



demonstrate that sequences homologous to the *P. aeruginosa* chromosomal *ars* operon can be detected in the chromosomes of *P. aeruginosa* strain PAK, another major wild type strain of *P. aeruginosa* [Minamishima et al., 1968; Takaya and Amako, 1966], and of *P. fluorescens*. However, no sequences homologous to the *P. aeruginosa* chromosomal *ars* operon were found in the chromosomal DNAs of *P. stutzeri*, *P. diminuta*, and *Bhurkholderia cepacia* (formerly *P. cepacia*) under the conditions used, suggesting that the *P. aeruginosa* chromosomal *ars* operon is conserved, at the DNA level, in some, but not all *Pseudomonas* species.

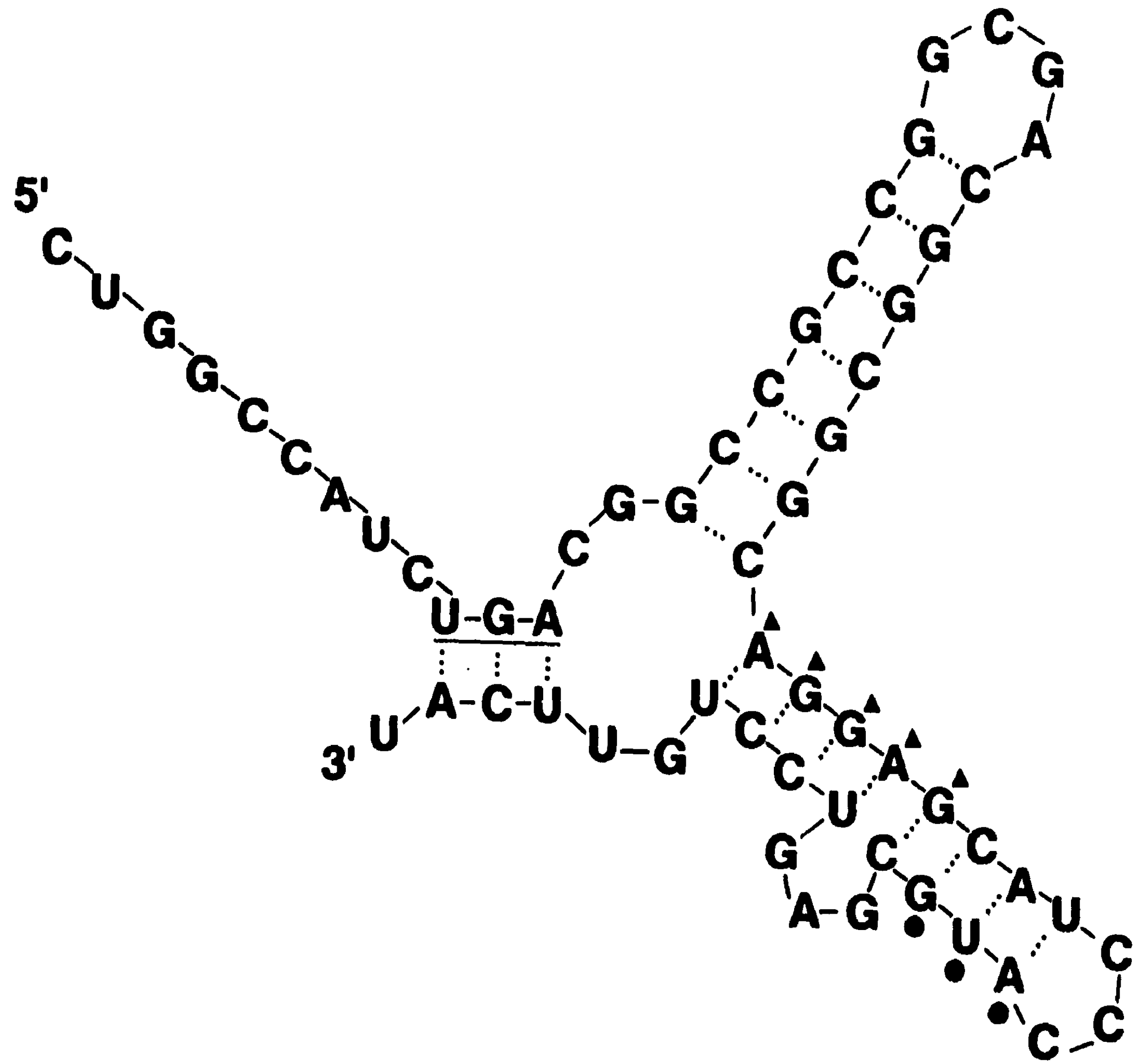
5.6 DISCUSSION

The genetic organization of the *ars* operons identified to date appears to be conserved. Almost all begin with the gene encoding the ArsR repressor, and end with the *arsC* gene encoding arsenate reductase, except that in the recently discovered *Y. enterocolitica* Tn2502 *ars* operon, an *arsH* gene is located upstream of *arsR* and transcribed in an opposite direction [Neyt et al., 1997]. The *arsB* gene is essential for arsenic detoxification, and acts either alone or in combination with an arsenite- and antimonite-specific ATPase [Dey and Rosen, 1995]. The present study describes the identification and preliminary characterization of the *P. aeruginosa* chromosomal *ars* operon, which is