Prokaryotic Arsenic Resistance - Studies in Bacillus subtilis

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PROKARYOTIC ARSENIC RESISTANCE - STUDIES IN *BACILLUS SUBTILIS*

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Abstract

Arsenic is a toxic metalloid which is found all over the globe. Due to its toxicity and wide abundance, all living organisms have evolved intrinsic arsenic resistance systems. In this study, three proteins that provide arsenic resistance in the Gram-positive bacterium *Bacillus subtilis* have been investigated. Acr3 is a trans-membrane protein that extrudes arsenite to the cell exterior. It belongs to the ACR family of arsenite transporters. Experimental data on the topology of Acr3, the first ever for a member of the ACR family, is provided and show that Acr3 has 10 transmembrane helices. Both the N- and C-terminal ends of Acr3 are located to the cytoplasm and the protein has unusually short loops connecting its helices. ArsR is an arsenite sensitive transcription regulator that controls the expression of genes that encode arsenic resistance proteins. Experiments show that the operator site for ArsR from *B. subtilis* consists of a 6-6-6 inverted repeat and that DNA binding by ArsR involves formation of higher order multimers of the protein. The ArsK (former YqcK) protein has an unknown function. The present study shows that the *arsK* gene contributes to resistance towards both arsenite and arsenate. The results indicate that the function of ArsK is important in an aerobic environment and that it decreases the inhibitory effect that rsenite has on the sporulation efficiency of *B. subtilis*. A function of ArsK that involves an enzymatic addition of low molecular weight thiols to arsenic is proposed.

Key words: *Bacillus subtilis*, arsenic resistance, arsenate reduction, arsenite transport, gene regulation, arsenite, arsenate, transmembrane topology, ArsR, ArsB, Acr3, ArsK

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Every day is a journey,
and the journey itself is home

Matsuo Basho

Table of contents

1 List of papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

I. Aaltonen, Emil K.J. and Silow, Maria. Transmembrane topology of the Acr3 family arsenite transporter from *Bacillus subtilis*. Biochimica et Biophysica Acta, 2008. 1778(4): p. 963-73.*

II. Aaltonen, Emil K.J., Cohn, Marita, and Silow, Maria. DNA binding of the *Bacillus subtilis* arsenite responsive transcription regulator ArsR. Manuscript.

III. Aaltonen, Emil K.J. and Silow, Maria. Deletion of *Bacillus subtilis arsK* (*yqcK/orf2*) affects aerobic growth and sporulation in the presence of arsenic. Manuscript.

* Reprinted from Biochimica et Biophysica Acta, 1778(4), Emil K.J. Aaltonen, Maria Silow, *Transmembrane topology of the Acr3 family arsenite transporter from* Bacillus subtilis, p. 963-973, 2008, with permission from Elsevier.

2 Introduction

This thesis is focused on arsenic and the mechanisms that confer arsenic resistance in prokaryotes. I have studied these mechanisms in the Gram-positive bacterium *Bacillus subtilis*. The main aim of the thesis is to both summarize and present the research I have done and to put it into context with what is known in the field. To accomplish this, I have structured the thesis in three parts; Part I, II and III. The first part gives the reader a general understanding of arsenic. It includes a brief introduction to arsenic, the history of the element and how arsenic affects an alarmingly large number of people. Following this short introduction, I present the main kinds of arsenic, how they are formed and how they affect living cells. The final sections of Part I deal with the important phenomenon of arsenic respiration and how arsenic is internalized in cells.

In Part II of the thesis I describe, in more detail, the proteins that are considered to be the main mediators of arsenic resistance in prokaryotes. Our research concerns three of these proteins and it has resulted in three papers (I-III). The main aim of Part II is to relate our results to, as well as integrate them with, the overall understanding of prokaryotic arsenic resistance. Part II ends with a summary of the papers presented in part III, together with a discussion on the future perspectives of our research.

Part III at the very end of the thesis is composed of the manuscripts that have resulted from our research. The papers give a detailed account for the methods that have been used, each individual result, as well as derived conclusions and discussions of these.

I would like to briefly comment on the choice of title for my thesis. Even though the title as well as the first sentence in this chapter specifies "prokaryotic", the following text includes examples from the Domain of *Eukaryota*, e.g. humans, rats and yeasts. This is simply because a vast amount of research has been conducted on the interaction between arsenic and eukaryotes. Thus, even though this is a thesis in microbiology, the present knowledge on eukaryotes is an invaluable asset when discussing the interactions between arsenic and prokaryotes. Also, the readers who possess knowledge about the phylogeny of microorganisms might wonder why I chose "prokaryotic" instead of "bacterial" – a valid question since I have focused my studies on the bacterium *Bacillus subtilis* and the term Prokaryote comprises both *Bacteria* and *Archaea*. The answer is that many features of arsenic resistance are similar between the two Domains, and the mechanisms discussed below are not only applicable to *Archaea*, they are also in some cases exemplified by them.

3 Part I – A Brief Background

The toxic metalloid arsenic (As) is element nr 33 in the periodic table of elements and belongs together with nitrogen, phosphorous, antimony and bismuth to group XV. Arsenic is the 20th most abundant element in the Earth's crust and has been present on our planet since its birth. Human activity such as coal combustion, mining and use of arsenic in agriculture has led to severe arsenic pollution. However, the main causes of spread throughout the biosphere are volcanic activity, weathering and leaching [1].

The occurrence of arsenic in the environment is of great concern, mainly because of contaminated drinking water. The World Health Organisation (WHO) has set the provisional guideline value of arsenic to 10µg/L, meaning that water with arsenic concentrations that exceed this limit is considered unsuitable for consumption [2]. However, at least twenty regions, dispersed across all continents, contain hydrologic sources with arsenic concentrations exceeding 10µg/L, and approximately 100 million people risk disadvantageous health effects due to arsenic ingestion [3]. The geographical location that harbours the largest population being exposed to dangerously high levels of arsenic is the Ganges-Brahmaputra-Meghna region in India and Bangladesh. Here, the arsenic concentration in waters can exceed 4000µg/L and 50 million people are estimated to be subjected to contaminated drinking water [3, 4]. The situation has been described as the "largest poisoning of a population in history" [5].

The vast human exposure to arsenic is of great concern due to the metalloids' inherent toxicity. In humans, arsenic ingestion can lead to arsenicosis, arsenic poisoning, which in its early stages manifests as melanosis and keratosis

(abnormal deposits of melanin and a hardening of the skin) on the hands and feet [6]. Prolonged exposure to arsenic has been shown to elevate the risk of developing several cancers, including lung cancer, skin cancer and bladder cancer [7, 8]. Arsenic is also known to increase the risk of developing type 2 diabetes [9] and it can cause severe neurological effects [10].

Although the main public topic of arsenic has recently been focused on contaminated drinking water, the story of arsenic use and abuse stretches further back in time and people have throughout history found practical uses of the metalloid. Under the names '*Poison of the Kings and the King of Poisons*' and '*inheritance powder*', arsenic trioxide (As_2O_3) has since ancient times been known to be a potent poison. People have also found other, less morbid, areas of use for arsenic. It was for long used in beauty products, paint and in tapestries. During the last century arsenic was used as a wood preservative, in electronics, in pesticides and as an additive in poultry food to protect against parasites and increase growth [11]. In contrast to its cause of mass poisoning via drinking water, arsenic also has a history of successful use in medicine. During the 18th century Thomas Fowler dissolved arsenic trioxide in potassium bicarbonate and the resulting Fowlers' solution was used for treatment of different infections and diseases [12]. In 1908 Paul Ehrlich won the Nobel Price for his invention of Salvarsan, an arsenic-containing ointment developed for treating syphilis [13, 14]. Today, arsenic has gained a lot of attention in the development of treatments for multiple myeloma [15], promyelocytic leukemia [12, 16] and it is presently under trial for treatment of solid tumors [17].

3.1 Arsenic species and their toxicity

Arsenite and arsenate

Arsenic is predominantly present as the trivalent arsenite (As(III)) and the pentavalent arsenate (As(V)). The biologically most important form of arsenite is arsenic trioxide (As_2O_3). Arsenic trioxide has a very high pKa (pKa=9.2) and is as a result uncharged at neutral pH [18-20]. Arsenite reacts strongly with thiols and is more toxic than arsenate. Many enzymes contain reactive thiol groups. Thus, these enzymes risk loosing their activity if they are exposed to arsenite [21]. Arsenite also forms strong bonds with the imidazolium nitrogens of histidine residues [19], which could possibly interfere with, for example, heme binding in cytochromes.

The biologically most important form of arsenate is arsenic acid (H_3AsO_4), which exists as the arsenate oxyanion at neutral pH [18-20]. Arsenate has a similar structure and similar chemical properties to phosphate. As a consequence, arsenate is able to compete with phosphate in chemical compounds. Of critical concern for living organisms is the ability of arsenate to induce arsenolysis, a process which uncouples the production of adenosine-5'-triphosphate (ATP) from respiration. In contrast to the stable, "high energy" P-O bonds in ATP, the corresponding As-O bond is not stable. This causes the bond to spontaneously hydrolyse, ultimately depleting the cell of ATP [21]. Arsenate has also been proposed to interfere with DNA integrity in a similar manner [22].

