ELUCIDATION OF MOLECULAR MECHANISMS CONFERRING ARSENIC TOLERANCE TO YEAST CELLS

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ABSTRACT

ELUCIDATION OF MOLECULAR MECHANISMS CONFERRING ARSENIC TOLERANCE TO YEAST CELLS

Arsenic is a highly toxic metalloid available in the environment mainly as arsenite or arsenate. These compounds' interference with many molecular mechanisms results in several diseases including cancer. Conversely, arsenic is used in therapeutic approaches, however, they are associated with drug resistance. Although some tolerance and toxicity mechanisms of arsenicals in yeast have been enlightened by previous studies, complete understanding, which is important for development of protection and therapy strategies, has not yet been achieved. Comprehensive genome-wide screening is a promising approach for the elucidation of novel genes involved in arsenic-associated mechanisms. The aim in this study was to screen a yeast genome library to characterize novel genes whose overexpression confers resistance to toxic concentrations of arsenate or arsenite in *Saccharomyces cerevisiae.* The plasmids from the colonies confirmed to be highly-resistant against arsenicals were sequenced to determine the genomic regions and seven genes were selected to clone into expression vectors. The overexpression of Pho86p and Vba3p provided yeast cells with the highest arsenate and arsenite resistance, respectively. Arsenate is a phosphate analogue and taken up by phosphate transporters. Pho86p is an ER-resident protein regulating ER-exit of the phosphate transporter. Therefore, it is reasonable that overexpression of Pho86p provides arsenate resistance. Vacuolar sequestration is a common route for the removal of toxic compounds from the cytosol and Vba3p is a vacuole-located transporter of basic amino acids with a likely role in arsenite resistance. Consequently, the screen in the current study revealed two genes with promising roles for tolerance mechanisms against arsenicals.

ÖZET

MAYA HÜCRELERİNE ARSENİK DİRENÇLİLİĞİ KAZANDIRAN MOLEKÜLER MEKANİZMALARIN AYDINLATILMASI

Arsenik doğada genellikle arsenit veya arsenat formlarında bulunan oldukça toksik bir metaloiddir. Bir yandan, birçok moleküler mekanizmayı etkileyerek, kanser de dahil olmak üzere çeşitli hastalıklara yol açarken, diğer taraftan bazı ilaçların içerisinde terapitik ajan olarak kullanılmaktadır. Fakat kanser hücreleri bu ilaçlara karşı direnç geliştirmektedirler. Şimdiye kadar yapılmış çalışmalar, arsenik dirençliliği ve toksisitesinin bazı yönlerini aydınlatmış olsa da, henüz tam bir anlayışa ulaşılamamıştır. Bu mekanizmaların her yönüyle anlaşılması, arsenikten korunma veya arseniği tedavilerde kullanabilme açısından çok önemlidir. Bu anlaşılmada, genom düzeyindeki taramalar umut vaadeden yaklaşımlardır. Bu çalışma, maya genom kütüphanesini tarayarak, yüksek ekpresyonu, toksik arsenik konsantrasyonuna direnç kazandıran genleri tayin etmeyi amaçlamıştır. Dirençli olduğu kanıtlanmış kolonilerden elde edilen plasmidler, plasmidlerdeki genomik bölgeleri belirlemek için sekanslanmış ve bu bölgelerdeki genlerin bir kısmı ekspresyon vektörlerine klonlamak için seçilmiştir. Seçilen yedi gen arasında, PHO86'nın arsenata, VBA3'ün ise arsenite oldukça dirençli olduğu görülmüştür. Arsenat bir fosfat analoğu olduğundan, ER'dan çıkışı Pho86 proteini tarafından yönetilen fosfat taşıyıcı proteininden faydalanarak hücre içine girmektedir. Dolayısıyla, PHO86 geninin yüksek ekpresyonunun direnç kazandırması mümkündür. Koful, toksik maddelerin sitozolden uzaklaştırılması için kullanılan bir organeldir ve VBA3 de kofulda bulunan ve bazik aminoasitleri kofula taşıyan bir taşıyıcı proteindir. Bilinen bu fonksiyonu, Vba3 proteininin dirençlilik mekanizmasında rol oynayabileceğine işaret eder. Sonuç olarak, yapılan bu tarama, arsenik dirençlilik mekanizmaları ile ilgili olabilecek ve umut vaaden iki tane geni tayin etmiştir.

