

## Phytoremediation for Arsenic Contamination: Arsenate Reductase

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### Abstract

Arsenic is a toxic pollutant present in the environment that causes serious health issues. Phytoremediation is one potential solution to mitigate arsenic contamination in either soil or water. Two main forms of arsenic exist in the environment, arsenite, [As(III)], and arsenate, [As(V)]. In plant mechanisms, arsenate reductase reduces arsenate to arsenite with the help of thioredoxins or glutaredoxins, which serve as electron donors. Arsenite can be subsequently stored in the plant vacuole. In one study, an *Arabidopsis thaliana* transgenic plant was engineered to express bacterial arsenate reductase and glutamylcysteine synthase, which enhanced its ability to store two to three times more arsenic per gram of tissue than the non-transgenic plant. Further investigation of the mechanism of arsenic uptake in plants is needed to provide a foundation to develop better transgenic arsenic hyperaccumulators.

### Introduction

Arsenic contamination of groundwater is a natural occurrence in which a high concentration of arsenic is found present in lower levels of groundwater. Recently, an infamous incident of arsenic poisoning involving the Ganges Delta in Bangladesh and West Mongol, India, brought the public's attention to the fatal consequences of arsenic contamination in the delta's soil and water.<sup>1</sup> Moreover, there are reports detailing that Thailand, Taiwan, Argentina, Chile and China have also been seriously contaminated by arsenic<sup>2</sup> and even some locations in the United States have been known to be contaminated with relatively high

arsenic concentrations.<sup>3,4</sup>

Arsenic exposure can cause a variety of health problems including anemia, neuropathies, hyperpigmentation and skin irritation.<sup>5</sup> In addition, arsenic exposure for a prolonged period of time can result in skin lesions and skin cancers as well as result in internal cancers likely to occur in the bladder and the lungs.<sup>6</sup> The latency for skin lesions from arsenic exposure appears to be about 10 years, and in the case of skin cancer, the latency seems to be about 20 years.<sup>7</sup> Skin cancers can be curable if the treatment is appropriately addressed immediately. However, internal cancers attributed to arsenic exposure are critical for human health, and it has been reported that in Taiwan and Chile there are marked increases in mortality from internal cancers.<sup>6</sup> Due to the health problems arising from arsenic toxicity, many nations have adopted regulatory standards to restrict the arsenic amount in drinking water.

Although arsenic has multiple oxidation states (+5, +3, 0, -3), arsenite As(III) and arsenate As(V) are the most prevalent forms. Both As(III) and As(V) ionic forms are toxic to living organisms; however, As(III) is the more toxic of the two. Because its neutral form (HAsO<sub>2</sub>) is the dominant form at neutral pH, As(III) is more mobile, proving it to be far more toxic than As(V). The As(V) form is more stable, so the vast majority of arsenic is found in the oxidized state, which is the arsenate form, AsO<sub>4</sub><sup>3-</sup>. The complete arsenate form in the soil is thought to be FeAsO<sub>4</sub>.<sup>10</sup>

A possibility for the remediation of such arsenic contamination, phytoremediation, or bioremediation mediated by plants, could be the best solution, as it is a cost-effective method. Physical alternatives such as soil removal and burial are not only expensive, but also environmentally destructive. To investigate phytoremediation further, it is necessary to understand the process by which plants take up arsenate from the soil. In this review, we will discuss several candidates for phytoremediation, as well as the detailed enzymatic mechanisms of arsenate reductase and its electron donors, thioredoxins and glutaredoxins.

### Phytoremediation

Plants that possess the intrinsic ability to accumulate higher than normal levels of toxic heavy metals are known as either hyperaccumulators or metallophytes. To be considered a hyperaccumulator, the plant must have the ability to store increased amounts of heavy metals in

its aboveground parts.<sup>11</sup> To date, more than 450 metallophytes have been identified, and they typically only accumulate one specific metal.<sup>11</sup> In order to determine the concentration of normally toxic metals, these plants have developed mechanisms by which such metals are incorporated into complexes and subsequently transported into and stored in the vacuole.<sup>11</sup> Plants use various protein chelators in this process, including phytochelatins and metallothioneins. Both these biomolecules are replete with cysteine residues capable of complexing a variety of heavy metals as metal thiolates.<sup>11</sup>