Methylated arsenic

It has for a long time been known that both mammals and bacteria can methylate arsenic to form non-volatile monomethylarsonic acid (MMA^v) and

dimethylarsenic acid (DMA^{V}), and volatile trimethylarsine oxide ($TMAO^{V}$), trimethylarsine (TMA^{III}) and dimethylarsine (DMA^{III}) [23]. The Cyt19 enzyme which methylates arsenite in rat has recently been identified [24]. The sequence of Cyt19 is very similar to that of proteins found in mouse and humans. Thus, these proteins have been suggested to be members of the Cyt19 family of arsenite methyltransferases. Cyt19 methylates inorganic arsenite to form MMA^{V} by addition of a methyl group originating from *S*-adenosylmethionine (AdoMet). MMA^{V} is then reduced by thioredoxin (Trx) to MMA^{III} which is methylated to DMA^{V} by Cyt19 [24]. As summarized in [25], further methylation and reduction steps converts DMA^{V} to $TMAO^{V}$ and TMA^{III}. DMA^{V} can also be reduced to DMA^{III}. Arsenite methylation was for a long time considered a way to detoxify arsenic [26]. The main reason for this was that the methylated end products are usually less toxic than inorganic arsenite [24]. However, several recent studies have shown that methylated arsenic is in fact more toxic than inorganic arsenic. MMA^{V}, for example, perturbs phospholipid bilayers more effectively than arsenate [27] and MMA^{III} is a stronger deactivator of thioredoxin and the nucleotide excision repair system of damaged DNA than arsenite [28, 29]. As a consequence, methylation of arsenic is no longer considered to be a mechanism of detoxification [29]. On the other hand, expression of *Rhodopseudomonas palustris arsM* has been shown to increase arsenic tolerance in *E. coli* [30]. ArsM was the first arsenite-*S*-adenosylmethyltransferase to be identified in bacteria. It mediates resistance by catalyzing the production of TMA^{III} which can leave the cell due to its volatility [30, 31]. Even though arsenic methylation yields highly toxic compounds, it is clearly too early to dismiss the methylation process as a way of detoxification.

Arsenic and oxidative stress

In several studies, arsenic has been shown to induce the formation of reactive oxygen species, ROS, in cells [32]. ROS have one or more unpaired electrons and two important forms are the superoxide ($O_2^{\cdot-}$), and the hydroxyl ($^{\cdot}OH$) radicals. Superoxide and hydroxyl radicals are produced at low levels under normal physiological conditions when oxygen accepts electrons from redox enzymes, e.g. terminal oxidases and xanthine oxidases [33]. They are also formed by ionizing radiation [34, 35]. Under normal conditions, superoxide is rapidly converted to hydrogen peroxide by superoxide dismutase [36] and hydrogen peroxide is subsequently removed by catalases and glutathione peroxidases [37]. However, arsenic can induce the formation of ROS that overload the ROS detoxification enzymes and other redox maintaining systems.

The toxicity of ROS is multifaceted. One significant effect is the induction of DNA damage, including DNA strand breaks and DNA oxidation [32]. More than 100 ROS-induced oxidized DNA-products have been identified, all of which are associated with mutagenesis and carcinogenesis in humans [35]. Reactive oxygen species can also initiate the autocatalytic oxidation of lipids, resulting in the highly toxic malondialdehyde (MDA) [38]. Formation of MDA can deplete the cell of lipids and result in DNA-DNA or DNA-protein crosslinking due to formation of MDA-guanine adducts [35]. There are several ways by which ROS can oxidize proteins and amino acid side chains [39-41]. The amino acid side chains most susceptible to ROS oxidation are Arg, Glu, His, Val, Tyr, Cys and Met. Oxidation of the last three can lead to protein crosslinking [42]. Cys and Met are especially vulnerable to oxidation by ROS [43]. In addition, superoxide can release iron from enzymes containing iron-sulfur clusters [44, 45]. The free iron is reduced by superoxide and becomes available for the Fenton reaction which generates hydroxyl radicals (Fe(II) +

H_2O_2 -> Fe(III) + $^{\cdot}OH$ + OH^-) [46]. The net yield is the formation of more ROS.

The mechanisms by which arsenic causes the formation of ROS can be either direct or indirect. Ahmad *et al* [47] showed that both DMA^V and DMA^{III} efficiently cause a release of iron from horse spleen ferritin and they proposed that the free iron could contribute to ROS formation by the Fenton reaction. It has also been demonstrated that DMA^V causes damage to DNA by the formation of superoxide, hydroxyl or peroxyl radicals [48]. The trivalent species As(III) and MMA^{III} are known to bind to thioredoxin and glutathione and inactivate glutathione- and thioredoxin reductases [28, 49-51]. The glutathione- and thioredoxin redox systems are involved in maintaining the intracellular redox homeostasis [52]. By disrupting these systems, arsenic cripples a vital part of the cells ROS detoxification scheme. It has also been suggested that by accepting electrons from either water or oxygen, As(III) can spontaneously be oxidized to As(V). Hydrogen peroxide is produced in the process [22]. In conclusion, the formation of ROS is an aspect of arsenic toxicity.

3.2 Arsenic respiration

In the process of cellular respiration, nutrients are converted to biochemical energy. In aerobic respiration, the oxidation of organic compounds is coupled to the terminal reduction of oxygen. Initially, a dehydrogenase oxidizes an electron donor. Electrons are then shuffled through a membrane bound electron transport chain and are finally used by a terminal oxidase to reduce molecular oxygen. Bacteria are known to utilize many different dehydrogenases and several terminal oxidases. In *B. subtilis*, the membrane bound type 2 NADH dehydrogenase and succinate dehydrogenase are main dehydrogenases

[53, 54]. Upon oxidation of either NADH or succinate, the enzymes transfer electrons to menaquinone which is reduced to menaquinol. Menaquinol is oxidized by cytochrome *bd* or cytochrome aa_3, both of which are terminal oxidases [55]. Alternatively, menaquinol can be oxidized by the cytochrome *bc*-complex which then reduces cytochrome caa_3. The cytochrome *bc* complex translocates protons [56, 57]. The reduced terminal oxidases transfer electrons to molecular oxygen which is reduced to water. This final reduction of oxygen by oxidases results in further proton translocation [55]. The net result is the formation of an electrochemical gradient across the membrane. This gradient can be utilized by the bacterium in several different ways, e.g. to actively transport solutes across the membrane (4.2 The arsenic resistance protein - Arsenite extrusion, p. 23), to energize flagellar motion [58] and most importantly, to drive the synthesis of ATP by ATP synthase [59].

Some prokaryotes are able to use arsenic in respiration, either as an electron donor (aerobic conditions) or acceptor (anaerobic conditions) [18, 60]. Arsenic respiration can influence both the speciation of arsenic and its mobility. This in turn can have a large impact on the environment. The importance of microbial arsenic respiration has been illustrated in Bangladesh, where a number of studies have shown that microorganisms at large contribute to arsenic contamination of water supplies [11, 61, 62]. The contamination is a result of the ability of bacteria to release arsenic from sediments, aluminum oxides, ferrihydrites and other minerals [18, 63].

Bacteria that use arsenate as terminal electron acceptor in the electron transport chain are referred to as dissimilatory arsenate-reducing bacteria. They comprise members of the *Crenarchaeota*, *Aquificae*, *Chrysiogenes*, *Deferribacteres*, low GC gram-positive bacteria, *Halanaerobacter* as well as the γ-, δ- and ε-Proteobacteria [60]. Dissimilatory arsenate reduction is mediated by a

heterodimeric enzyme consisting of a catalytic ArrA subunit and a smaller electron transferring ArrB subunit. The ArrAB-complex can be either soluble and located in the cytoplasm [64] or anchored to the cytoplasmic membrane [65, 66]. The enzyme belongs to the family of DMSO reductases, which are all members of the mononuclear molybdate class of enzymes [67]. The ArrA polypeptide from *Shewanella* sp. Strain ANA-3 (95.2kD) contains an iron-sulfur cluster coordinating domain (C-x_2-C-x_3-C-x_{27}-C), a molybdopterin dinucleotide binding domain, and a TAT-export (twin-arginine translocation) signal sequence. The corresponding ArrB subunit (25.7kD) and contains four putative iron-sulfur cluster domains [68]. ArrAB-complexes from other prokaryotes exhibit similar characteristics [64, 65]. The *Shewanella* sp. Strain ANA-3 *arrA* gene is only expressed under anaerobic conditions when arsenic is present and when electron acceptors with more favorable redox potentials, e.g. nitrate (the midpoint potential of arsenate/arsenite is +130mV compared to +420mV of nitrate/nitrite) are absent [69]. In contrast to the ArsC arsenate reductases, which receive their reducing equivalents from glutathione or thioredoxin (see 4.2 The arsenic resistance proteins – Arsenate reduction, p. 19), the dissimilatory arsenate reductases reduce arsenate using electrons from the respiratory electron transport chain. In *Shewanella* sp. Strain ANA-3 the electrons are transferred from the quinone pool to the Arr-complex by the CymA periplasmic *c*-type cytochrome [70].

Bacterial arsenite oxidizers are phylogenetically diverse and include α-, β-, and γ-proteobacteria, *Thermus* [11], and *Archaea* [71, 72]. However, in contrast to anaerobic arsenate reduction, respiration by arsenite oxidation is mainly aerobic and coupled to the reduction of molecular oxygen [18]. Even though the arsenite oxidases are members of the DMSO family of reductases they are phylogenetically separate from arsenate reductases. The arsenite oxidases are heterodimers consisting of AoxA and AoxB polypeptides. The catalytic ~90kD

AoxB subunit contains an iron-sulfur cluster and a molybdenum atom coordinated by four sulfur ligands [73, 74]. The smaller ~14kD AoxA subunit contains one Rieske-type iron-sulfur cluster and a TAT-signal sequence [18, 73]. Even if arsenite oxidation is usually coupled to metabolism (chemolithoautotrophic arsenite oxidizers), some species do not use arsenite as an electron donor in respiration (heterotrophic arsenite oxidizers). This suggests that arsenite oxidation also can be exclusively used a mechanism of arsenic resistance [1, 75, 76].