the second of the chromosomally-located *ars* operons of bacteria identified so far. DNA sequencing analysis revealed three potential ORFs (Fig. 1), which share significant homology to the *arsR*, *arsB*, and *arsC* ORFs of other known *ars* operons, and are oriented in an identical fashion to those in other known *ars* operons. Multiple alignments of the amino acid sequences of the putative *P. aeruginosa* ArsR sequence with the ArsR proteins of other known *ars* operons, using the Clustal program [Higgins and Sharp, 1988; 1989], revealed the conservation of the putative metal-binding motif and DNA binding H-T-H motif in the *P. aeruginosa* ArsR protein. This further supports the fact that the *P. aeruginosa* chromosomal *ars* operon is a member of the arsenic oxyanion efflux family. The putative ArsB protein of the *P. aeruginosa* chromosomal *ars* operon is the most conserved among the *ars* polypeptides, consistent with what has been observed for other known *ars* operons [Silver et al., 1993; Diorio et al., 1995; Silver, 1996]. Both

ArsR and ArsB share greater homology to their respective proteins from Gram-negative *ars* operons, which is not unexpected since *P. aeruginosa* is a Gram-negative bacterial species. Surprisingly, the putative ArsC protein of the *P. aeruginosa* chromosomal *ars* operon shares greater homology to the ArsC polypeptides of Gram-positive *ars* operons.