TABLE OF CONTENTS

LIST OF FIGURES viii

LIST OF TABLES xii

CHAPTER 1. INTRODUCTION 1

1.1. Arsenic: as an Element.. 1

1.2. Forms of Arsenic.. 1

1.2.1 Arsenate.. 2

1.2.2. Arsenite.. 2

1.3. Abundance of Arsenic in the Environment........................ 2

1.4. Effects of Arsenicals.. 3

1.5. Arsenic: as a Therapeutic Reagent................................ 4

1.6. Studies on Molecular Mechanisms of Arsenic..................... 5

1.6.1. Yeast as a Model Organism in Arsenic Research.......... 5

1.7. Arsenic Uptake into Yeast Cells.................................. 6

1.7.1. Phosphate Transporters.................................. 6

1.7.2. Aquaglyceroporins.. 7

1.7.3. Hexose Transporters...................................... 7

1.8. Toxic Effects of the Arsenicals in Yeast Cells.................. 8

1.9. Detoxification of Arsenicals from Yeast Cells.................. 10

1.9.1. Arsenic Efflux through Acr3p............................ 11

1.9.2. Sequestration into the Vacuole.......................... 12

1.9.3. Role of Fps1p as an Arsenite Efflux..................... 12

1.10. Aim of the Current Study.. 13

CHAPTER 2. MATERIALS AND METHODS 14

2.1. Approach Used in the Current Study.............................. 14

2.1.1. Reproducibility.. 15

2.2. Yeast Strains and Their Growth Conditions....................... 15

2.3. Transformation of Plasmids into the Yeast Cells……………….. 16
2.4. Bacterial Transformation, Plasmid Isolation and Sequencing…... 16
2.5. Cloning of the Selected Candidate Genes…………………….. 17
2.6. Spotting Assay……………………………………………….... 17
2.6.1. MIC Determination…………………………………... 17
2.6.2. Sensitivity Assay……………………………………... 18
2.6.3. Overexpression Analysis…………………………….. 18
2.6.4. Cross-resistance Analysis……………………………. 18
2.7. Growth Curve…………………………………………………. 19
2.7.1. MIC Determination…………………………………... 19
2.7.2. Overexpression Analysis…………………………….. 19

CHAPTER 3. RESULTS …………………………………….……...... 20
3.1. Identification of Minimum Inhibitory Concentration of Arsenical 20
3.2. Genomic Library Screening on Arsenic-containing Selective Plate….. 24
3.3. Validation of Tolerance in the Resistant Transformants………… 25
3.4. Further Confirmation in Yeast-back Transformants…………….. 28
3.5. Sequencing of the Plasmids for the Identification of Genomic Cassettes…………………………………………………………. 30
3.6. Sensitivity Assays for Diploid Deletion Mutants of Candidate Genes………………………………...……………………… 32
3.7. Cloning of the Selected Genes into Expression Vectors………… 33
3.8. Validation of the role of the selected genes in arsenic tolerance…. 34
3.8.1. Spotting Assay……………………………………….. 34
3.8.2. 24-hour Growth Analysis…………………………….. 36
3.9. Cross-resistance Analysis for the Candidate Genes……………... 38