Oremland and Stolz [11] have proposed a model of how prokaryotic arsenate reduction and arsenite oxidation may contribute to arsenic mobilization and contamination of water supplies. In summary; initial activity of both heterotrophic and chemolithoautotrophic arsenite oxidizing prokaryotes mobilize arsenic from arsenite-containing minerals. Over time, this results in accumulation of arsenate which is subsequently adsorbed to oxidized minerals. Various human activities elevate the waters' oxygenation which leads to increased bacterial growth and arsenite oxidation and a subsequent accumulation of microbial organic material. Following the increase in biomass, anoxic conditions arise. This in turn supports the activities of prokaryotic arsenate reducers that release arsenite into the aqueous phase. Over time, these processes increase the arsenite concentration to toxic levels and result in heavily contaminated waters.

3.3 Mechanisms of arsenic uptake

Not much is known about how extracellular arsenic affects bacterial cells. However, considering the mechanisms of toxicity discussed above and the fact that almost all components and reactions which sustain the life of the cell are enclosed within the phospholipid bilayer, it is reasonable to believe that

intracellular arsenic exerts a larger threat to the cells' viability compared to extracellular arsenic. The phospholipid bilayer is a semi permeable barrier which prohibits the free diffusion of hydrophilic molecules, including arsenic. Movement of molecules across the membrane occurs instead via a large number of trans-membrane transporters with varying specificity. Since there are no known proteins that are used to specifically transport arsenic into the cell it is of interest to discuss how the metalloid is able to enter the cytoplasm.

Phosphate is of large importance to the cell. In *Escherichia coli* phosphate is known to be internalized via two different transport systems; the Pit- and the PhoS-PstABC systems. Because of the chemical similarity between arsenate and phosphate, both systems mediate the uptake of also arsenate [77, 78]. Similarly, the yeast *Saccharomyces cerevisiae* phosphate transporters Pho84, Pho86 and Pho87 have been shown to internalize arsenate [79]. *B. subtilis* has both a Pst- and a Pit transport system. In contrast to the situation in *E. coli*, the *B. subtilis* Pst system does not mediate uptake of arsenate [80]. However, *B. subtilis* sensitivity to added arsenate is decreased by phosphate in the medium (E. Aaltonen and M. Silow, unpublished) which suggests that arsenate enters the cell via a phosphate transporter. Since only two phosphate transport systems have been described in *B. subtilis*, arsenate uptake in this organism most likely occurs via by the Pit transporter.

Arsenite is not internalised by phosphate transporters. It has been suggested that $As(OH_3)$ can form six membered rings similar to that of hexose [20]. Indeed, studies have shown that arsenite internalization in *S. cerevisiae* is facilitated by the hexose permeases [20]. In addition, the structure of $As(OH_3)$ is similar to that of glycerol which explains why the glycerol facilitators Fps1p in *S. cerevisiae* and GlpF in *E. coli* have been found to transport arsenite to the cytoplasm [81, 82]. Array studies show that the *B. subtilis* glycerol facilitator

gene *glpF* [83] is downregulated upon arsenic exposure (M. Silow, unpublished). A deletion of *glpF* has also been shown to decrease *B. subtilis* arsenite sensitivity [84]. This suggests that GlpF is the entry point of arsenite in *B. subtilis*. Glucose is mainly transported by the *B. subtilis* glucose-PTS system and the GlcP permease [85] but it is not known if either of these systems also transports arsenite.

4 Part II - Arsenic Resistance

Because of the toxicity and ubiquity of arsenic most living organisms have evolved intrinsic systems for arsenic resistance. Since the article describing an attempt to construct an arsenic resistant strain of *Spirochaeta pallida*, the causative agent of syphilis, in 1948 [86], the scientific community has managed to identify and characterize many of the systems that mediate bacterial arsenic detoxification. Most of the prokaryotic arsenic resistance systems share three main features: 1) reduction of arsenate to arsenite, 2) extrusion of arsenite to the outside of the cell, 3) control of functions 1 and 2 by regulation of gene expression. Features 1-3 are mediated by three proteins. ArsC reduces arsenate to arsenite. ArsB and Acr3 are integral membrane proteins that transport arsenite across the cell membrane. ArsR is a negative regulator which represses the expression of the resistance genes in the absence of arsenite. In addition to these three core components, there are several proteins which have been found to contribute to arsenic resistance. Some of these work together with the above proteins, e.g. ArsD in *E. coli* [87] whereas other proteins seem to function independently, e.g. ArsM in *Rhodopseudomonas palustris* [30]. The genes for the proteins with peripheral function are usually encoded together with the genes for the core proteins. Interestingly, even though the functions of the resistance proteins are conserved throughout the *Bacteria*, their amino acid sequences as well as the structural- and mechanistic properties vary. There is also a wide diversity in the genomic location and context of the genes encoding the resistance proteins. Resistance genes are most often clustered together in what are called *ars* operons [88] but they can also be dispersed in the genome [89]. Operons and individual resistance genes are located on chromosomes [88, 90], on plasmids [91-93] or on transposons [94]. In addition, even though the

order of the genes in identified *ars* operons often is *arsR, arsB, arsC*, the number of resistance genes, their order and their orientation can differ. *Bacillus clausii* KSM-k16, for example, has two *arsR* genes within the same operon, *Thiobacillus ferrooxidans* has two unusual divergently transcribed *arsC-arsR* and *arsB-arsH* operons [95] and the orientation of the *arsR* gene in the *ars* operon of *Nostoc* sp. PCC7120 is reversed with respect to the other resistance genes.

Arsenic resistance genes are essential for the survival of prokaryotes in an arsenic contaminated environment. The disruption of a single gene can drastically affect the arsenic tolerance of the organism (c.f. Paper III) and the removal of an entire resistance operon is usually fatal if the cell encounters even low levels of the metalloid. The opposite is true with respect to adding resistance genes. A general rule is that more resistance genes confer a higher tolerance towards arsenic [94, 96]. One of the most arsenic resistant bacteria isolated so far is *Corynebacterium glutamicum* [96]. It contains two separate resistance operons and two additional isolated resistance genes, comprising a total of 9 resistance genes. *C. glutamicum* can grow at up to ~12mM arsenite, compared to ~5mM for *E. coli* [96] and ~4mM for *B. subtilis* (Paper III). When multiple copies of its resistance operons were introduced, *C. glutamicum* cells were viable at 60mM arsenite, a previously undescribed level of resistance [89].

4.1 Arsenic resistance in *Bacillus subtilis*

B. subtilis is an aerobic, spore-forming bacterium with soil as its natural habitat. It belongs to the family *Bacillaceae* but unlike its relatives *Bacillus cereus* and *Bacillus anthracis* it is not pathogenic. The bacterium has served as a model organism for the Gram positive order for the past 50 years and its

biochemistry, genetics and physiology has been extensively mapped. The genome of *B. subtilis* strain 168 was completely sequenced in 1997 [97].

Arsenic resistance in *B. subtilis* is mediated by two resistance systems. The four-gene *ars* operon, located in the *skin* element on the bacterial chromosome, is responsible for resistance to arsenate and arsenite. It consists of *arsR*, *arsK* (*yqcK*/ORF2), *acr3* (*arsB)* and *arsC* [88]. The *ars* operon accounts for approximately 50% of the arsenite and 100% of the arsenate tolerance. The other resistance system is encoded by the chromosomal two gene *ase* operon. It encodes AseR, a negative transcriptional regulator, and AseA, an arsenite extrusion pump [98, 99]. The Ase system provides 40% of the resistance to arsenite but since it contains no arsenate reductase it does not provide resistance to arsenate [98]. The proteins encoded by the *ars* and *ase* operons are considerably different. Acr3 belongs to the ACR family of transporters and its amino acid sequence shares only ~10% identity with that of AseA. In contrast, the amino acid sequence of AseA shares 57% identity to that of ArsB from *E. coli* (E. Aaltonen, and M. Silow, unpublished), the founding member of the dual mode transporters which are phylogenetically distant from the ACR family. ArsR and AseR both belong to the ArsR/SmtB family of repressor proteins [98](Paper II). However, they are only ~27% identical on the sequence level (E. Aaltonen, and M. Silow, unpublished). Whereas both repressors contain putative α3 arsenite binding sites, ArsR also contains an α5 site (see 4.2 The arsenic resistance proteins – Transcription regulation, p. 29). Additionally, the proposed AseR DNA binding site [99] is different from the ArsR DNA binding site (Paper II). It is believed that the *ars* operon is a recent lateral acquisition [88, 100] while the *ase* operon may be ancestral and originating from before the event of oxygenation of the atmosphere [98].

4.2 The arsenic resistance proteins

The following sections describe what is known about the individual components that contribute to arsenic resistance. The first three sections discuss the individual core components of the resistance machineries, i.e. arsenate reduction, arsenite extrusion and transcription regulation. The last section deals with additional proteins that have been found to play a role in arsenic resistance in prokaryotes.

Arsenate reduction

Arsenate reductase catalyzes reduction of arsenate to arsenite [101]. Excluding the respiratory arsenate reductases and methylation reductions discussed in the Arsenic species and their toxicity-section above, three classes of bacterial arsenate reductases have been described [102]. They are all relatively small proteins (~135 amino acid residues) and act as soluble monomers in the cytoplasm. They all reduce arsenate to arsenite via several steps of thiol-mediated redox-reactions. The mechanisms of reduction, however, have evolved independently of each other [103].

The first class is composed of Trx-coupled arsenate reductases. The most well studied ArsC proteins are found in *B. subtilis* and *Staphylococcus aureus* (encoded on the pI258 plasmid). These enzymes are similar both in their structure and their reduction mechanism and they are related to the low molecular weight protein phosphotyrosine phosphatases [104, 105]. The Trx-coupled reductases are mostly found in Gram positive bacteria, but they have also been identified in two Gram negative species [95, 106] and possibly also in the Archaeon *Archaeoglobus fulgidus* [102].