A functional analysis of *P. aeruginosa* chromosomal *ars* operon in *E. coli* suggests that the operon can provide enhanced resistance to arsenic and antimony oxyanions in *E. coli* when cloned in a multicopy plasmid in a manner similar to that observed for the *E. coli* chromosomal *ars* operon [Diorio et al., 1995; Carlin et al., 1995]. However, when the *P. aeruginosa* chromosomal *ars* operon was introduced into wild type *E. coli*, only increased resistance to arsenite and antimonite was observed, and no detectable increase was found in arsenate resistance. This is in contrast to our previous observation that the *E. coli* chromosomal *ars* operon, when cloned in a multicopy plasmid and introduced into wild type *E. coli*, resulted in increased resistance to both arsenite and arsenate ions [Diorio et al., 1995]. The lack of increase in arsenate resistance in wild type *E. coli* containing a cloned *P. aeruginosa* chromosomal *ars* operon could be due to one or more of the following reasons. First, previous studies have shown that the Gram-positive ArsC protein of the *Staphylococcus aureus* plasmid pI258 *ars* operon requires thioredoxin and thioredoxin reductase for proper function [Ji and Silver, 1992b; Ji et al, 1994], whereas the *in vivo* enzymatic activity of the Gram-negative ArsC protein from the *E. coli* plasmid R773 *ars* operon requires glutathione, and glutathione reductase [Oden et al., 1994]. This reflects the existence of inherent differences between Gram-negative and Gram-positive ArsC proteins. This difference may account for an inefficient function of the *P. aeruginosa* ArsC protein in *E. coli* cells, since the *P. aeruginosa* ArsC protein more closely resembles that of Gram-positive bacteria. In addition, due to the closer relationship of the ArsC protein of the *P. aeruginosa* chromosomal *ars* operon to that of Gram-positive *ars* operons, the *arsC* gene of the *P. aeruginosa* chromosomal *ars* operon may not be well expressed in *E. coli*. Analysis of the DNA sequence of the *P. aeruginosa* chromosomal *ars* operon revealed a relatively large intergenic region between the *arsB* and *arsC* genes, compared with that of other known *ars* operons and that between the *arsR* and *arsB* cistrons of the *P. aeruginosa* chromosomal *ars* operon (Fig. 1). It is possible that either

Figure 5. Sequence and prediction of a potential mRNA secondary structure in the intergenic region between *arsB* and *arsC* of the *P. aeruginosa* chromosomal *ars* operon. The termination codon, UGA, of the *arsB* gene is underlined. The putative ribosome binding sequence (SD) is marked with triangles. The translation initiation codon, AUG, of the *arsC* gene is marked with dots. The free energy of formation of the indicated secondary structure is -29.3 kcal/mol, as estimated by the Hibio DNAsis™ program (Hitachi Software Engineering Co., Ltd. Yokohama, Japan).


5'
3'

the *arsC* gene is controlled by another promoter, which may not be active in *E. coli*, or it is not efficiently translated by ribosomes in *E. coli*. Previous studies have shown that an optimal aligned spacing of 5 nt between Shine-Dalgarno (SD) sequence and the translation initiation codon is required for ribosomes to efficiently translate mRNAs in *E. coli* [Chen et al., 1994a], and long-range secondary structures involving the Shine-Dalgarno sequence and internal complementary sequences were found to inhibit translation initiation [Chang et al., 1995]. Secondary structure predictions of the *P. aeruginosa* chromosomal *ars* mRMA in the intergenic region between the *arsB* and *arsC* cistrons revealed the presence of a potential secondary structure as shown in Fig. 5, and this structure involves both the putative SD sequence and the initiation codon of ArsC. Thus, it is possible that the formation of this secondary structure in *arsC* mRNA could hinder the binding of ribosomes to the SD sequence and inhibit initiation of translation. Because of the above reasons, the *P. aeruginosa arsC* gene may be expressed at a low level or have a low activity in *E. coli* cells. Nonetheless, in an *ars*$^-$ background, this low level of ArsC activity may still lead to significant reduction of arsenate to arsenite, resulting in the observed increase in arsenate resistance in strain LF20012(pJC801), compared with that in strain LF20012(pBR322) (Fig. 3B). However, in wild type *E. coli* this low level expression (or activity) of ArsC from pJC801 may have been disguised by the fully-induced endogenous *E. coli* chromosomal *arsC* gene. Thus, no significant increase of arsenate resistance was observed in wild type *E. coli*.

