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Proteomic Response to Arsenic Stress in *Chromobacterium violaceum*

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ABSTRACT

Exposure to arsenic, whether acute or chronic, is a public health problem in many parts of the world and is associated with various types of deleterious effects on human health. One way that risk of contamination with this metalloid can be reduced is through bioremediation of areas contaminated with arsenic. Natural resistance mechanisms are widely distributed in microorganisms, meriting further study in an effort to improve their efficiency. Chromobacterium violaceum is a betaproteobacterium found in tropical and subtropical regions; its resistance to arsenic is controlled by an operon, arsRBC. The proteins expressed by the operon ars have been well studied; however, the overall cell response that determines resistance to this metalloid is little understood. We investigated changes in protein expression in response to arsenite. This was done through two-dimensional differential gel electrophoresis (2D-DIGE). Quantities of 26 proteins were altered after treatment with arsenite, 23 of which increased. The differential spots were analyzed with MS and MS/MS; eight proteins were identified that are involved in response to oxidative stress (SOD, GST, Grx), in DNA repair and in the metabolism of lipids, amino acids and coenzymes. We conclude that the response of C. violaceum to arsenite involves defense mechanisms against oxidative stress and alterations in cell metabolic cycles.

Keywords: *Chromobacterium violaceum*; Arsenic; Proteomics; Bioremediation.

1. Introduction

Arsenic is a metalloid that is widely distributed on the earth's surface. The forms that are most important biologically are arsenate (As^{5+}) and arsenite (As^{3+}) [1]. The arsenic found in water and soil is there principally due to natural processes, such as volcanic emissions and leaching; though it is intensified by human activities, including mining and arsenical-containing fungicides, pesticides, herbicides, and wood preservatives [2].

Exposure to arsenic in drinking water is a public health problem that affects various parts of world. Inorganic arsenic is a human carcinogen and is associated with chronic and cardiovascular diseases [3]. Arsenite bonds strongly to thiol groups of proteins, while arsenate is a structural analog of phosphate and competes with this element in many phosphorylation reactions [4].

Currently, remediation of arsenic in water and soil is done through physical-chemical interventions; however, these are high cost, energy-intensive measures, and the resulting waste still needs to be discarded or eliminated [5]. Microorganisms have developed various strategies to counter the toxicity of arsenic that can be used in bioremediation, through bioprocesses of adsorption, precipitation, complexation and bioaccumulation [6]. Improvement of the capacity of these bacteria requires a better understanding of the resistance mechanisms of these organisms.

In nature, microorganisms respond to arsenic in different ways, but the most common pathway in bacteria is mediated by the operon *ars* [7], which codes for three proteins: an arsenate reductase (ArsC), an efflux pump (ArsB) and a transcription regulator (ArsR). Additionally, in some bacte-

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ria, the operon can include genes that code for an ATPase (ArsA) and a metallochaperone (ArsD) [8]. Arsenate reductase uses glutaredoxin or thioredoxin to reduce the arsenate to arsenite, which is expulsed from the cell by ArsB or by the ArsAB pump [1].

Chromobacterium violaceum is a Gram-negative, facultative anaerobic betaproteobacterium, found in the water and soil of tropical and subtropical regions [9]. It is an opportunistic pathogen and has a very versatile metabolism, making it capable of confronting diverse types of environmental conditions [10].

The most notable phenotypic characteristic of *C. violaceum* is violacein, a violet pigment that has antibacterial, antifungal, cytotoxic and antiviral activity [9]. This bacterium produces chitinases [11], polyhydroxyalkanoates [12], cellulase [13], and it is capable of solubilizing metals [14].

Genome analysis of *C. violaceum* demonstrated an operon *ars* of the type *ars*RBC [9, 15]. This operon was shown to be functional by Azevedo and colleagues [16], who demonstrated that expression of the gene *ars*R is increased in response to arsenite and expression of this gene is correlated with resistance to this metalloid. The arsenical resistance tests in *C. violaceum* indicated that this bacteria was able to tolerate micromolar concentrations of arsenite, and the IC50 value for arsenite was determined to be approximately 500 μM [Rocha et al., unpublished data]. However, the global effects of arsenite on metabolism were yet not reported. Here, we used a differential proteomic approach focused on metabolic cycles involved in the adaptation of *C. violaceum* to arsenite that are not directly correlated with this resistance.

2. Materials and Methods

2.1 *Bacterial growth and treatment with arsenite*

We used *C. violaceum* ATCC 12472 in our study. The bacteria were grown on Luria-Bertani (LB) medium containing 0.1 mg/mL ampicillin, at 28°C with 200 rpm agitation, according to [16]. One hundred microliters of pre-inoculum were diluted in 10 mL of fresh medium containing 10 μM sodium arsenite and incubated for 16 h. This inoculum was then diluted in 50 mL of fresh culture medium containing 100 μM sodium arsenite, order to obtain an optical density (OD) of 0.05 at 720 nm [17], and incubated until it reached an exponential growth phase (DO_{720} = 1.00). As a control, the bacteria were grown under the same conditions, but without arsenite.