Trx-coupled arsenate reductases contain three conserved cysteine residues (Cys10, Cys82 and Cys89 in *B. subtilis* and *S. aureus* ArsC). The cysteines are critical for the enzymatic function [107] and they function in a disulfide cascade mechanism (Figure 1) [108]. The reaction cycle is initiated with a nucleophilic attack of Cys10 on arsenate which results in a covalent Cys10-As(V) intermediate. Cys82 then attacks Cys10 and forms a Cys10-Cys82 intermediate with the subsequent release of As(III). Finally, Cys82 is attacked by Cys89 which leads to the formation of a Cys82-Cys89 intermediate [107]. Oxidized ArsC is then regenerated and converted into its reduced and active state by intermolecular thiol-disulfide exchange reactions with Trx [109, 110].

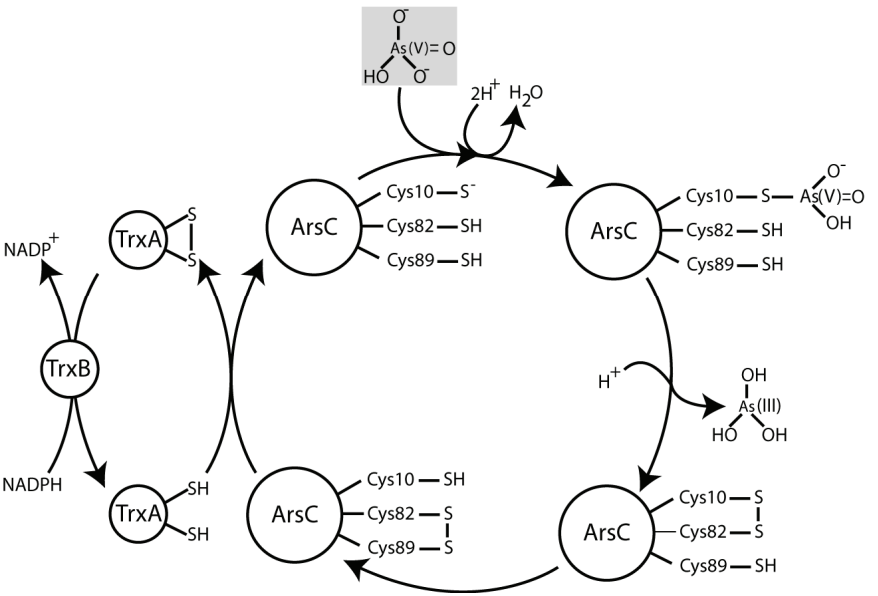

Figure 1. Trx-coupled arsenate reduction by ArsC. Grey arsenate marks the startpoint. *B. subtilis* thioredoxin A (TrxA) and thioredoxin reductase B (TrxB) are used in this illustration. Adopted from [102].

The *B. subtilis* thioredoxin A (TrxA)-ArsC interaction occurs via a nucleophilic attack of TrxA-Cys29 on ArsC-Cys89 which regenerates the ArsC-C82 and -89 thiols. A subsequent attack of TrxA-Cys29 on TrxA-Cys32 results in the formation of an intramolecular Cys29-Cys32 disulfide bond [109]. Trx is in turn recycled to its reduced state by thioredoxin reductase and NADPH [109-111]. Thioredoxin is known to only interact with oxidized ArsC, but how it discriminates between the two oxidative states of ArsC is under some debate. Based upon studies of *S. aureus* ArsC it has been proposed that the Cys82-Cys89 region in oxidized ArsC loops out to the surface of the protein and interacts with Trx [112]. However, a recent study on *B. subtilis* ArsC has shown that the loop does not contribute to the TrxA-ArsC interaction. Instead, upon formation of the Cys82-Cys89 disulfide bridge, a conformational change moves the segment spanning between Lys88 and Val99. Residues Lys88-Pro94 were seen to constitute the main contact points with Trx. In addition, the conformational change exposes Cys89 which at this stage points towards the surface of the protein. Thus, it is likely that the conformational change as well as the position and reactivity of Cys89 cooperate in mediating the ArsC-TrxA reaction [109].

The second class of arsenate reductases use glutathione (GSH) as a reductant. The model enzyme for this class is encoded by *arsC* on the *E. coli* plasmid R773. The sequence similarity between the *E. coli* ArsC protein and ArsC in *B. subtilis* and *S. aureus* is low (about 10%) and there are few structural similarities between the enzymes [102, 113]. Moreover, only one of the two cysteines found in *E. coli* ArsC, Cys12, is essential for catalysis. The other two thiols used in the disulfide cascade mechanisms are present in glutathione (GSH) and glutaredoxin (Grx) [102]. The proposed thiol-disulfide reaction cascade therefore differs significantly from the Trx-ArsC mediated arsenate reduction (Figure 2). The reaction cycle is initiated by a nucleophilic attack of

Cys12 on arsenate which results in a covalent Cys12-As(V) intermediate. GSH then binds to the arsenic by displacing a hydroxyl group. The resulting glutathionylated As(V) intermediate is subsequently reduced to a covalently

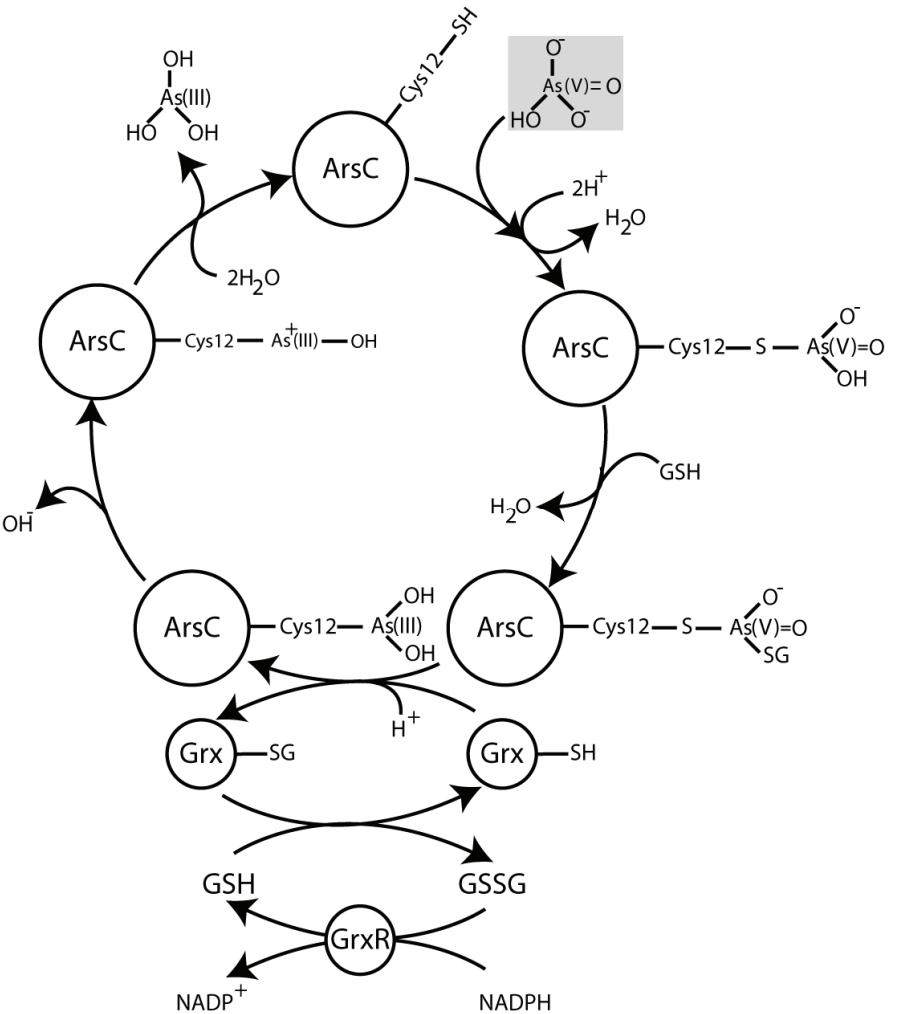

Figure 2. GSH-coupled arsenate reduction. Grey arsenate marks the startpoint. GSH; glutathione, Grx; glutaredoxin, GrxR; glutaredoxin reductase. Adopted from [102].

bound dihydroxy As(III) intermediate by Grx and oxidized Grx-SG is released. Then in two steps the dihydroxy As(III) intermediate is first converted to monohydroxy As(III) and then to free As(III) by hydroxylation [114, 115]. Glutaredoxin is regenerated by glutaredoxin reductase and NADPH [116].

The third class of reductases is exemplified by ArsC from *Synechocystis* sp. Strain PCC 6803. The amino acid sequence of this enzyme is highly similar to those of ArsC from *S. aureus* and *B. subtilis*. However, it shares characteristics with both the Trx- and the GSH- coupled ArsC enzymes [117]. Although the activity of ArsC from *Synechocystis* sp. strain PCC 6803 is dependent on three reactive cysteine residues (Cys8, Cys80 and Cys82), similar to ArsC from *S. aureus* and *B. subtilis*, the enzyme uses GSH and Grx as reductants. The proposed reduction mechanism is similar to the first steps of *E. coli* ArsC, up to the formation of the ArsC-glutathionylated As(V) intermediate. However, instead of the following formation of a dihydroxy arsenate intermediate, the arsenate is reduced and released as arsenite and a Cys8-GSH disulfide remains. After a subsequent shuffle of GSH to Cys80 or Cys82, ArsC is reduced and reactivated by Grx [117].

Although not being a bacterial reductase, it is worth mentioning the eukaryotic Acr2p arsenate reductase from *S. cerevisiae*. Acr2p is evolutionarily independent of the bacterial enzymes. It is very similar to ArsC from *E. coli* with respect to the use of GSH and Grx and the reaction schemes are rather similar. However, it differs from the previously discussed arsenate reductases in that it functions as a homo-dimer [102, 118].