It has been shown that both the *E. coli* chromosomal and plasmid R773 *ars* operons are transcribed upon arsenic exposure, and the transcripts are processed within 1 h [Owolabi and Rosen, 1990; Cai and DuBow, 1996]. Evidence also indicated that overexpression of the *E. coli* ArsR or ArsBC proteins is toxic to the cells [Cai and DuBow, 1996]. Therefore, a control on the upper level of *ars* mRNA (provided, where presented by the ArsD protein) may protect the cells from *ars* polypeptide-induced toxicity. In the present study, the *P. aeruginosa* chromosomal *ars* operon is found to be transcribed upon cellular exposure to subinhibitory levels of arsenite ions, and that the level of *ars*-specific RNA decreases with prolonged induction time (Fig. 4A), consistent with what has been observed for other *ars* operons. A Southern blotting analysis (Fig. 4B)

revealed the presence of *ars* homologous sequences in *P. aeruginosa* strain PAK, and *P. fluorescens*, but not in *P. stutzeri*, *P. diminuta*, and *B. cepacia*, a species formerly classified as *P. cepacia*, the later three species being more distally related than *P. fluorescens*. Further genetic sequencing and identification of chromosomal *ars* operons in other bacteria will shed light on the evolution of this highly-conserved and important bacterial operon. Moreover, recent results have shown that this type of arsenic resistance mechanism has been observed in mammals [Wang and Rossman, 1993; Wang et al., 1994, 1996], supporting the notion that the protective function of *ars* operons has been strongly conserved in the course of evolution.

CHAPTER 6

SUMMARY, CONCLUSIONS, AND FUTURE PROSPECTS

Arsenic is well known as a toxicant, with little evidence for its nutritional requirements [Nielsen, 1991]. The ubiquitous existence of arsenic in the environment due to both natural and man-made sources has become an unavoidable source of exposure to humans and other living organisms. Various detrimental effects caused by arsenic exposure to living systems have been observed and investigated, as discussed in Chapter 1. During evolution, living organisms have evolved various genetically-programmed mechanisms to cope with environmental stresses, including the elevated levels of arsenic. A fundamental mechanism by which living cells adapt to the changes in the external environment is to modulate gene expression. Therefore, a crucial step towards understanding these mechanisms is to monitor gene expression upon exposure to these toxicants. In many cases, however, it is inconvenient, or impossible, to assay the gene product directly as a means of monitoring gene expression. This problem is frequently circumvented by generating transcriptional fusions between the promoter of interest and a reporter gene, whose product is easy to assay. Many reporter gene systems have been developed. The most widely used reporter genes are *lacZ* (for ß-galactosidase) [Silhavy and Beckwith, 1985], *cat* (for chloramphenicol acetyl transferase), *galK* (for galactokinase) [McKenney et al., 1981], and *lux* (for bacterial luciferase) [Engebrecht et al., 1985].

Since all living organisms follow the central dogma for the expression and replication of genetic information, we chose *Escherichia coli*, whose genetics is well understood in addition to other advantages shared by all microorganisms, as a model system to study the alterations in gene expression upon cellular exposure to environmental stresses. Our investigation began with the creation of a random *lacZ* gene fusion library of *E coli*. This was accomplished by infecting a *lac*-deleted *E. coli* strain (40) with a transposable bacteriophage MudI, which carries a promoterless *lacZ* reporter gene and a selection marker (Ap^r). By screening the library in the presence and absence of various environmental toxicants, including arsenic salts, a clone, which turned blue in the presence of arsenic but remained white in its absence, was isolated and designated LF20001 (Chapter 2). Analysis of the *lacZ* reporter gene expression revealed that expression of this

gene can be induced by sodium arsenite, sodium arsenate and antimony oxide, at the levels just above environmental background, but not by cacodylic acid even at the high concentrations of arsenic in this organoarsenic pesticide (Chapter 2, Figure 3). Induction of gene expression by inorganic arsenic and antimony was found to be concentration dependent. Cloning and sequencing analyses showed three open reading frames (ORFs) that share strong homology to the *arsR*, *arsB*, and *arsC* ORFs of the plasmid encoded arsenic resistance operons (*ars*) from both Gram-positive and Gram-negative bacterial species (Chapter 2, Figure 2). Therefore, the operon we identified was named the chromosomal *ars* operon. The MudI insertion in the strain LF20001 was found to be within the coding region of the *arsB* gene. Arsenic sensitivity assays showed that the *arsB*::*lacZ* gene fusion strain is 10- to 100-fold more sensitive to arsenate and arsenite than the wild type cells, whereas wild type *E. coli* containing the cloned *ars* operon on a multicopy plasmid showed increased resistance to these arsenic ions (Chapter 2, Figure 4), suggesting that the *E. coli* chromosomal *ars* operon plays a role in protection against arsenic toxicity.