2.2 *Protein Extraction*

The bacteria were harvested by centrifuging the culture medium at 5,000 rpm, for 10 min, at 4°C. The liquid medium was discarded, and the bacterial pellet was washed three times with 50 mM Tris-HCl pH 7.5 and resuspended in 1 mL of lysis buffer (7 M urea, 2 M thiourea, 2% CHAPS, 75 mM DTT, and a cocktail of protease inhibitors). Cells were sonicated on ice for five cycles of 10s, with 10 s intervals. The lysate was centrifuged for 40 min, at 14,000 rpm, at 4°C. The soluble proteins were stored at -70°C until use. The protein samples were quantified using the 2D Quant kit (GE Healthcare).

2.3 *2D-DIGE*

The proteins in the extracts of three replicates from arsenic -treated and control bacteria were precipitated with methanol/chloroform and dissolved in sample buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris-HCl pH 9). Analytical gels were prepared with 50 μg of protein labeled with fluorescent dyes Cy3 or Cy5 (400 pmol), according to manufacturer's instructions (GE Healthcare). An internal control was made with a mixture of treated and control group material, totaling 50 μg of protein, labeled with Cy2. The labeling reaction was stopped by adding 1 μL of 10 mM lysine for 10 min. The differentially labeled samples were combined and diluted with rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 1% ampholytes pH 4-7, 75 mM DTT and 0.002% bromophenol blue), were used to rehydrate immobilized pH gradient (IPG) gel strip for isoelectric focusing (IEF) (pH 4-7, 18 cm, GE Healthcare). The IEF was performed in *Ettan IPGphor* (GE-Healthcare) until 80,000 Vh. After equilibration for 15 min in a 50 mM Tris HCl (pH 8.8) buffer solution containing 6 M urea, 2% SDS, 30% glycerol, 0.001% bromophenol blue and 10 mg/mL DTT, the strips were equilibrated for 15 min in the same solution, except that the DTT was replaced by 25 mg/mL iodoacetamide. The SDS-PAGE was performed on a 15% polyacrylamide gel using an Ettan DALTsix device (GE-Healthcare). DIGE gels were scanned using a Ettan DIGE Imager (GE Healthcare), and image analysis was performed with ImageMaster 2D Platinum 7.0 software (GE Healthcare). Spots were considered to be differential when the mean ratio between volumes was ± 1.3 times and $p < 0.05$ in the ANOVA test.

For the preparative gels, 600 μg of proteins from extracts of bacteria that had been treated or not with arsenite were resolved by 2DE as previously described. The proteins were stained with colloidal Coomassie blue. The gels were digitized using ImageScanner (GE Healthcare), and image analysis was performed with ImageMaster 2D Platinum 7.0 software (GE Healthcare).

2.4 *Digestion in gel with trypsin*

The differential spots were excised from the preparative gels (three for each condition), using SpotPicking Ettan (GE Healthcare). The digestion was performed according to a previously described protocol [18], with some modifications. The spots were destained with 25 mM ammonium bicarbonate /50% acetonitrile (v/v), treated with acetonitrile for 5 min and completely dried. The gel pieces were digested with modified trypsin (Promega) 20 ng/μL in 25 mM ammonium bicarbonate, at 37°C for 16 h. The peptides were extracted

with 2.5% formic acid/50% acetonitrile (v/v). The peptide solution was concentrated in a SpeedVac (Savant, USA) to a volume of about 10 iL, and desalted using P10 ZipTip C18 (MilliPore).

2.5 *Mass Spectrometry and Protein Identification*

For identification of the proteins, 0.5 µL of the peptide solution and 0.5 µL of the alpha-cyano-4-hydroxycinnamic acid matrix 10 mg/mL were mixed on the target. Analysis through PMF was made in an AXIMA-CRF MALDI-TOF mass spectrometer (Shimadzu). The acquisition parameters were: positive reflector mode, laser repetition rate of 5 Hz, 20 kV acceleration voltage, m/z range of 500 - 4,000 Da; 200 shots were accumulated per spectrum and spectra were processed usisng the MALDI-MS Application software tool (Shimadzu). The MS/MS analysis was done using a AutoFlex III MALDI-TOF/TOF mass spectrometer (Bruker Daltonics). The acquisition parameters were: positive reflector mode, laser repetition rate of 50 Hz, 25 kV acceleration voltage, 500 to 4,000 Da m/z range; spectra were processed using FlexControl software tool (Bruker Daltonics). Protein identification was performed using the Mascot software (http://www.matrixscience.com/) to search the genome of *C. violaceum* deposited in the NCBI data bank (http://www.ncbi.nlm.nih.gov/NCBInr). The search parameters were: other proteobacteria as the taxonomic group, monoisotopic mass, tolerance of 0.5 Da, 1 missed cleavage, carbamidomethylation of cysteine as a fixed modification, and oxidation of methionine as a variable modification.