Arsenite extrusion – Paper I

The central feature of arsenic resistance in prokaryotes is the transport of arsenite from the cytoplasm to the outside of the cell. This extrusion is

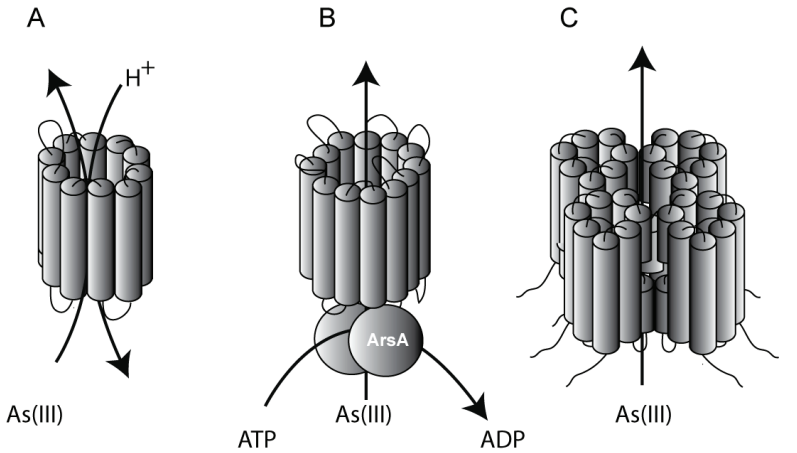

Figure 3. Cartoon representations of three types of arsenite transporters found in bacteria. A) An ACR transporter with 10 trans-membrane (TM) helices and with a putative H^+/As(III) antiport mechanism (Paper I). B) An ArsB transporter with 12 TM-helices associated with dimeric ArsA ATPase. C) Hypothetical organization of the AqpS channel with four AqpS monomers with 6 TM-helices each. It should be noted that the organization of the TM helices has not been determined for any of the transporters. Thus, the cartoons are simplified illustrations.

accomplished by specialized trans-membrane proteins that can be divided into three distinct families depending on the protein structure and the mechanism of pumping (Figure 3).

The ACR family of arsenite transporters is represented by the 346 residue Acr3 (previously ArsB, Paper I) from *B. subtilis* and the 404 residue Acr3p from the eukaryote *S. cerevisiae* (Figure 3A) [88, 119, 120]. Since these proteins are unrelated to ArsB from *E. coli* and *S. aureus* we have renamed *B. subtilis* ArsB as Acr3 (Paper I). Members of the ACR family are phylogenetically wide spread and are found in Gram-positive and Gram-negative bacteria as well as in *Archae* and *Eukaryota* [121, 122]. The ACR proteins belong to the BART (bile acid/arsenite/riboflavin transporter) superfamily [121]. It was previously believed that ArsB-type transporters were more common than those of the

ACR family. However, a recent analysis of bacteria in soil samples showed a significantly larger prevalence of ACR-type transporters [122]. Interestingly, the use of either an ACR- or ArsB-transporter does not seem to be a factor in determining the level of arsenic tolerance [122].

Members of the ACR family have been much less studied compared with the ArsB-type of transporters. Little is known about their structure or mechanism of transport. Previous studies predicted that the ACR transporters have 10 trans-membrane (TM) α-helices. Since they have not been found to be coupled to ATPase activity it is speculated that the extrusion of arsenite is driven by the proton motive force by a $As(OH)_3/H^+$ antiporter mechanism [88, 119].

We contributed to the knowledge of this large class of arsenite transporters by a study of the trans-membrane topology of *B. subtilis* Acr3 (Paper I). The results

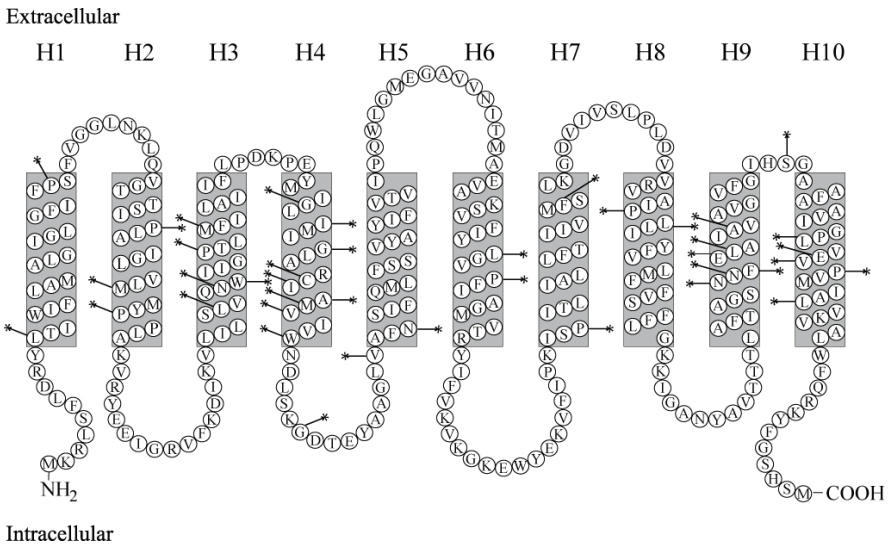

Figure 4. Topological model of *B. subtilis* Acr3 in the cytoplasmic membrane (Paper I). Residues conserved in six ACR family members are marked with an asterisk (*). H1 to H10 indicate trans-membrane helices.

of the study provided the first experimentally determined structural information on a member of the ACR family of arsenite transporters. By combining *in silico* topology modeling with activity of green fluorescent protein (GFP) and alkaline phosphatase (PhoA) reporter proteins fused to a range of C-terminally truncated Acr3 variants, we concluded that Acr3 has 10 TM helices and both the N- and C-terminus located in the cytoplasm (Figure 4). The protein has short extracellular loops and slightly longer intracellular loops. A comparison between *B. subtilis* Acr3 and ACR representatives from a range of Gram-positive and Gram-negative bacteria as well as from yeasts showed that all proteins share a similar topology. Interestingly, 38 or 39 of the 41 conserved residues are located within TM helices, including the single cysteine which is found in TM helix 4. In addition, 8 of the conserved residues are prolines which may indicate that several of the helices in the ACR proteins contain multiple helix kinks [123].

Nothing is yet known about how Acr3 extrudes arsenic or which residues are actively involved in the transport mechanism. It is interesting that TM helices 4, 5, 9 and 10 contain a significant amount of the residues that are conserved in the ACR family members analyzed in Paper I. These residues could be intimately involved in arsenite transport. In the "Future perspectives" section (p. 39) I describe ongoing work to further characterize the *B. subtilis* Acr3 transporter.

The so called dual mode transporters, such as ArsB from *E. coli* plasmid R773 (Figure 3B), were the first family of arsenite transporters to be characterized. They are also the most studied transporters. Homologs of *E. coli* ArsB can be found in both Gram-positive and Gram-negative bacteria [122]. These proteins belong to the Major Facilitator Superfamily (MFS) and extrude arsenite driven by the electro chemical gradient or by hydrolysis of ATP by a

second protein, the ArsA ATPase [124, 125]. The *E. coli* ArsB and ArsA proteins form an $ArsA_2B$ complex which is able to extrude arsenite at a much higher rate than ArsB alone [125]. The 429 amino acid ArsB has been shown to have 12 trans-membrane α-helices. Both the N- and C-termini are oriented to the cytoplasm [126]. The 583 residue ArsA protein contains two homologous segments, A1 and A2, with a 25 amino acid linker in between. Both halves contain nucleotide binding domains (NBD). Segment A1 contains two cysteine residues (C113 & C172) whereas segment A2 contains one cysteine (C422). These three cysteines function as a binding site for arsenite. Upon binding of one arsenite, the A1 and A2 segments are brought together. This in turn brings the NBDs in close proximity to each other. As a result, ATP is hydrolyzed and energy is provided for the extrusion of arsenite through the ArsB-membrane channel [127]. In the absence of ArsA, the extrusion of arsenite by ArsB is powered by the charge difference across the membrane and the protein functions as a $As(OH)_3/H^+$ antiporter [81, 125].

It has recently been found that a third protein, ArsD, also participates in arsenite extrusion by the *E. coli* ArsAB complex. The homodimeric ArsD protein has been found in *E. coli* [126], *Shewanella* species strain ANA-3 [128], *Acidithiobacillus caldus* [94], and *Halobacterium* sp. strain NRC-1 [129]. It was previously thought that ArsD functions as a transcription regulator controlling the 'upper limit' of transcription of the arsenic resistance genes [130]. However, it was recently found that ArsD acts as a metallochaperone that scavenges the cytosol for free arsenite and delivers it to ArsA [87]. The interaction between ArsD and ArsA enhances the catalytic affinity of ArsA. I.e., the transfer of arsenite from ArsD to ArsA increases the efficiency of arsenite extrusion. Because ArsD has a higher affinity for arsenite than ArsA, ArsD confers more efficient resistance at lower concentrations of arsenite [87].

The amino acid sequence of ArsB from *E. coli* is 58% identical to the sequence of ArsB encoded by *Staphylococcus aureus* plasmid pI258 [125] and 57% identical to the *B. subtilis* AseA sequence [99]. The latter two are encoded by operons that do not encode ArsA homologs. However, when *arsA* from *E. coli* was expressed *in trans* together with *arsB* from *S. aureus* it was found that ArsA and ArsB functioned together and provided a higher level of arsenite extrusion than ArsB alone [125]. It is possible that ArsA homologs are encoded elsewhere on the chromosomes of *B. subtilis* and *S. aureus* or that ArsB from *S. aureus* and AseA from *B. subtilis* might be coupled to other ATPases and transport substrates other than arsenite. This does not seem likely however since ArsB from *S. aureus* is specific for arsenite [125].

