Biochimica et Biophysica Acta 1833 (2013) 997–1005

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

# Yeast protective response to arsenate involves the repression of the high affinity iron uptake system

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ARTICLE INFO

*Article history:*
Received 3 November 2012
Received in revised form 19 December 2012
Accepted 23 December 2012
Available online 4 January 2013

*Keywords:*
Arsenic uptake
Fet3–Ftr1
Iron deficiency

ABSTRACT

Arsenic is a double-edge sword. On the one hand it is powerful carcinogen and on the other it is used therapeutically to treat acute promyelocytic leukemia. Here we report that arsenic activates the iron responsive transcription factor, Aft1, as a consequence of a defective high-affinity iron uptake mediated by Fet3 and Ftr1, whose mRNAs are drastically decreased upon arsenic exposure. Moreover, arsenic causes the internalization and degradation of Fet3. Most importantly, *fet3ftr1* mutant exhibits increased arsenic resistance and decreased arsenic accumulation over the wild-type suggesting that Fet3 plays a role in arsenic toxicity. Finally we provide data suggesting that arsenic also disrupts iron uptake in mammals and the link between Fet3, arsenic and iron, can be relevant to clinical applications.



## 1. Introduction

Arsenic (As), the 20th most abundant element in the earth's crust is a highly toxic metalloid with respect to human health [1]. Although often synonymous to a poison, it is one of the oldest drugs in history of mankind, first used to treat cutaneous ulcers, later periodic fever and malaria, and currently as treatment for acute promyelocytic leukemia (PML) [2]. Due to its paradoxical biological effects, As has been the object of intense scrutiny, with regards to its cellular metabolism and molecular biological impact. However to date, these studies have never established a link between As and iron (Fe) homeostasis.

Cells require Fe for a wide array of metabolic functions, which include oxygen transport, cellular respiration, lipid metabolism, gene regulation and DNA replication and repair, yet Fe is toxic when present in excess [3]. *Saccharomyces cerevisiae* expresses three distinct transport pathways for Fe, two reductive systems and a non-reductive one. The reductive pathways consist of low- and high-affinity uptake systems operated by Fet4, and by a protein complex composed of the multicopper ferroxidase Fet3 and the permease Ftr1, respectively. The Fet3–Ftr1 complex is specific for Fe and is regulated both transcriptionally and post-transcriptionally by Fe [4–6]. The non-reductive Fe uptake pathway is mediated by the ARN family (Arn1-4) of membrane permeases that transport siderophore-ferric iron complexes [7]. In *S. cerevisiae*, these Fe uptake systems are all induced under Fe scarce conditions by the Aft1 transcription factor and its homologue Aft2, as part of the Fe regulon [8].

We have here conducted a genome-wide mRNA profiling of the *S. cerevisiae* response to arsenate (AsV), which revealed a potent induction of the Fe gene regulon. However, neither *FET3* nor *FTR1* appeared induced by this treatment. Strikingly, AsV caused the immediate internalization of Fet3 in the endoplasmic reticulum (ER) and the apparent lack of *FET3* transcriptional induction was in fact the consequence of the degradation of its mRNA by the major pathway for mRNA decay mediated by the 5′-3′ exonuclease Xrn1. Moreover, increased AsV tolerance in strains with null mutations of either *FET3* or *FTR1*, together with a decrease AsV cellular uptake in these mutant strains, indicate a role of Fet3 and Ftr1 in mediating AsV cellular uptake and toxicity. Taken together this work provides a molecular connection between As toxicity and Fe homeostasis, which should be relevant to further understand the toxic and therapeutic effects of As at the molecular level.

## 2. Materials and methods

### 2.1. Yeast strains and growth conditions

The plasmids and yeast strains used in this study are listed in supplementary Table S3. Spot assays were carried out by spotting 5 μl of early exponential phase cultures ($A_{600} = 0.4$) sequentially diluted (approximately $5 \times 10^3$ to 10 cells) in medium containing 2 mM $Na_2HAsO_4$ (AsV), 100 μM $Fe_2(SO4)_3$ ($Fe^{3+}$) or 50 μM BPS. Growth was recorded after incubation for 2 days at 30 °C.

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http://dx.doi.org/10.1016/j.bbamcr.2012.12.018

### 2.2. DNA microarray analysis

Wild-type (WT) cells were grown in triplicate in media containing 2 mM of AsV for 1 hour, and RNA was extracted, labeled, and hybridized to Affymetrix Yeast Genome S98 arrays. (For further information visit the Duke Microarray Core Facility at http://www.genome.duke.edu/cores/microarray/). All data were analyzed using both Partek® Genomics Suite™ and dChip softwares. Data have been deposited in the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE33427.

### 2.3. RNA blot analysis

Total yeast RNA was isolated from cells untreated or treated with either 2 mM of AsV, 100 μM of $Fe_2(SO4)_3$ ($Fe^{3+}$), 100 μM of BPS or 400 μM $H_2O_2$. PCR-amplified fragments were radiolabeled with $^{32}$P-dCTP to be used as probes. *U3*, a small nuclear RNA (SNR17A), was used as loading control.

### 2.4. Real-time PCR analysis

RNA was extracted from early log-phase cultures that were either untreated or exposed during 60 min to 2 mM AsV. DNA was removed by on-column DNAse I digestion (RNase-Free DNase Set; Qiagen). Total RNA (1ìg) was reverse transcribed with Transcriptor Reverse Transcriptase (Roche Diagnostics). qPCR reactions were performed in the LightCycler 480 Instrument (Roche), using LightCycler 480 Green I Master (Roche) and the oligonucleotides are listed in supplementary Table S4. Actin (*ACT1*) was used as a reference gene. All assays were made in triplicate.

