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Two distinct arsenite-resistant variants of *Leishmania amazonensis* take different routes to achieve resistance as revealed by comparative transcriptomics

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ABSTRACT

Genome-wide search for the genes involved in arsenite resistance in two distinct variants A and A′ of *Leishmania amazonensis* revealed that the two variants used two different mechanisms to achieve resistance, eventhough these two variants were derived from the same clone and selected against arsenite under the same conditions. In variant A, the variant with DNA amplification, the biochemical pathways for detoxification of oxidative stress, the energy generation system to support the biochemical and physiological needs of the variant for DNA and protein synthesis and the arsenite translocating system to dispose arsenite are among the primary biochemical events that are upregulated under the arsenite stress to gain resistance. In variant A′, the variant without DNA amplification, the upregulation of aquaglyceroporin (AQP) gene and the high level of resistance to arsenate point to the direction that the resistance gained by the variant is due to arsenate which is probably oxidized from arsenite in the arsenite solution used for selection and the maintenance of the cell culture. As a result of the AQP upregulation for arsenite disposal, a different set of biochemical pathways for detoxification of oxidative stress, energy generation and cellular signaling are upregulated to sustain the growth of the variant to gain resistance to arsenate. From current evidences, reactive oxygen species (ROS) overproduced by the parasite soon after exposure to arsenite appear to play an instrumental role in both variants to initiate the subsequent biochemical events that allow the same clone of *L. amazonensis* to take two totally different routes to diverge into two different variants.

1. Introduction

Leishmania is a protozoan parasite, of which many species are human pathogens. Carried by sandfly in the form of promastigotes, it replicates obligately as amastigotes in host macrophages [1,2]. Though it is endemic in some countries in the world, there are many new cases of Leishmaniasis diagnosed each year. The clinical symptoms of the disease include cutaneous, mucocutaneous, and the lethal visceral infections if untreated. As currently a valid and effective vaccine against the parasite is still not available, chemotherapy becomes the major way to treat the disease [3,4]. Pentavalent antimony-containing drugs have long been used as a frontline drug in the treatment, but resistance to this type of drugs has become a serious problem [5,6]. Mechanisms of drug resistance remain obscure although resistance to arsenite has been studied extensively in *Leishmania* as well as other organisms such as prokaryote *Escherichia coli* and eukaryote *Saccharomyces cerevisiae*; arsenite, a heavy metal shares similar chemical properties with antimony [7,8]. Since the occurrence of resistance in the field is gradually arising, it is suggested that more than one mutation or mechanism is involved [9].

Drug uptake, efflux and sequestration have been proposed to involve in drug resistance, and aquaglyceroporins (AQPs) that have been identified as channels for the transport of neutral solutes in mammalian cells [10] are subsequently identified to be actively responsible for transporting arsenite into *E. coli*, *S. cerevisiae* and *Leishmania* [7,11]. A loss or reduction of AQP activity can lead to resistance whereas an increased expression of the gene can restore sensitivity to the drug. In the efflux system that has been characterized, ArsAB complex in bacteria, and Acr3p protein in the yeast were identified as the proteins for arsenite-translocating ATPases, which confer arsenite resistance by direct extrusion of arsenite from the cells. In the yeast, there exists yet another system, one of the ABC transporter, multidrug-resistant protein A (MRPA) that transports arsenite in the form of arsenite-thiol (glutathione) conjugates into the vacuole for arsenite disposal [7,12]. AQPs and sequestration system have been studied in arsenite-resistant *Leishmania* though a direct efflux or extrusion system similar to ArsAB in bacteria or Acr3p in yeast has not been described. Current evidence indicated

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that extrusion of arsenite and antimony from metal-resistant *Leishmania* involves the conjugation of metals to thiols (trypanothione) in a complex which is then recognized by ABC transporter MRPA (pgpA) and sequestered intracellularly for disposal [14,15]. This system is quite similar to those described for yeast. Although influx and efflux systems such as those described are most frequently used by the cells to detoxify arsenite, in view of the complex nature of arsenite resistance, several mechanisms may exist and act additively or synergistically in the cell to gain resistance.

Inorganic trivalent arsenite (ASIII) and pentavalent arsenate (ASV) are the two most important forms of inorganic arsenics that exist in nature, the former being more toxic than the later [15]. Arsenite is readily oxidized to arsenate when exposed to oxygen [16]. Due to the structural similarity to phosphate, arsenate can compete with phosphate in a number of metabolic reactions [17]. Thus resistance to arsenate has not been well explored. Recent finding in legume symboint *Sinorhizobium meliloti* that the $As(OH)_3/H+$ antiporter ArsB of the Ars operon in this bacterium was replaced by an aquaglyceroporin gene (aqpS) for direct extrusion of arsenite resulted from arsenate reduction in the arsenate-resistant cells [18], it is beginning to realize that arsenate imported into *S. meliloti* through the phosphate system was first reduced to arsenite and then disposed through the aqpS channel to gain resistance. Therefore AQP can act in both ways as a transporter for arsenite uptake as well as arsenite efflux depending on the resistant status of the cells. During the processes to acquire resistance to heavy metals, genes or pathways are elaborately controlled or regulated.