3. Results and Discussion

Exposure of *C. violaceum* to arsenite altered the expression of 26 spots on the DIGE, 23 of which were increased and 3 reduced (Fig. 1). The range of the different ratio of spot intensities of bacteria treated with arsenite in comparison to control levels is between 1.3 and 2.4. The differential spots were cut, digested with trypsin and submitted to analysis by MS and MS/MS. Eight proteins were identified in 10 spots, two of which were identified by PMF, four by MS/MS and four by PMF and MS/MS (Table 1).

The differential proteome of *C. violaceum* in response to arsenite revealed that the major alterations caused by this metalloid are associated with oxidative stress. Three proteins directly involved in the cellular response to oxidative stress had their expression increased in response to arsenite: superoxide dismutase (SOD), glutathione-S-transferase (GST) and glutaredoxin (Grx). Spots 907 and 909, identified as the enzyme SOD, had the same molecular weight and a small difference in pI, which could be due to posttranslational

modifications. The same was found for spots 891 and 901, identified as a protein of the GST family.

Oxidative stress occurs when reactive oxygen species (ROS) are elevated, which could cause lipid peroxidation, DNA damage and protein oxidation [19], as well as inactivation of iron-sulfur centers, which are essential for many electron transfer proteins.

The enzyme SOD converts the superoxide ion into hydrogen peroxide, which is then reduced to H_2O by peroxidases, such as the peroxiredoxins (Prx), which are dependent on the thioredoxin system [20]. The GSTs are enzymes that catalyze the conjugation of glutathione with toxic agents and participate in the regeneration of S-thiolated proteins through oxidative stress [21]. The GST CV_0289 of *C. violaceum* belongs to the beta class, specific to bacteria, and can aid in the response to arsenic-induced oxidative stress. Induction of these enzymes observed in *C. violaceum* would be due to the generation of ROS within the cell, induced by arsenic, and has been reported from other bacteria, including *Comamonas sp.* [22], *Herminiimonas arsenicoxydans* [23] and *Leptospirillum ferriphilum* [24]. Arsenic disturbs the redox equilibrium through mechanisms such as activation of NADPH oxidase, inhibition of glutathione-peroxidase and binding of thiol groups of regulatory proteins [3, 25].

An increase in the expression of a single-strand DNA-binding protein (SSB), CV_1889, was also observed in the proteome of *C. violaceum*. The SSBs have various functions involved in the replication, repair and recombination of DNA [26]. CV_1889 is part of DNA repair pathways through recombination and the SOS response of *C. violaceum* [27]; and increased expression of this protein could have protective role against the DNA damage caused by oxidative stress due to arsenic.

Arsenic also affects metabolic pathways that are important for cell maintenance, increasing the expression of two enzymes involved in lipid metabolism, acyl-CoA dehydrogenase (ACAD) and enoil-CoA hidratase (ECAH), an enzyme involved in amino acid metabolism, 3-hydroxyisobutyrate

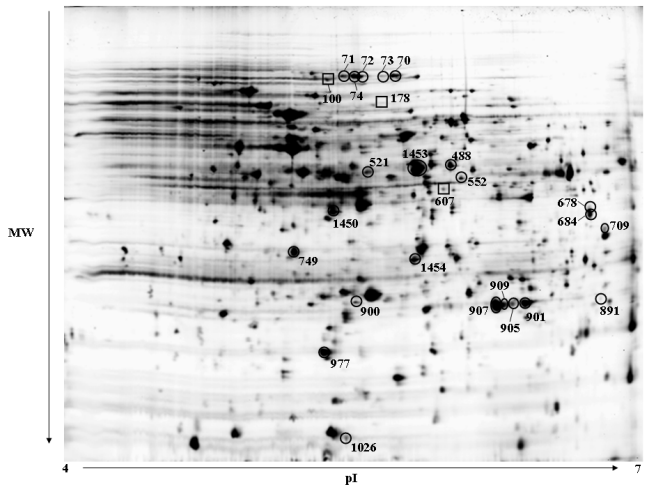

Figure 1. 2D-DIGE image of proteins of C. violaceum treated with arsenite. The circles show proteins whose expression increased and squares show proteins whose expression decreased with arsenite treatment.