To elucidate transcriptional regulation of the *E. coli* chromosomal *ars* operon, two more chromosomal transcriptional gene fusions, in *arsR* and *arsB*, respectively, were constructed, using the *luxAB* genes from a marine bacterium, *Vibrio harvyi*. Luciferase has been used extensively as a reporter gene due to its unique advantages of high sensitivity, rapidity, and ease of detection. It has not only been used for monitoring gene expression, but also in other aspects of microbiology and biotechnology. Exposure of the cells from the *arsB*::*luxAB* gene fusion strain, LF20012, to sodium arsenite elicited a concentration-dependent luminescent response (Chapter 3, Figure 1A), similar to that observed in the *arsB*::*lacZ* gene fusion strain, indicating that the observed results were not due to reporter gene-specific effects, and confirmed the inducible expression of the operon by arsenite. Interestingly, insertion of the *luxAB* reporter gene into *arsR*, which is the first gene in the operon, resulted in a constitutive expression of luciferase (Chapter 3, Figure 1B), suggesting that the *arsR* gene product may function as a repressor, which negatively regulates expression of the operon, including itself. It is known that the *E. coli* plasmid

R773 encoded *arsR* gene product functions as a *trans*-acting repressor that acts by binding to an operator sequence as a dimer [Wu and Rosen, 1993b]. The chromosomal ArsR shares 77% amino acid identity with that of the R773 *ars* operon (Chapter 2, Figure 2B). Therefore, it is possible that the chromosomal ArsR protein functions in a similar manner as that of the R773 plasmid. To demonstrate that the chromosomal ArsR protein can function in *trans* to repress expression of the chromosomal *ars* operon, a cloned chromosomal *arsR* gene was introduced into the *arsR::luxAB* gene fusion strain, and cellular luminescence was assayed upon exposure to arsenite. It has been shown that the constitutively expressed *arsR::luxAB* chromosomal gene fusion can be repressed by the cloned *arsR* gene in *trans* (Chapter 3, Figure 2B). Moreover, the chromosomal and the R773 plasmid ArsR proteins are functionally interchangeble since the R773 ArsR can also repress the expression of the chromosomal *arsR::luxAB* gene fusion when provided in *trans* (Chapter 3, Figure 2C). These results indicate that both chromosmal and plasmid *ars* operons of *E. coli* are regulated via a similar mechanism, and that these *ars* operons are evolutionarily related. Northern hybridization and primer extension analyses showed that the operon is transcribed as a single transcript, which is about 2.1 kb in size, and appears to be processed into two smaller RNA molecules (Chapter 3, Figure 3A). Transcription of the operon initiates at 27 nt upstream of the *arsR* initiation codon (Chapter 3, Figure 3B). The individual genes of the *E. coli* chromosomal *ars* operon have been cloned into an expression vector, pKK223-3, in which the cloned genes are controlled by a P_{tac} promoter. The gene products have been visualized in an *in vivo* expression system using a chloramphenicol release assay (Chapter 3, Figure 3C).