### 2.5. β-Galactosidase assay

WT cells and the isogenic *aft1* mutant were transformed with a β-galactosidase reporter plasmid containing the promoter sequence of *CTH2* previously described [9], pCM64-*CTH2*-FeRE-*CYC1*-LacZ. WT was also co-transformed with the plasmids pEG202LexA-*AFT1* and pSH18-34. β-Galactosidase was measured in triplicate following the degradation of the colorimetric substrate ONPG (o-nitrophenyl-b-D-galactopyraniside) at $A_{420}$ and normalized against total protein concentration.

### 2.6. Measurement of total iron and arsenic

Strains were grown in YPD media with 1 mM of AsV for 2 or 4 hours, collected by centrifugation and washed with 10 mM EDTA and metal-free water. The total Fe and As were measured by inductively coupled plasma (ICP) atomic emission spectroscopy.

### 2.7. Protein analysis

Protein extracts were generated from cell cultures using cell lysis buffer supplemented with protease inhibitors (Roche). Protein was resolved by SDS–PAGE, and immunoblotted with Anti-HA and Anti-cMyc (Roche) Anti-TAP-tag (Life Technologies). Anti-Pgk1 (Life Technologies) was used as loading control.

### 2.8. Fluorescence microscopy

Microscopy experiments were carried out on live cultures using LEICA DMRA2 Microscope coupled with a CoolSNAP™ HQ Photometrics camera (Roper Scientific). The analysis of fluorescence intensity was done using the MetaMorph software package (MDS Analytical Technologies). Overnight liquid cultures expressing Aft1-GFP, Fet3-GFP or Sec63-GFP were re-inoculated to an optical density $A_{600} = 0.1$ in YPD medium containing 1 mM of AsV, 100 μM of BPS (Bathophenanthroline disulfonate), 100 μM BSC (Bathocuproine disulphonate) or 10 μM $Cu_2SO_4$. After washing with phosphate-buffered saline (PBS) cells were resuspended in DABCO solution and visualized.

### 2.9. Cells and media

Mousse embryonic fibroblasts (MEF) cells and HeLa cells were Fe loaded by addition of Ferric ammonium citrate ($Fe^{3+}$, 100 μM) and Fe depleted by addition of desferroxamine 5 μM and treated with arsenic trioxide (AsIII, 5 μM) for 24 hours. To extract protein, cells were solubilized in 1.0% of Triton X-100, 150 mM NaCl, 10 mM EDTA, and 10 mM Tris (pH7.4) with a protease inhibitor cocktail (Roche). Protein was resolved by 12% SDS–PAGE and transferred to a nitrocellulose membrane (GE Healthcare). Ferritin levels were detected using Mouse Anti-Ferritin (Abcam), Ccs1 levels using Rabbit Anti-Ccs1 (Santa Cruz Biotechnology, Inc), and Rat Anti-Tubulin (Novus Biologicals) was used as a loading control.

## 3. Results

### 3.1. The Saccharomyces cerevisiae genome-wide response to arsenic exposure

In *S. cerevisiae*, resistance to AsV has been ascribed to Yap1 and Yap8, two AP-1 like transcription factors that regulate the expression of genes involved in redox homeostasis and As detoxification processes, respectively [10]. To identify novel As tolerance pathways, we analyzed the genome-wide mRNAs profile of yeast cells exposed to AsV. Fig. 1 summarizes the results of these experiments (see data deposit in http://www.ncbi.nlm.nih.gov/geo/). In total, 1608 genes had their expression significantly altered upon AsV exposure. Classification of these genes by Gene Ontology showed that they belonged to many functional categories, including the response to metals and oxidative stress, cell cycle, mRNA processing, mitochondrial related-processes, transcriptional and translational activity, transporter activity, endoplasmic reticulum related-processes, protein folding, among others (Fig. 1A). Many of these genes have already been shown to respond to As [11,12]. However expression of genes responsive to metals, such as, zinc, iron (Fe) and copper (Supplementary Fig. S1A) has never been shown to be altered by As. Within the cellular Fe homeostasis category, 82 genes showed changes of at least 1.2-fold in their expression levels in response to AsV stress (Fig. 1B and Supplementary Table S1). Among these, many of the genes of the Aft1/2 regulon [13–15] were present, such as *CTH2*, the ARN family genes and *FIT3* that encode cell wall mannoproteins involved in siderophore-$Fe^{3+}$ uptake (Fig. 1C and Supplementary Table S2). Surprisingly, despite a large induction of the Aft1/2 regulon, *FET3* and *FTR1* mRNA levels appeared significantly decreased upon AsV exposure (Fig. 1C and Supplementary Table S1). Moreover the same was observed for *FET3* mRNA levels upon arsenite (AsIII) exposure (Supplementary Fig. S1B).

### 3.2. Arsenic activates Aft1 and causes Fe scarcity

Except for *FET3* and *FTR1*, the mRNA profiling analysis indicated that AsV exposure mimics the genomic response to Fe deficiency. Yeast cells respond to Fe deprivation by the Aft1/2-dependent transcriptional activation of the Fe regulon that encodes proteins involved in Fe uptake, trafficking and utilization [16]. Aft1 is controlled at the level of its nucleocytoplasmic distribution. To confirm that AsV can activate Aft1/2, we monitored by fluorescence microscopy the kinetics cellular localization of an Aft1-GFP fusion in cells exposed to AsV (Fig. 2A). After 40 minutes of exposure to AsV, Aft1-GFP fluorescence was indeed shifted from a diffuse to a nuclear pattern, similar to what is seen in cells treated with the iron chelator BPS. Subcellular localization of a Myc-Aft1 fusion by cellular fractionation similarly showed that in AsV-treated lysates, Aft1 was almost exclusively seen in the