CHAPTER 4. DISCUSSION ……………………………………………. 40

CHAPTER 5. CONCLUSION ……………………………………….... 52

REFERENCES ……………………………………………………….. 54

LIST OF FIGURES

Figure		Page
Figure 1.1	Two major species of arsenic in the nature (A) arsenate and (B) arsenite………………………………………………………...	2
Figure 1.2.	Aquaglyceroporins and hexose transporters are utilized by arsenite and phosphate transporters are used by arsenate to enter yeast cells…………………..………………………….	6
Figure 1.3.	The structure constructed by the polymerization of three $As(OH)_3$ molecules resulting in the similarity to hexose sugars………………………………………………………...	8
Figure 1.4.	After entrance into the cell, arsenite and arsenate target a wide range of cellular mechanisms as exemplified in the figure…………………………………………………………	9
Figure 1.5.	The main routes for the removal of arsenic from the cytosol are indicated with red arrows…………………………………	10
Figure 2.1.	The workflow followed throughout the current project………………………………………………………..	14
Figure 3.1.	Spotting assay analysis for W303-1A, GK4, BY4741 and BY4743 in YPD plates containing given concentrations of arsenate. ……………………………………………………...	20
Figure 3.2.	Spotting assay analysis for W303-1A, GK4, BY4741 and BY4743 in YPD plates containing given concentrations of arsenite..……………………………………………………...	21
Figure 3.3.	Growth curve analysis of A) W303-1A and B) GK4 cells in treatment with given concentrations of arsenate, As(V)…………………………………………………………	22

Figure 3.4. Growth curve analysis of A) W303-1A and B) GK4 cells in treatment with given concentrations of arsenite, As(III)………..…………………………..………………… 23

Figure 3. 5. Genomic DNA library screening……………………..……… 24

Figure 3.6. Spotting assay analysis for GK4 colonies resistant to [As(V)]=1 mM…….…………………………………………… 25

Figure 3.7. Spotting assay analysis for W303-1A colonies resistant to [As(V)]=2 mM……………………...…………………….. 26

Figure 3.8. Spotting assay analysis for W303-1A colonies resistant to [As(V)]=4 mM………………...…………………………….. 27

Figure 3.9. Spotting assay analysis for GK4 colonies resistant to [As(III)]=0.1 mM……………………...………………….. 27

Figure 3.10. Spotting assay analysis for yeast-back transformants of W303-1A colony from [As(V)]=2 mM and GK4 colony from [As(V)]=1 mM (5-day) ……………………...………………. 28

Figure 3.11. Spotting assay analysis for yeast-back transformants of W303-1A colony from [As(V)]=4 mM (5-day) …………….. 29

Figure 3.12. Spotting assay for yeast-backs transformants of GK4 colony from [As(III)]=0.1 mM………………………………………. 30

Figure 3.13. The sequence analysis of the plasmid from colony W303-1A, 4#6………………………………………………………… 30

Figure 3.14. Sensitivity assay for diploid deletion mutants of candidate genes in gradient plates (A) with the highest concentration of [As(V)]= 15 mM and (B) with up to 3 mM…………………... 32

Figure 3.15. Gel electrophoresis result……………………………………. 33

Figure 3.16. Overexpression analysis for W303-1A, GK4 and haploid deletion mutant of PHO84 cells transformed with empty plasmid, pAG425, and PHO86-pAG425 on gradient plates 34

with up to 2, 12 and 15 mM arsenate, respectively. (OD600=0.02 for all spots)…………………………………...

Figure 3.17. Overexpression analysis for W303-1A cells transformed with empty plasmid, pAG425,, AIM26-pAG425 and UGP1-pAG425 in gradient plates with the highest concentration of [As(V)]= 12 mM (OD600=0.02 for all spots) ……………….. 35

Figure 3.18. Overexpression analysis for GK4 cells transformed with empty plasmid, pAG425,, YAP1-pAG426 and VBA3-Yep13 in gradient plates with the highest concentration of [As(III)]= 0.2 mM (OD600=0.02 for all spots) ……………………… 35

Figure 3.19 Overexpression analysis for GK4 cells transformed with empty plasmid, pAG425, or ERG6-pAG425 in gradient plates with the highest concentration of [As(III)]= 0.2 mM (OD600=0.02 for all spots)…………………….…………. 36

Figure 3.20 24-hour-growth analysis for W303-1A cells transformed with empty plasmid, pAG425, and PHO86-pAG425 plasmids in given concentrations of arsenate………….…………………. 37

Figure 3.21. 24-hour-growth analysis for GK4 cells transformed with empty plasmid, pAG425,, VBA3-Yep13 and ERG6-pAG425 plasmids in given concentrations of arsenite………………… 37