Several hyperaccumulators are specific for arsenic and could potentially be utilized as bioremediators. *Lemna gibba* is one such species that has already been determined to naturally accumulate high levels of arsenic, and it is currently used both to monitor the degree of arsenic contamination and remove said contamination.<sup>11</sup> In addition, *Pteris vittata*, *P. cretica*, *P. longifolia*, and *P. umbrosa* are also able to accumulate high levels of arsenic.<sup>11</sup> *P. vittata* is one particularly efficient arsenic hyperaccumulator, being able to survive in arsenic-contaminated soil up to a concentration of 1.5 mg arsenic/g soil, and sequester 95% of absorbed arsenic in its aboveground tissues; other arsenic metallophytes average approximately 20% aboveground accumulation.<sup>12</sup> As of yet, however, no research has uncovered the enzymatic and genetic mechanisms by which this plant is able to accumulate arsenic to such a great extent.<sup>12</sup> Because of this lack of research, *P. vittata* has yet to be utilized for phytoremediation, although it could potentially perform this task exceedingly well.

Another potential arsenic phytoremediator is a desert plant, *Prosopis*. In a recent experiment, this plant species, commonly known as mesquite, was grown on separate media containing As(V) and As(III), respectively. The seedlings that grew on media containing As(V) were found to contain higher percentages of As and, furthermore, the arsenic was determined to be in its trivalent form: As(III).<sup>13</sup> This study concluded that mesquite could be a good phytoremediator for dry environments with high concentrations of arsenic since it was able to reduce absorbed arsenate.<sup>13</sup>

Although several excellent arsenic metallophytes exist naturally, transgenic organisms may offer more potential in terms of accumulating greater concentrations of arsenic over larger regions. In a recent experiment, Dhankher et al. transformed *Arabidopsis thaliana* with the *E. coli* gene R773 ArsC encoding arsenate reductase and the *E. coli*  $\gamma$ -ECS gene encoding  $\gamma$ -glutamylcysteine synthase in an attempt to create a transgenic

arsenic hyperaccumulator. The arsenate reductase was used in the plant to reduce arsenate to arsenite and the  $\gamma$ -glutamylcysteine synthase was added to catalyze the formation of  $\gamma$ -glutamylcysteine, which complexes with As(III) through its thiol groups, thereby rendering it inert and capable of being stored away in the vacuole. In this experiment, the ArsC gene was linked to the well-characterized soybean ribulose biphosphate carboxylase (rubisco) SRS1 gene, whose expression is light-induced.<sup>12</sup> The end result of this strategy was that ArsC was only produced in plant tissues exposed to light, i.e. aboveground tissues, meaning that, ultimately, arsenite could only be stored aboveground.<sup>10</sup> Through the course of the experiment, Dhankher's group found that when these two proteins were co-expressed, the transgenic plants were able to accumulate 2-3 times more arsenic than wild type plants.<sup>12</sup> Interestingly, the singly transgenic plants, those that only expressed either R773 ArsC or  $\gamma$ -glutamylcysteine synthase, fared little better than wild type plants when grown in high arsenic concentrations (200 M).<sup>10</sup> This finding is in line with the fact that merely reducing arsenate is of no benefit to the plant, especially since arsenite is more toxic than arsenate; the arsenite must also be complexed (in this case by  $\gamma$ -glutamylcysteine) and then stored away in the vacuole. Figure 1 illustrates the process by which a plant accumulates arsenic in the form of arsenite. Although this illustration shows arsenite-thiolate compounds being stored in both root and leaf vacuoles, ideally more arsenic should be stored in aboveground tissues so that it can be more readily harvested and removed from the environment.

### Enzymatic mechanism: arsenate reductase

Arsenate reductases, which catalyze the essential reduction reaction in the process seen in Figure 1, have been characterized for a wide array of organisms. In general, the operons responsible for conferring arsenic resistance in an organism consist of three genes. In *E. coli*, they are labeled as follows: *arsC*, which codes for the reductase enzyme; *arsB*, which codes for the arsenite transporter; and *arsR*, which codes for a repressor used in gene regulation.<sup>14</sup> Although the genes within this operon are labeled differently for different species, they are strikingly similar in function. It is important to note that, while this process does increase the toxicity of the As compound for the organism, As(III) is able to complex with free thiol groups, which detoxifies the compound.<sup>10</sup>