The third family of bacterial arsenite transporters is represented by AqpS, the major arsenite transporter in *Sinorhizobium meliloti* (Figure 3C). The 233 residue amino acid sequence of AqpS is similar to the bacterial glycerol facilitator (GlpF) and the yeast aquaglyceroporin Fps1. AqpS facilitates unidirectional diffusion of arsenite. It only confers resistance towards arsenite. The *aqpS* gene is situated in an operon that contains an *arsC* gene, the product of which is similar to the arsenate reductase ArsC from *E. coli*. In a proposed detoxification scheme, arsenate enters *S. meliloti* through phosphate transporters, is reduced to arsenite by ArsC and the subsequent increase in the intracellular concentration of arsenite drives its transport through AqpS to the cell exterior [131]. The trans-membrane topology of AqpS has not been investigated. However, considering its sequence similarity with *E. coli* GlpF the possibility exists that it also has the structure of a tetramer in which each monomer has 6 TM-helices and two helices which span through half the membrane [132, 133].

There is also a fourth family of arsenite efflux proteins. However, it is not found in prokaryotes. It belongs to the multidrug resistance-associated protein (MPR) subgroup of the ABC transporter superfamily and is represented by Ycf1p protein from *S. cerevisiae* [134]. Ycf1p is a close relative to the human MRP, and has been shown to pump a wide diversity of metal substrates conjugated to glutathione. The energy for pumping is provided by hydrolysis of ATP. In contrast to the dual mode- and ACR transport families, which transport arsenite to the exterior of the cell, Ycf1p transports glutathione-metal conjugates into the vacuole for sequestration [135]. Ycf1p is predicted to contain three trans-membrane domains (Tmd1-3). Tmd1 has 4-6 α-helices whereas Tmd 2 and 3 each have 6 α-helices [136]. Ycf1p contains two nucleotide binding folds (NBF) which together constitute the ATP hydrolyzing domain. One NBF is located in the cytosolic region between Tmd2 and Tmd3 together with a regulatory domain and the other NBF is located in the cytosolic domain following Tmd3 [137]. The exact mechanism of ATP hydrolysis and substrate pumping is unknown. It is thought that the binding and hydrolysis of ATP induces a conformational change which allows substrate to pass through the protein [138].

Transcription regulation – Paper II

Proteins that confer arsenic resistance are not constitutively produced by their host cells. The cells have the ability to "sense" arsenite and as a response "turn on" the expression of resistance genes. The sensing is mediated by negative transcription regulators named ArsR. In the absence of arsenite this dimeric regulator is bound to a region of DNA located just upstream of the *ars* genes (the operator sequence) and thereby it inhibits the binding of RNA-polymerase. If arsenite enters the cell it binds to specific sites on the ArsR

protein, causing the protein to dissociate from the DNA. This allows gene transcription and production of arsenic resistance proteins.

ArsR is a founding member of the ArsR/SmtB family of metalloregulatory proteins [139]. All members of this family are homodimeric metal sensitive transcriptional repressors that control the expression of specific metal resistance genes. Although the structure of ArsR has not been solved, it is believed to be similar to that of several other members of the family; i.e. to have five α-helices and two β-strands arranged into a α1-α2-α3-α4-β1-β1-α5-fold. The α3-turn-α4 region constitutes a helix-turn-helix (HTH) DNA binding motif of which the α4 helix is speculated to interact with the major groove. Helices α1 and α5 from one monomer interact in an antiparallel manner with the corresponding helices of the second monomer to form the dimer interface domain [140].

ArsR/SmtB regulators bind to DNA at specific inverted repeats which are usually organized in a 12-2-12 pattern. These repeats are located near to, or overlapping with, the transcriptional start site of the regulated gene or operon. A comparison of inverted repeats shows that the ArsR/SmtB proteins SmtB, ZiaR, CzrA and NmtR bind to a consensus sequence of aAtAxxTGAaca-xx-tatTCAxaTxtt (capital letters; conserved residues, lower case letters; semiconserved residues, x; non-conserved residues). The operator consensus sequence of ArsR from *E. coli* ($ArsR_E$) and CadC from *S. aureus* ($CadC_S$) is TAxAxTCAAAta-xx-taTTTGaxTxTA [140]. Although monomeric SmtB binds weakly to DNA [141, 142], the ArsR/SmtB regulators most often bind as multimeric complexes consisting of two ($CadC_s$, [143]) or four homodimers (CzrA, [140]).

B. subtilis ArsR binds to a 50 bp double stranded oligonucleotide that includes a previously identified [88] 8-2-8 inverted repeat (IR1) as well the *ars* operon

Figure 5. Operator sequence alignment. A) Alignment of the operator sequences of the *B. subtilis ars* operon and of operons encoding ArsR$_B$ homologs. Asterisks (*) indicate conserved nucleotides. Boxed regions on the *B. subtilis* sequence show IR1. Arrows and shaded nucleotides indicate IR2. B) Comparison between the operator consensus sequence of ArsR$_E$/CadC$_S$ and ArsR$_B$ homologs. The ArsR$_E$ contact motifs are shaded.

-35 promoter region (Paper II). The binding is reversed by adding arsenite but not arsenate. A previous study [88] suggested that IR1 is the ArsR$_B$ binding site. However, experiments with mutant variants of the 50 bp oligonucleotide show that only the first half of IR1 is essential for ArsR$_B$-DNA binding and that neither the -35 region nor the second half of IR1 is important (Paper II). IR1 is not similar to known operator sequences for other ArsR/SmtB regulators, and is not conserved in the operator regions of *ars* operons that encode ArsR$_B$ homologs (Paper II). We identified a 6-6-6 inverted repeat (IR2) in the DNA region located upstream from the *ars* operon where it overlaps with the first half of IR1. IR2 is conserved in other bacteria encoding ArsR$_B$ homologs (Figure 5A). IR2 is strikingly similar to the operator sequences of CadC$_S$ and ArsR$_E$, including the TCAT and TTTG motifs identified as the DNA contact points for ArsR$_E$ [139] (Figure 5B). These findings strongly

suggest that IR2 is the $ArsR_B$ operator site (Paper II). Interestingly, a comparison between the operator consensus sequences of the $ArsR_B$ homologs and $ArsR_E$/$CadC_S$ shows that there is a difference in the number of nucleotides that separates each half of the inverted repeats (Figure 5B). This may reflect a yet undetermined factor in protein-DNA specificity (Paper II).

DNA binding by $ArsR_B$ involves formation of higher order multimers of $ArsR_B$ (Paper II). Although the phenomenon is expected and known for other ArsR/SmtB proteins, this is the first example of formation of higher order multimers in an autoregulated arsenic responsive ArsR repressor. It may indicate that multimerization plays an important role in transcription regulation by ArsR proteins.

Metal recognition and binding by ArsR/SmtB proteins is conferred by two different sites in the protein. The α3, or type 1, binding site is composed of one core motif and one variable component. The core component is a highly conserved Cys-Val-Cys-Asp (CVCD) motif located in the α3 helix. The $C^{58}VC^{60}D$ core motif in $CadC_S$ is complemented by an N-terminal C^{7}-X_3-C^{11} motif. Metal binding in $CadC_S$ is conferred by C^{7} and C^{11} from one monomer and the C^{58} and C^{60} residues from the other monomer [144]. The CVCD core component of *E. coli* plasmid R773 ArsR ($ArsR_E$) is instead complemented by a third cysteine, forming the binding motif $C^{32}VC^{34}DLC^{37}$. The $ArsR_E$ monomers bind arsenite independently of each other by using all three cysteines to coordinate the ion [145]. *Mycobacterium tuberculosis* CmtR ($CmtR_M$) binds cadmium with its type 1 site, but uses a C-terminal cysteine, C^{102}, from the second monomer to coordinate the ion [146]. Metal binding in $CadC_S$, $ArsR_E$ and $CmtR_M$ is presumed to induce a conformational change in the DNA binding HTH domain which leads to protein dissociation from the DNA.

The α5, or type 2, binding site of ArsR/SmtB proteins is located on the α5-helix and is thus situated in the dimerization domain. Until recently only one main type of α5 binding site had been identified. This consists of an Asp-X-His-X_{10}-His-X_2-Glu/His (DxHx10Hx_2E/H) motif. The use of this α5 metal binding site is exemplified by *Synechococcus* PCC7942 SmtB ($SmtB_S$) where D^{104} and H^{106} from one monomer together with H^{117} and E^{120} from the second monomer coordinate a zinc ion [147]. However, a recent study of ArsR from *Acidithiobacillus ferrooxidans* ($ArsR_A$) identified a novel ArsR α5 arsenite binding site consisting of a $C^{95}C^{96}X_5C^{102}$ motif. $ArsR_A$ lacks the α3 binding site found in $ArsR_E$ and in contrast to $SmtB_S$ the three cysteine residues of $ArsR_A$ coordinate arsenite within each monomer [148]. It is speculated that a conformational change induced by α5 metal binding results in regulator dissociation from the DNA.

Some ArsR/SmtB family members contain only a type 1 metal binding site (e.g. $ArsR_E$). Other proteins of the family contain only a type 2 binding site (e.g. $ArsR_A$, CzrA and NmtR), whereas still others contain both types (e.g $CadC_S$ and $SmtB_S$). In the latter case it seems as if only one of the sites is essential for metalloregulation. The only known exception is *Synechocystis* ZiaR ($ZiaR_S$) which depends on both type 1 and type 2 binding sites to sense zinc [140]. Interestingly, *B. subtilis* ArsR contains at least two putative arsenite binding sites, one α3 and one α5 (E. Aaltonen and M. Silow, unpublished). Although these sites have not been studied, $ArsR_B$ may be the first identified arsenite responsive ArsR regulator with two arsenite binding sites. The α3 site has a conserved $C^{38}VC^{40}D$ motif and an additional C^{32} residue. A cysteine corresponding to $ArsR_B$ C^{32} is also found adjacent to the CVCD motif of $CadC_S$, but is not involved in metal binding. Also, although C^{37} of the $ArsR_E$ $C^{32}VC^{34}DLC^{37}$ motif is involved in arsenite coordination, it is not essential.