Figure 3.22 Spotting assay for the yeast cells overexpressing candidate genes to test their growth on antimonite…………….……… 38

Figure 3.23 Spotting assay for the cross-resistance test on boric acid……………………………………………………... 38

Figure 3.24 Spotting assay for the yeast cells overexpressing candidate genes for arsenite resistance to test their growth on arsenate… 39

Figure 3.25 Spotting assay for the yeast cells overexpressing candidate genes for arsenite resistance to test their growth on arsenate.. 39

Figure 4.1. The region of chromosome X available in the green-coded cassettes. Each green bar indicates the region covered by the corresponding cassette. ………………………………………… 42

Figure 4.2. ER-resident protein Pho86 is required for the ER-exit of Pho84p, which imports inorganic phosphate and arsenate into the cell, through unknown process. ………………………….. 45

LIST OF TABLES

Table **Page**

Table 2.1. The strains of *S.cerevisiae* used in this study……………… 16

Table 3.1. The number of colonies which were obtained on the given concentrations of arsenite or arsenate within 72 hours……… 20

Table 3.2. The genomic regions and the genes available in cassettes of the plasmids isolated from (A) arsenate-resistant and (B) arsenite-resistant colonies. ………………………………….. 33

CHAPTER 1

INTRODUCTION

1.1. Arsenic: as an Element

Arsenic is an element with the symbol of "As" and the atomic number of 33. It is located in the fourth period and 15A group of the periodic table. This location is part of the stair-step diagonal line which bears metalloids (semi-metals) separating metals and nonmetals in the periodic table. This intermediate location reflects the fact that metalloids have physico-chemical properties resembling both metals and nonmetals (Dilda & Hogg, 2007; Ralph H. Petrucci, F. G. H., Jeffry D. Madura, 2011).

Arsenic is generally found in a yellow solid form (As_2S_3, orpiment) or gray solid form having metallic lusters (Ralph H. Petrucci, F. G. H., Jeffry D. Madura, 2011). The sodium, calcium and potassium salts of arsenite or arsenate are the naturally available forms in the environment, in addition to compounds of arsenicals constructed with oxides or sulfides which are known to be instable.

While the elemental arsenic is not utilized for humans, its salts, oxides and compounds with copper or lead are used in several fields. For example, chromated copper arsenate used to be applied to wood to keep away insects and diminish decomposition of the wood. After comprehension of its toxic effects on health, its usage has been decreasing (Dilda & Hogg, 2007; Tamás, Labarre, Toledano, & Wysocki, 2006).

1.2. Forms of Arsenic

Although it is possible to describe many arsenic forms available in the soil and air, there are two forms which are the most abundant in the environment, arsenate and arsenite (Thorsen et al., 2012).

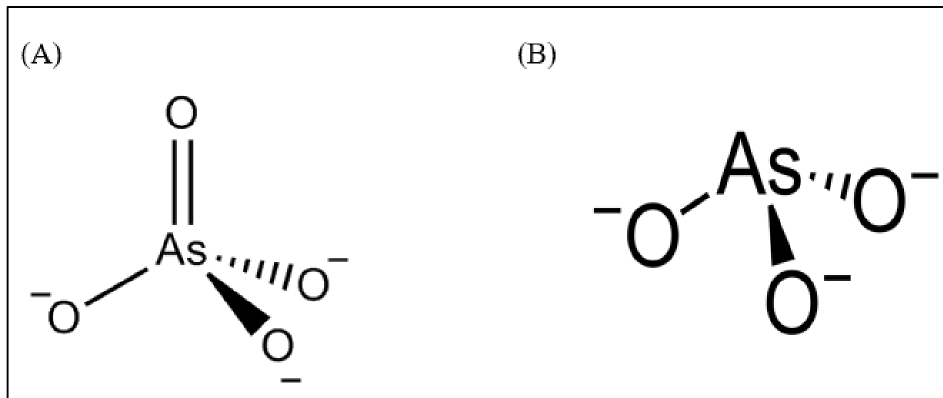


Figure 1.1. Two major species of arsenic in nature (A) arsenate and (B) arsenite (Source: https://commons.wikimedia.org)

1.2.1. Arsenate

The form of arsenic ample in aerated soil is the pentavalent form that is also known as arsenate (AsO_4^{3-} or As(V)). Arsenate is an analog of phosphate which means it resembles the phosphate group (Figure 1.1.A) and this property provides arsenate with entrance into the cell by utilizing phosphate transporters (Bienert, Thorsen, et al., 2008; Talemi et al., 2014).