The active site of arsenate reductase contains a pair of cysteine

residues which are essential to its catalytic action. One of these cysteine residues is part of a highly conserved sequence known as the P-loop (Cys-X<sub>5</sub>-Arg); in general, this cysteine residue forms a thioester bond with As(V), and the arginine residue assists in stabilizing the intermediate.<sup>15</sup> <sup>16</sup> In order to perform this vital function, the enzyme itself must receive electrons from an outside source to regenerate its active form; that is, it must be reduced. Depending on the species of organism, this reducing agent is either a thioredoxin or a glutaredoxin (we will discuss later these two proteins in detail). Based on the type of reducing agent utilized, as well as protein structure, arsenate reductases can be separated into three distinct groups, which are exemplified by R773 ArsC from *E. coli*, Acr2p from *Saccharomyces cerevisiae*, and pI258 ArsC from *Staphylococcus aureus*.<sup>16</sup> Both R773 ArsC and pI258 ArsC are monomers, whereas Acr2p is a homodimer. However, R773 ArsC and Acr2p use glutathione and glutaredoxin as reducing agents, while pI258 ArsC uses thioredoxin.<sup>16</sup> Relatively recently, a unique arsenate reductase, which combines aspects of the above mentioned enzymes, was isolated from *Synechocystis* PCC 6803; that is, this enzyme's amino acid sequence, and therefore structure, is similar to that of pI258 ArsC, yet it uses glutaredoxin as a reducing equivalent.<sup>16</sup>

#### Electron donor protein: thioredoxin

Thioredoxins (Trx) and glutaredoxins (Grx), which are used by arsenate reductases to regenerate their active form, regulate disulfide bond formation and degradation in proteins in all organisms. Trx and Grx are characterized, in general, by a structural motif known as the thioredoxin fold.<sup>17</sup> This distinguishing structure, which consists of a four-stranded beta sheet surrounded by three alpha helices, is common to a variety of oxidoreductases but is primarily used to identify both thioredoxins and glutaredoxins. In addition to the thioredoxin fold, both groups of proteins also contain a Cys-X-X-Cys sequence in the active site, which is located between the first beta sheet and the first alpha helix.<sup>15, 17</sup> The two residues between these catalytically active cysteines vary greatly between proteins of different species, and the identity of these amino acids affects the redox potential as well as the efficiency of the individual protein.<sup>17, 18</sup> Trx and Grx are also essential in ensuring that proteins are folded correctly.<sup>17</sup> This finding should come as little surprise, since both Trx and Grx affect disulfide bond formation, which

is integral to correct protein structure and function.<sup>17</sup>

Thioredoxins have been studied extensively, and the mechanism by which they reduce disulfide bonds is well-characterized. In the redox reaction between the reduced or dithiol form of Trx and the oxidized or disulfide form of the target protein, the N-terminal active site cysteine first nucleophilically attacks one of the sulfur atoms of the target disulfide. The result is the formation of a mixed disulfide between Trx and the target protein. Finally, the Trx C-terminal active site cysteine attacks the N-terminal residue, which yields a disulfide bond between the two Trx cysteines. This step necessarily breaks the mixed disulfide bond and consequently leaves the target protein's cysteines in their thiol form.<sup>17</sup> In order to prepare Trx for continued catalysis, it must be reduced by thioredoxin reductase (TrxR), which converts the disulfide bond back to thiol groups.<sup>17</sup> This typical mechanism is shown in Figure 2.

Due to steric effects, Trxs are most easily able to reduce surface disulfide bonds. However, Messens et al. describe a process by which Trx can also reduce internal structural disulfide bonds by means of an intra-protein disulfide bond shuffle.<sup>19</sup> Essentially, Trx first reduces the external disulfide according to the mechanism described above. After this reduction, one of the newly reduced thiol groups bonds with one of the internal thiols to produce a mixed disulfide; this bond is then transferred to the exterior, where Trx easily reduces it again, thereby reducing the interior bond in the process.<sup>19</sup> The following figure, Figure 3, displays diagrams of this mechanism, where C<sub>10</sub>-C<sub>15</sub> represents the interior disulfide and C<sub>82</sub>-C<sub>89</sub> represents the exterior disulfide found in pI258 ArsC.