Using the same clone of *L. amazonensis* for selection of arsenite resistance *in vitro*, we have identified two arsenite-resistant variants that one is the arsenite-resistant variant consisting of an amplified H circle DNA (variant A) and the other is a variant having no DNA amplification (variant A′). Subsequent characterizations of these two variants identified: (1) the composition of the mitochondrion (kinetoplast) as well as the mitochondrial DNA sequences were drastically altered in A variant as compared to variant A′ [19–22]; (2) variant A showed low accumulation and a quick release of arsenite as opposed to accumulation of arsenite in variant A′ and the wild-type (W), and the quick release of arsenite correlated well with the presence of a multidrug-related protein, MRPA (pgpA) which is amplified in the H circle [22,23]; (3) variant A′ is cross-resistant to heavy metals such as cadmium (Cd^{2+}) and lead (Pb^{2+}) while variant A is not (unpublished data); (4) recent work identified that cytosolic tryparedoxin peroxidase (cTXNPx) and mitochondrial tryparedoxin peroxidase (mTXNPx) are distinctly overexpressed in the arsenite-resistant variants A and A′, respectively. The distinct overexpression of these peroxidases is linked to arsenite selection, resulting in differential resistance and sensitivity of these variants to the oxidants H_2O_2, *t*-butyl hydroperoxide and nitroprusside [24]. The phenomenon was further demonstrated to be due to ROS which was overproduced by the parasite soon after the exposure to arsenite [25]. Based on the kinetic differences in the overexpression of cTXNPx and mTXNPx after arsenite treatment and the differences in the detoxification of H_2O_2 and *t*-butyl hydroperoxide, respectively, by cTXNPx and mTXNPx in variants A and A′, it was postulated that different species of ROS may have been overproduced in A or A′ during arsenite selection for resistance that caused the divergence of the trypanothione-dependent tryparedoxin cascade into cytosolic and mitochondrial pathways due to functional and biochemical needs [25]. In order to understand how the same clone of *Leishmania* respond to arsenite to diverge into different resistant variants, genome-wide screening by comparative transcriptomic approaches were employed to provide a general picture of the genes involved in the mechanisms of arsenite resistance in these variants of *L. amazonensis*.

2. Materials and methods

2.1. Cells

L. amazonensis (LV78) promastigotes were cultured at 25 °C in medium 199 supplemented with 10% heat-inactivated fetal bovine serum (HIFBS) and 25 mM Hepes (pH 7.4), 100 units/ml penicillin and 100 g/ml streptomycin [19,20]. The promastigotes were cloned by limiting dilution, and then re-cloned on agar plates as described previously [19,20]. Clone 2–23 was used in this study.

2.2. Drug-resistance selection

Different samples of wild-type *L. amazonensis* clone 2–23 were independently stepwise selected against arsenite until a final concentration of 30 μM was reached. The status of arsenite-resistant variants with DNA amplification (A) and without DNA amplification (A′) were determined by hybridization with DNA probe specific to the H-circular DNA by Southern on total DNA isolated from these variants following the method described previously [19,20]. Therefore variants A and A′ were derived from the same clone 2–23. The DNA amplified in A is an extrachromosomal circular H DNA of approximately 69 kB containing a multidrug-resistant homologue MRPA (pgpA) gene and a PTR1 gene (GenBank accession No. AF001865).

2.3. Isolation of mRNA and Nothern hybridization

Messenger RNA was purified using Oligotex mRNA Mini Kit (Qiagen, Germany) from total RNA, which was isolated in a single-step method [26]. Northern hybridizations were performed according to the standard methods [24] under high stringency of hybridization and washing. Five micrograms of mRNA from wild-type, A and A′ variants were used for Northern blottings. Cloned cDNA sequences labeled with [^{32}P] were used as probes and α-tubulin and actin were used as internal controls.

2.4. cDNA synthesis and cDNA subtractive hybridization

The purified mRNA was reverse transcribed to cDNA using Universal RiboClone cDNA Synthesis System (Promega) and polyT as an extension primer, according to manufacturer's procedures. The cDNA subtractive hybridization was performed using PCR-Select cDNA Subtraction Kit (Clontech) by following the manufacturer's protocol. Each of the cDNA from variants A and A′ of *L. amazonensis* was used as a tester cDNA, and the cDNA from wild-type (W) *L. amazonensis* was used as a driver cDNA. The tester and driver cDNAs were first digested by RsaI to generate blunt-ended cDNA fragments. Each tester cDNA (A or A′) was divided to two populations, each of which was ligated with a different cDNA adaptor (adaptor 1 and adaptor 2R). Two hybridizations were carried out for each tester cDNA (A or A′). In the first hybridization, an excess of driver cDNA (W) was added to the two populations of tester cDNA that were then denatured and allowed to anneal. In the second hybridization, the two primary subtractive hybridization samples were mixed together without denaturing, and fresh denatured driver cDNA (W) was added to anneal again. This step is to further enrich the double-stranded tester molecules with different adaptors, adaptors 1 and 2R, at each end, which was then filled in by DNA polymerase. The entire subtractive hybridization mix was subsequently subjected to two rounds of PCR amplification. The first round PCR is suppression PCR that only double-stranded tester cDNA with different adaptors could be amplified. The second round PCR was to further amplify differentially expressed sequences and reduce background PCR products.