Thus, it is possible that the $ArsR_B$ α3 site binds arsenite either with or without the C^{32} residue. The α5 binding motif corresponds to the $ArsR_A$ binding site and consists of two conserved cysteine pairs, $C^{93}C^{94}$ and $C^{104}C^{105}$, that are located at the C-terminus. These residues may function as an α5 binding site, but it is also possible that they work similar to $CmtR_M$ C^{102}, i.e. by delivering arsenite to the α3 binding site.

Other resistance proteins

Many arsenic resistance systems in prokaryotes include proteins other than arsenate reductases, arsenite transporters and transcription regulators. Some of the proteins are without known exact function whereas others have functions that have been characterized in greater detail.

ArsK – Paper III

The *arsK* (previously *yqcK* or ORF2) gene was first identified in *B. subtilis* [88] and genes encoding proteins with sequences similar to that of ArsK are present in *ars* operons of at least twelve other species belonging to the firmicutes, cyanobacteria, actinobacteria and proteobacteria (Paper III). *arsK* encodes the 146 residue ArsK protein and has in two separate studies, by an in-frame deletion (Paper III) and a plasmid insertion disruption [88], been shown to contribute to resistance against low levels of arsenite, as well as high levels of arsenate. Interestingly, an *arsK* deletion mutant and wild type *B. subtilis* cells grow similarly for some time before the arsenic sensitivity phenotype of the mutant becomes apparent (Paper III). This indicates a delayed need for ArsK after the onset of arsenic stress.

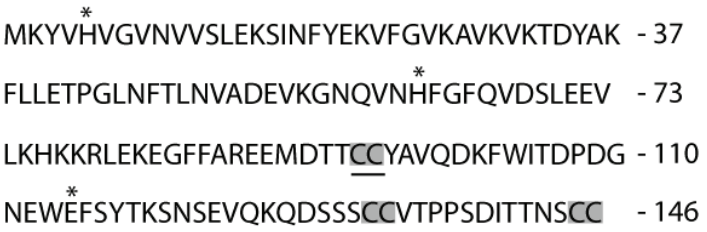

Figure 6. Amino acid sequence of ArsK. Asterisks (*) indicate putative metal coordinating residues. The cysteine pair found to be conserved in all ArsK homologs is underlined and shaded. Remaining cysteine residues are shaded.

The amino acid sequences of ArsK and its homologs are similar to that of members of the Vicinal Oxygen Chelate (VOC) superfamily (Paper III). The dimeric VOC-family proteins catalyse a diverse set of reactions, including isomerizations (glyoxalases) and nucleophilic subsitutions (fosfomycin resistance proteins). All of them, except the bleomycin resistance proteins, contain a metal ion cofactor [149]. Interestingly, three of the four residues known to coordinate a Ni^{2+} ion in *E. coli* glyoxalase I (GlxI) [150] are conserved also in all ArsK homologs (Figure 6) (Paper III). The same three residues have been proposed to function as metal coordinators in the fosfomycin resistance proteins [149]. The similarity between ArsK and members of the VOC-family indicates that ArsK may be an enzyme containing a tridently coordinated metal ion cofactor (Paper III). Another interesting feature of ArsK is that it contains three pairs of cysteine residues (Figure 6), all of which are absent in the known VOC proteins. One pair is conserved in all the investigated ArsK homologs. When projected and modelled onto the structures of *E. coli* GlxI [151] and *Pseudomonas aeruginosa* fosfomycin resistance protein A (FosA) [152], this cysteine pair is located on a loop with close access to the active sites of the enzymes (Paper III). Considering the strong affinity of arsenite for dithiols [51], this may indicate that the cysteines

are involved in the function of ArsK (Paper III). All but one of the ArsK homologs also contains one, two, or three additional cysteine pairs in their C-terminal part. Although these residues cannot be projected or modelled onto the structures of GlxI and FosA due to low sequence similarity, they may serve a specific function, e.g. scavenge free arsenite for transfer to the active site or sequester arsenite in a manner similar to ArsD [87].

Based on the functions of various VOC-family enzymes, we hypothesise that ArsK functions similarly to the fosfomycin resistance proteins (Paper III). These inactivate the antibiotic fosfomycin by coupling it to cysteine or glutathione [153, 154]. Since *B. subtilis* does not encode genes needed for glutathione biosynthesis, we speculate that ArsK uses cysteine, coenzyme A (CoA) or the newly identified thiol U12 [155, 156]. The possession of ArsK may allow the cell to specify which thiol is to be used in the detoxification process and thereby limit the toxic effects of arsenic, including that on the intracellular thiol pool.

In support of this hypothesis we found that an *arsK* deletion mutant is more sensitive to arsenite in the presence of oxygen (Paper III). As discussed above, arsenic is known to induce oxidative stress and low molecular weight thiols play an important role in the protection against oxidative stress. Thus, it is possible that the *arsK* deletion mutant is impaired in its thiol homeostasis which renders it more sensitive to arsenite induced oxidative stress. Our experiments also showed that an *arsK* deletion mutant has reduced sporulation efficiency in the presence of arsenite (Paper III). The sporulation process includes a complex set of events, some of which depend on the formation and breaking of disulfide bonds [157]. In parallel with the oxidative stress experiment, the sporulation deficient phenotype of the *arsK* mutant may be an effect of a disrupted thiol homeostasis.

ArsK is undeniably an important part of the *B. subtilis* resistance machinery. However, one may validly argue that any disruption of the arsenic detoxification process could result in oxidative stress- and sporulation phenotypes similar to that of the *arsK* deletion mutant. No matter what the function of ArsK is, its removal is likely to result in elevated levels of intracellular arsenic. This can by itself increase the degree of oxidative stress and affect the sporulation process. Thus, even if the results support our hypothesis, we need to confirm it with more experiments. Some of these experiments are described in the "Future perspectives" (p. 41) section.

ArsM

The *arsM* gene was initially found coupled to an *arsR* gene in the haloarchaeon *Halobacterium* sp. strain NRC-1. A deletion of *arsM* resulted in increased arsenite sensitivity [129]. The amino acid sequence of ArsM is strikingly similar to those of both human methyltransferases and *Magnetospirillum magnetotacticum* S-adenosylmethionine-dependent methyltransferase. This led to the speculation that ArsM may function as an arsenite-methyltransferase [129]. A later study showed that a hypersensitive *E. coli* strain expressing *arsM* from *R. palustris* had an increased arsenic tolerance and that arsenic in this strain was converted to volatile TMA^{III} gas with DMA^{V} and $TMAO^{V}$ as intermediates [30, 31]. A subsequent analysis of purified ArsM showed that it catalyzes the transfer of methyl groups from *S*-adenosylmethionine (AdoMet) to arsenite [30]. This suggests that the production of volatile arsenic species by ArsM may be a detoxification mechanism [30, 129].

ArsH

The ArsH proteins were first identified in *Yersinia enterocolitica* [93] and later in *Acidothiobacillus ferrooxidans* [95], *Synechocystis* sp. strain PCC6803 [158] and *S. meliloti* [131]. ArsH has been shown to be necessary for resistance to both arsenite and arsenate in *Y. enterocolitica* and *S. meliloti* [93, 131], but it is not critical for resistance in *Synechocystis* sp. or *A. ferrooxidans* [95, 158]. The amino acid sequence of ArsH shows similarities to those of NADPH-dependent mononucleotide reductases [131, 158] and it produces hydrogen peroxide by reducing oxygen via a NADPH:FMN oxidoreductase mechanism [159]. The 241 residue ArsH from *S. meliloti* was recently crystallized and found to be a tetramer [159]. The exact mechanism by which ArsH contributes to arsenic detoxification is not known. It is speculated that it either oxidizes arsenite to the less toxic arsenate [158, 159] or reduces arsenite to volatile arsines [159].

ArsO and ArsT

Two novel arsenic resistance coupled genes were recently found on the linear plasmid pHZ227 in *Streptomyces* sp. strain FR-008 [160]. The *arsT* gene encodes a putative thioredoxin reductase and is cotranscribed with an *arsR* gene. The *arsO* gene encodes a putative flavin-binding monooxygenase and is localized in an *arsRBO* operon. The *arsT* gene is essential for arsenic resistance and the ArsT protein is likely to partake in arsenate reduction together with ArsC which has sequence similarities with Trx-coupled arsenate reductases. In contrast, the *arsO* gene is not essential for arsenic resistance. Considering its similarity to flavin-binding monooxygenases it may have a function similar to ArsH [160].

4.3 Summary of papers and future perspectives

Paper I – Acr3

We have analyzed the transmembrane topology of the ACR family arsenite transporter Acr3 from *B. subtilis*. Using *in silico* topology modeling we initially constructed two models with 9 and 10 TM helices, respectively. Subsequent experimental analysis of 32 translational fusions of a*cr3* with genes for alkaline phosphatase and green fluorescent protein showed that both the N- and C-termini are located in the cytoplasm. The experiments provided information about the locations of the majority of the cytoplasmic and extracellular loops of Acr3. Two data points indicated a possible 8 TM helix topology. However, the results from 10 additional translational fusions did not support this alternative topology. We concluded that Acr3 contains 10 TM helices. The results also indicate that helices 5 and 6 may be tilted within the membrane. A majority of the residues which were found to be conserved in six Acr3 homologs are located in TM helices.