#### Electron donor protein: glutaredoxin

As seen in these diagrams, thioredoxins are perfectly capable reducing agents. Glutaredoxins, however, also perform the same function although they operate according to a completely separate mechanism. Whereas thioredoxins are reduced by thioredoxin reductase, glutaredoxins are reduced by the tripeptide  $\gamma$ -glutamylcystylglycine, or glutathione (GSH); the oxidized form of glutathione (GSSG) is in turn reduced by glutathione reductase.<sup>17</sup> Additionally, Grxs may act by either dithiol or monothiol mechanisms, depending on their active site sequence; monothiol Grxs have a serine residue in place of the second cysteine.<sup>17</sup> Monothiol Grxs are of particular interest since they are the

primary reducers of protein-glutathione mixed disulfides.

In the first mechanistic step, the As(V) bonds with the free thiol group of Cys<sub>8</sub>, producing an arsenoenzyme intermediate. The enzyme begins with a disulfide bond between Cys<sub>80</sub> and Cys<sub>82</sub>. Next, glutathione attacks the arsenate portion of the intermediate; this structure devolves into a glutathione-enzyme compound, releasing arsenite in the process. These first three steps comprise the actual reduction of arsenate to arsenite; the last three are involved with the regeneration of the active form of arsenate reductase.<sup>16</sup> The disulfide bond that existed between Cys<sub>80</sub> and Cys<sub>82</sub> is then transferred to Cys<sub>8</sub>, which moves the cysteine-glutathione mixed disulfide to a more accessible point on the protein's exterior.<sup>16</sup> The next step uses glutaredoxin to reduce this mixed disulfide. In this monothiol mechanism, only one of Grx's cysteine residues is utilized. In addition, the series of redox reactions presented in this step ultimately draw on energy and electrons supplied by NADPH (Nicotinamide adenine dinucleotide phosphate, a reducing agent) for the end reduction of the enzyme. Finally, the disulfide bond is transferred back to Cys<sub>80</sub> and Cys<sub>82</sub>, reactivating the enzyme for the next round of catalysis; recall that both thioredoxin and dithiol glutaredoxins are able to accomplish this intra-protein disulfide shift.<sup>16</sup> Although this mechanism has yet to be verified, it provides an excellent example of the means by which Grx functions in tandem with GSH to reduce disulfide bonds. Figure 4 illustrates the mechanism by which it is supposed that Grx functions in the reduction of the *Synechocystis* sp PCC6803 ArsC.

### Conclusion

Having discussed in some detail the mechanism by which arsenate reductase functions, we return to the pressing issue of arsenic contamination in the environment mentioned earlier. In general, plants import As(V) from the soil via phosphate carriers since the two compounds are structurally similar, and then reduce it to As(III) using arsenate reductase.<sup>12</sup> Upon reacting with free thiol groups, arsenite can be stored as an arsenite-thiolate complex. To date, several studies have been conducted on potential means of purifying contaminated areas via phytoremediation. In general, these studies fall into two categories: those which examine naturally occurring candidates for phytoremediation strategies and those which study the efficacy of genetically modified plants as bioremediators.

Dhanker's group demonstrated that it is possible to increase the amount of arsenic stored in aboveground tissues. Ideally, these transgenic hyperaccumulators should have deep and extensive root systems, large above-ground biomasses, and the ability to be easily harvested.<sup>11</sup> If Dhanker's results could be replicated in such an organism, arsenic contamination might be easily solved and become nothing but a relic of the past.

To reach this point, much additional research must be conducted in several different areas. The means by which the arsenite-thiolate compound moves into the vacuole is still unknown, although it clearly involves some sort of active transport. This transport protein should be identified and studied so that its mechanism can be understood for the purpose of streamlining the bioremediation process. Furthermore, while Dhanker *et al.* did successfully create efficient transgenic, arsenic hyperaccumulating plants, other plants are capable of naturally accumulating arsenic to much higher concentrations.<sup>12</sup> The mechanisms by which these plants accomplish this action should be studied so that they too could be maximized. On a related note, the thermochemistry of the glutaredoxin-glutathione-arsenate reductase reaction should be studied in greater depth. With increased research, the glutaredoxin most effective in reducing arsenate reductase could be isolated and incorporated into a transgenic metallophyte, further increasing the purifying capabilities of the organism. In short, the more we understand the details of each aspect of this purification process, the better our efforts will be for the removal of arsenic contamination.