Table 1. *C. violaceum* proteins that are differentially expressed in response to arsenite, identified by MS and MS/MS .

Spot[a]	RefSeq[b]	Protein	Theoretical MW / pI	Score[c]	Ars/Ctr[d]
Replication, recombination and repair[h]					
977[e]	NP_901559	CV_1889 Single-strand DNA-binding protein	17.11 / 5.25	94	1.73
Posttranslational modification, protein turnover, chaperones[h]					
749[f]	NP_901706	CV_2036 Peroxiredoxin/glutaredoxin family protein	26.97 / 5.09	68	1.35
891[f]	NP_899959	CV_0289 Glutathione S-transferase family protein	22.60 / 5.91	248	1.45
901[g]	NP_899959	CV_0289 Glutathione S-transferase family protein	22.60 / 5.91	89	1.71
Inorganic ion transport and metabolism[h]					
907[g]	NP_902174	CV_2504 Superoxide dismutase	21.63 / 5.87	77	1.59
909[f]	NP_902174	CV_2504 Superoxide dismutase	21.63 / 5.87	148	1.59
Coenzyme transport and metabolism[h]					
900[e]	NP_902057	CV_2387 Riboflavin synthase subunit alpha	22.28 / 5.34	107	1.31
Lipid transport and metabolism[h]					
488[g]	NP_901754	CV_2084 Acyl-CoA dehydrogenase	42.00 / 5.64	85	1.41
1454[g]	NP_901753	CV_2083 Enoyl-CoA hydratase	29.40 / 6.34	88	1.30
Amino Acid transport and metabolism[h]					
709[f]	NP_901751	CV_2081 3-Hydroxyisobutyrate dehydrogenase	30.10 / 6.24	68	1.34

[a.] Spot number refers to Fig. 1. [b.] RefSeq = NCBI access number. [c.] Score = MASCOT *Mowse score*. [d.] Ratio of spot intensities of extracts of *C. violaceum* treated with arsenite, compared to control levels. [e.] Spot identified by PMF; f. spot identified by MS/MS; [g.] spot identified by PMF and confirmed by MS/MS. [h.] Functional classification according to COG (Clusters of Orthologous Groups) .

dehydrogenase (3-HIBADH), and an enzyme involved in coenzyme metabolism, riboflavin synthase.

The two beta-oxidation enzymes that had increased expression, ACAD and ECAH, catalyze, respectively, the first and second steps of the reaction cycle that liberates acetyl-CoA, directed towards the citric acid or glyoxylate cycles, and electrons, carried by the coenzymes NADH and $FADH_2$ for the electron transport chain of energy generation. The activity of beta-oxidation enzymes is low when fatty acids are lacking; its genes are regulated by transcription factor FadR, cAMP, long-chain fatty acids and the life-cycle [28]. Expression of these enzymes can respond to stress conditions, such as salinity [29] and biosynthesis of polyhydroxyalkanoates [30].

Riboflavin synthase catalyzes the final step of riboflavin biosynthesis (vitamin B2), precursor of the coenzymes FMN and FAD, which are flavoproteins that are essential for energy metabolism, oxidation-reduction reactions and biosynthetic pathways, including the violacein biosynthetic pathway [31]. The riboflavin biosynthesis pathway responds to stress provoked by superoxides [32], metals and low phosphate levels [33]. In *C. violaceum*, expression of the riboflavin synthase alpha subunit was increased by arsenic or by arsenic-induced oxidative stress; this could be a response to a need for coenzymes for oxidation-reduction reactions. The riboflavin biosynthesis pathway also consumes ribulose-5-phosphate, produced by the pentose phosphate pathway, together with NADPH.

C. violaceum treated with arsenite had increased expression of 3-HIBADH, a key enzyme in the metabolism of valine, which catalyzes the reversible oxidation of 3- hidroxyisobutyrate to methylmalonate semialdehyde. This reaction produces NADPH and can have L-serine, D-threonine and other 3-hydroxy acid derivatives as substrates [34]. The function of 3-HIBADH can be understood together with that of other enzymes of the oxidative pathways that had increased expression, suggesting an overall effort by the bacteria to supply redox repair reactions, recruiting equivalent reducers normally involved in the oxidation of amino acids and fatty acids.

4. Conclusions

This is the first proteomic analysis of resistance of *C. violaceum* to arsenite, demonstrating the complexity of bacterial adaptation to a stress condition. The results suggest that this bacterium develops an ample response to oxidative stress provoked by arsenite, activating various enzymes involved in the elimination of oxygen radicals and repair of damage caused by these radicals, both at protein and DNA-repair levels. Arsenite in the culture medium also induces the expression of various proteins involved in cell processes, including energy metabolism and production of equivalent reducers.

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