The studies described in Chapter 2 and Chapter 3 demonstrated that the gene fusion technique is a powerful tool in identifying unknown genes, whose expression alters upon environmental changes. The reporter gene systems are also proven to be very useful in elucidating regulation of gene expression. These studies not only allow us to better understand how bacterial cells respond to external changes, but also provide us with sensitive molecular tools that can be exploited in environmental toxicology. Chapter 4 has focused on exploring the potential of the *E. coli arsB::luxAB* gene fusion strain, LF20012,

as a luminescent bacterial biosensor in monitoring the bioavailable levels and toxicity of arsenic compounds of environmental concern. The arsenic compound that is still used in vast amount is the wood preservative chromated copper arsenate (CCA), in which arsenate is mixed with two other toxic compounds, chromate and cupric oxide. Our study showed that the arsenic component in this mixture can be selectively detected by the luminescent bacterial gene fusion strain at the levels of arsenic within the environmentally relevant level. Whereas the mixture of cupric oxide and chromate, without arsenate, failed to induce the expression of the *arsB*::*luxAB* gene fusion (Chapter 4, Figure 1). These observations indicate that the *arsB*::*luxAB* gene fusion strain has a great potential to be used as a specific and sensitive biosensor for monitoring the bioavailable levels of arsenic.

So far, a number of plasmid-based *ars* operons have been isolated, and found to share homology amongst themselves. To address the question of whether the chromosomal *ars* operon is also a common genetic determinant shared by different bacterial species, we have conducted a Southern hybridization analysis with the chromosomal DNAs from a variety of Gram-negative bacterial species using the *E. coli* chromosomal *ars* operon as a probe. We found that, indeed, the homologous sequences of this operon can be detected in the chromosomes of many other bacterial species, including *Pseudomonas aeruginosa* (Chapter 2, Figure 5). To further extend our understanding of the chromosomally-encoded *ars* operons and the evolutionary relationship between them, the *ars* homolog of *P. aeruginosa* was cloned from a wild type strain, PAO1, and isolated by complementation of an *E. coli ars* mutant. Through DNA sequencing analysis, three ORFs have been identified in this operon based on their homology to the *arsR*, *arsB*, and *arsC* genes of other known *ars* operons. Comparison of the predicted protein sequences of the *P. aeruginosa* chromosomal *ars* operon with those of other known *ars* operons revealed a greater homology between the ArsR and ArsB of the *P. aeruginosa* chromosomal *ars* operon and those of Gram-negative bacterial *ars* operons. In contrast, a higher homology was found between the ArsC protein of *P. aeruginosa ars* operon and that of the Gram-positive *ars* operons. These observations indicate that the *ars* operons found in both Gram-positive and Gram-negative bacterial species may have arisen from a

common ancestor. Functional analysis showed that the PAO1 *ars* operon increased resistance to arsenite and antimonite, but not to arsenate, in wild type *E. coli*, probably due to inefficient expression of the *P. aeruginosa* ArsC protein and, perhaps, inappropriate function of the *P. aeruginosa* ArsC in *E. coli* cells. RNA dot blot hybridization showed an induced expression of the operon by cellular exposure to a subinhibitory concentration of sodium arsenite, and the regulation mainly takes place at transcription and post-transcription levels. Since pseudomonads represent a group of organisms with great genetic diversity, we also examined the presence of this chromosomal *ars* in the genomes of other *Pseudomonas* species, or those previously defined as pseudomonads, and found that the homologous sequences can be detected in some, but not all *Pseudomonas* species.

The study described in this thesis provided valuable information on identification and elucidation of genetically-programmed responses to the prevalent environmental pollutant, arsenic, in bacteria. Identification of the chromosomal *ars* operons in both *E. coli* and *P. aeruginosa* revealed that the efflux system is a prevalent mechanism involved in protection against arsenic toxicity. The homology between the *P. aeruginosa* chromosomal *ars* operon and other *ars* operons implied a common origin for these genetic determinants. The molecular approach used in this study can be exploited in other similar studies in both prokaryotic and eukaryotic systems for identifying genes responsive to external stimuli. In addition, the resulting gene fusion constructs have a great potential to be developed as efficient and specific biosensors for biomonitoring the levels and toxicity of various environmental toxicants or monitoring gene expression under given conditions.

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