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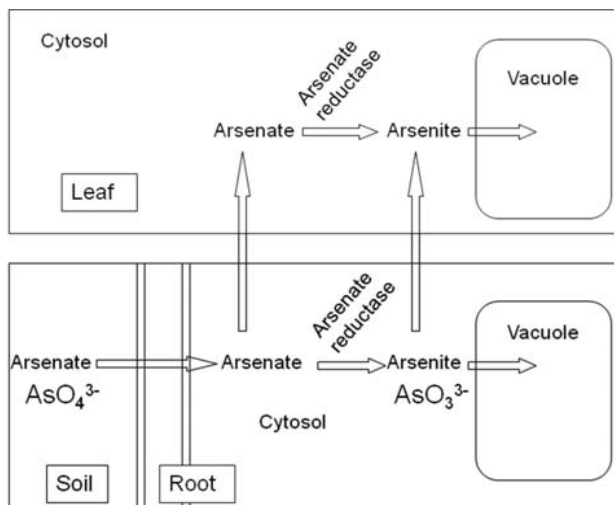
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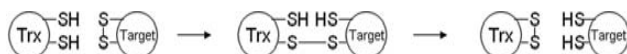
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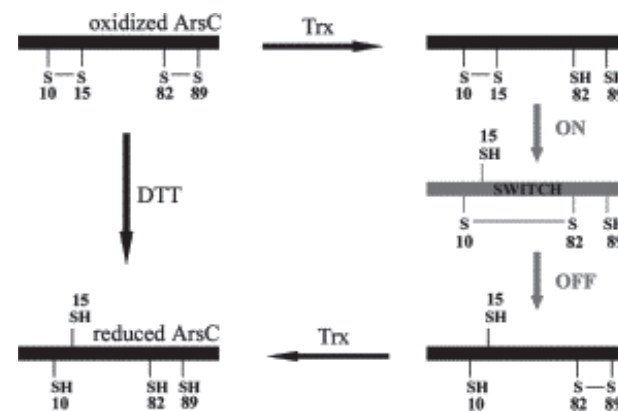
## APPENDIX OF FIGURES



**Figure 1.** The outline of arsenate uptake from a plant. The majority of arsenic form present in soil is arsenate ( $\text{AsO}_4^{3-}$ , possibly  $\text{FeAsO}_4$ ). Arsenate is taken up into the roots, passing through the plant cell walls, and arsenate is converted to arsenite by an arsenate reductase. The product arsenite is finally stored in the plant vacuole (modified from ref <sup>10</sup>).

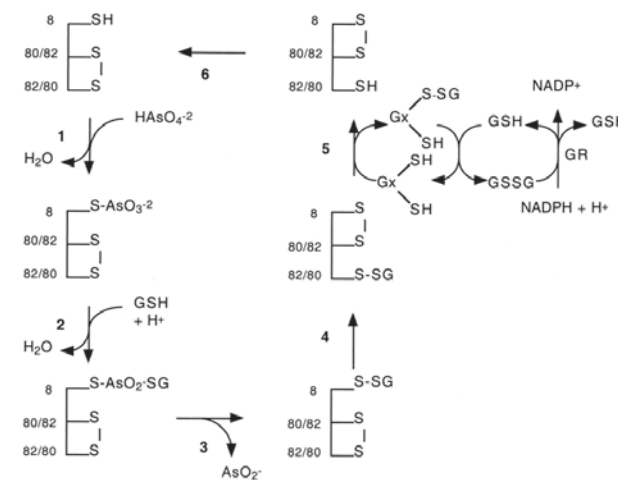


**Figure 2.** Typical interaction of Trx with a Trx-dependent protein (a target protein). A reduced Trx approaches an oxidized form of target protein, and subsequently a transient covalent bond is formed via thiol groups from each protein. Finally the target protein becomes reduced and the Trx becomes oxidized.



**Figure 3.** The possible mechanism of inter- and intra-molecular thiol/disulfide exchange in the *E. coli* arsenate reductase (taken from ref <sup>19</sup>).

**Figure 4.**



**Figure 4.** The plausible catalytic mechanism for the *Synechocystis* sp. Strain PCC 6803 arsenate reductase with an electron donor Grx. GR: glutathione reductase; GSH: reduced form of glutathione; GSSG: oxidized form of glutathione; Gx: glutaredoxin (taken from ref <sup>16</sup>).