Our future studies will focus on three aspects of Acr3; 1) the source of energy required for arsenite pumping, 2) the residues involved in arsenite pumping, and 3) the 3-dimensional structure. It has been speculated that Acr3 functions as an H^+/arsenite antiporter similar to $ArsB_E$ without the ArsA ATPase. We plan to investigate this possibility by studying Acr3 arsenite pumping in membrane vesicles. We are currently at a stage of purifying His_6-tagged *B. subtilis* Acr3 produced in *E. coli*. With the membrane vesicle system one can also assay the efficiency of arsenite pumping by Acr3. By mutating specific amino acid residues in Acr3 the assay will allow us to probe residues important for arsenite extrusion. An *in vivo* assay with the same purpose is also currently under development and aims to monitor arsenic sensitivity in a *acr3* deficient

strain which expresses mutated *acr3*. Although difficult, solving the crystal structure of Acr3 would give detailed insight into how the protein is organized and how it functions. The purification of heterologously produced Acr3 protein is our first step towards this goal.

Paper II – ArsR

Using a combination of modelling based on homologs and electrophoretic mobility shift assays we identified the operator site of $ArsR_B$ from *B. subtilis*. It consists of a single 6-6-6 inverted repeat and is located close to the -35 promoter element of the *ars* operon. The binding site is very similar to reported binding sites for ArsR/SmtB repressor proteins. $ArsR_B$ binds to double stranded DNA in a highly specific manner and the results indicate that the binding involves formation of higher order multimers of $ArsR_B$. This is the first time multimerization is reported for an autoregulated arsenic responsive ArsR repressor and the investigation is the first detailed study of DNA binding by an ArsR/SmtB repressor from *B. subtilis*.

We report a single 6-6-6 inverted repeat as the $ArsR_B$ operator site, but it remains to be determined which residues constitute the protein-DNA contact points. These residues are likely to include the conserved ATC and TGA motifs in each respective half of the repeat. However, this needs to be confirmed with footprinting and EMSA experiments. Our results suggest that the number of nucleotides that bridge the contact points may vary between different operator sequences. To our knowledge the plasticity of the spacing between the contact points recognized by ArsR/SmtB proteins has not been investigated. Thus, it would be interesting to determine how the spacing and sequence variables of the *ars* operon operator affect the binding of $ArsB_B$.

Another interesting feature of $ArsR_B$ is the presence of two putative arsenite binding sites. To distinguish if $ArsR_B$ makes use of only one or use all of the possible binding sites we have initiated a study to investigate which of the seven cysteine residues in $ArsR_B$ that are involved in arsenite binding. We have so far constructed a set of $ArsR_B$ mutants with different cysteine substitutions and we are currently studying the interaction between these variants and arsenic as well as between the variants and DNA.

Although the structures of several ArsR/SmtB proteins have been solved, there is no published structure of an arsenite responsive repressor. There is also no available structure of an ArsR/SmtB repressor bound to its cognate operator sequence. We are presently in the process of determining the structure of both apo $ArsR_B$ and $ArsR_B$ bound to DNA in solution using nuclear magnetic resonance (NMR) spectroscopy.

Paper III – ArsK

By constructing an in-frame deletion of *arsK* (*yqcK*) in *B. subtilis* and analyzing the arsenic tolerance of resulting strains we have shown that *arsK* contributes to arsenic resistance. The *arsK* deletion mutant is more sensitive to arsenite in the presence of oxygen and suffers decreased sporulation efficiency when arsenite is present. It is possible that these phenotypes are an effect of a disrupted low molecular weight (LMW) thiol homeostasis in the deletion mutant. On the sequence level, ArsK is similar to proteins belonging to the VOC-family of metalloenzymes. The three metal coordinating residues in the fosfomycin resistance proteins (Fos) are conserved in ArsK and its homologs. The function of Fos detoxification is to enzymatically couple cysteine or glutathione to fosfomycin. Considering the high affinity of arsenite for thiols we propose a similar detoxification mechanism for ArsK.

The results of the complementation of the *arsK* deletion mutant needs to be confirmed. By repeating the experiments at a lower level of arsenite we hope to increase the resolution of the results. Also, by acquiring and using antibodies directed against ArsK, we will investigate the presence of the ArsK protein in the complemented strains.

We are currently investigating the proposed function of ArsK. Using HPLC we analyze the low molecular weight thiol content of *B. subtilis* and how it changes during growth, both with and without arsenic in the medium. The results will be interesting by themselves since they may give insight into both normal and stress affected LMW thiol metabolism in real time. Comparing the results with those of similar experiments with the *arsK* deletion mutant could allow us to identify which LMW thiol compound is utilized by ArsK.

5 Populärvetenskaplig sammanfattning

Arsenik är ett naturligt förekommande och mycket giftigt grundämne. Det har i årtusenden nyttjats av människor i olika sammanhang och flera av dessa användningsområden, exempelvis gruvindustrin, har bidragit till enorma utsläpp av arsenik i vår miljö. Det är dock naturliga fenomen som ansvarar för den största frisättningen och spridningen av arsenik och man brukar räkna vulkanisk aktivitet, kemisk urlakning och erosion som de största orsakerna. Arseniks utbredda förekomst har på senare tid uppmärksammats som en allvarlig hälsorisk för omkring 100 miljoner människor runt om i världen. Det största hotet för människan är arsenikförorenat dricksvatten och man har funnit att människor som exponeras för arsenik löper hög risk att utveckla bland annat olika sorters cancer. Arsenik förekommer huvudsakligen i två former; som arsenit och som arsenat. Båda formerna kommer in i cellen genom proteiner som vanligtvis transporterar andra ämnen som cellen behöver. Arsenit reagerar med svavelgrupper i proteiner. Svavelinnehållande aminosyror är viktiga beståndsdelar i många enzym. Arsenit har en hög affinitet för aminosyrornas svavelgrupper och binder därför ofta till dessa. Inbindningen gör att enzymen förlorar sin aktivitet och arsenit kan på så sätt stänga av ett stort antal cellulära funktioner. Arsenat är däremot kemiskt likt fosfat och har förmågan att ersätta fosfat i ett antal viktiga molekyler. Problemet är att arsenat är instabilt när det används i molekyluppbyggnad och effekten är att dessa molekyler blir defekta och ofta förlorar sin funktion.

Lyckligtvis är alla levande organismer försedda med inbygda resistenssystem mot arsenik. De enklaste resistenssystemen finns i bakterier och består vanligtvis av tre olika proteiner; ArsB, ArsC och ArsR. ArsB är en pump som

sitter i bakteriens cellmembran. Pumpen har till uppgift att transportera arsenit från cellens insida (cytoplasma) till dess utsida. ArsB kan dock enbart transportera arsenit och proteinet ArsC har därför till uppgift att omvandla intracellulärt arsenat till arsenit. För att cellen ska veta när den ska producera ArsB och ArsC har den ArsR, som fungerar som en arseniksensor. I frånvaro av arsenik sitter ArsR bundet till ett område på cellens DNA som ligger precis bredvid de gener som kodar för resistensproteinerna. Denna inbindning gör att cellen inte kan ”läsa” resistensgenerna och därmed inte heller producera de kodade proteinerna. ArsR är mycket känsligt för arsenik och reagerar med det så fort det har tagit sig in i cellen. Resultatet av ArsR-arsenik-interaktionen är att ArsR lossnar från DNA:t och att cellen då kan producera ArsB och ArsC.

Vi studerar arsenikresistens i den jordlevande bakterien *Bacillus subtilis.* Dess resistens medieras av fyra proteiner, ArsR, AsrK, Acr3 och ArsC, varav vi har studerat de tre förstnämnda. Acr3 motsvarar den ovan beskrivna ArsB, men till skillnad från ArsB har man tidigare inte känt till hur Acr3-proteinet ser ut eller hur det fungerar. Vi har visat att Acr3 består av 10 stycken segment som vart och ett löper genom cellmembranet. Acr3 skiljer sig från det tidigare karakteriserade ArsB som har 12 st transmembrana segment. Vår strukturbestämning ligger till grund för framtida analyser av hur pumpen fungerar. Vi har även studerat ArsR från *B. subtilis* och kartlagt exakt var på DNAt proteinet binder för att blockera produktionen av de andra resistensproteinerna. DNA-sekvensen för inbindningsstället liknar tidigare identifierade inbindningsställen för ArsR från andra bakterier. Det finns däremot skillnader i hur sekvensen är strukturerad vilket kan innebära att vi har upptäckt en ny faktor som avgör var på DNAt som ArsR kan binda. Våra resultat visar även att flera ArsR proteiner kan binda till samma inbindningsställe på DNAt. Detta fenomen har man inte sett för andra ArsR

proteiner och det antyder att ArsR fungerar på ett mer komplicerat sätt än vad man tidigare har funnit.

Även ArsK är ett vanligt förekommande bakteriellt resistensprotein. Vi har visat att *B. subtilis*-celler som saknar den gen som kodar för ArsK är mycket känsligare för arsenik än celler som har genen kvar. Reultatet visar att ArsK har en roll i arsenikresistensen. Sekvensen av de aminosyror som bygger upp ArsK liknar den sekvens man hittar hos proteiner som tillhör en stor familj av enzymer som heter VOC. ArsK sekvensen är speciellt lik ett VOC-enzym som skyddar bakterier från ett visst antibiotikum. VOC-enzymet inaktiverar antibiotika genom att addera svavelrika molekyler. Med tanke på att arsenit ofta binder svavelrika aminosyror föreslår vi att ArsK-proteinet fungerar med en liknande mekanism, dvs att binda arsenit till specifika svavelinnehållande molekyler. En sådan funktion skulle minska mängden fri intracellulär arsenit och därmed skydda cellen.

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