1.2.2. Arsenite

The second form of arsenic is the trivalent one, arsenite, which is abbreviated as $As(OH)_3$ or As(III) (Figure 1.1.B). This form exerts the severest toxic effects on living organisms (Bienert, Thorsen, et al., 2008; Talemi et al., 2014; Thorsen et al., 2012).

1.3. Abundance of Arsenic in the Environment

Arsenic is highly abundant in the soil, water and air as can be understood from the values indicating that there is 2 mg of arsenic in a 1 kg soil. It was stated that 200-250 mg of arsenic cause lethality for humans (Bienert, Sch??ssler, & Jahn, 2008). This abundance

and toxicity makes arsenic an environmental toxin threating the health of living organisms throughout the world (Thorsen et al., 2012)

Both forms of arsenic are known to contaminate drinking water due to interaction of water with the rocks containing arsenic or action of mining and industry (Dilda & Hogg, 2007; Z. Liu et al., 2002). The contamination of drinking water with arsenicals which are available on the Earth's crust is a vital threat for millions of people, particularly those living in Bangladesh and West Bengal (Dilda & Hogg, 2007; S. X. Liu, Athar, Lippai, Waldren, & Hei, 2001; Thorsen et al., 2007). Reports prepared for many countries indicated that the concentration of arsenicals in the drinking water are higher than the values accepted as safe level. Additionally, those reports also confirmed the deleterious impacts of arsenic in the human health, as can be exemplified by the rise in the events of poisoning and cancer in Bangladesh where 50 million people are faced with the hazard of arsenic in the drinking water (Bienert, Sch??ssler, et al., 2008; S. X. Liu et al., 2001).

1.4. Effects of Arsenicals

The World Health Organization (WHO) and the US Agency for Toxic Substances and Disease Registry (www.atsdr.cdc.gov) declared that arsenic threatens the health of people from different regions of the world (Matia-González & Rodríguez-Gabriel, 2011). It was determined as a group I human carcinogen by International Agency for Research of Cancer and it was recorded that 200-250 mg arsenic is lethal for humans (Bienert, Thorsen, et al., 2008).

People may directly interact with arsenic-contaminated soil or they may consume water or food contaminated with arsenical compounds (Bienert, Thorsen, et al., 2008; Bienert, Sch??ssler, et al., 2008; S. X. Liu et al., 2001; Thorsen et al., 2009). When coal containing arsenic is burned, or soil contaminated with the arsenic is processed for the production of semiconductors or glasses, direct contact or inhalation leads to arsenical exposure. The finding that there was 10 times more incidence of lung cancer in miners working in areas contaminated with arsenic indicated the health risk of direct exposure to arsenic for occupational reasons (Dilda & Hogg, 2007; S. X. Liu et al., 2001).

Considering the toxic effects of arsenic even on the cells, continued exposure of people to arsenical compounds may result in poisoning or severe health issues as can be exemplified by neurological disorders, cardiovascular diseases, injuries in the liver, and

several cancer types (Jacobson et al., 2012; Matia-González & Rodríguez-Gabriel, 2011; Talemi et al., 2014; Thorsen et al., 2007, 2009). Mainly observed types of cancers are skin, liver, bladder and lung cancer (S. X. Liu et al., 2001)

1.5. Arsenic: as a Therapeutic Reagent

Arsenic has been used in medicine as a therapeutic reagent for many years starting from usage of potassium arsenite in Fowler's solution for treatment of several diseases (Dilda & Hogg, 2007; Tamás et al., 2006). The introduction of the term of "chemotherapy" to medicine was by Paul Ehrlich and his colleague Sahachiro Hata who discovered "Compound 606," a drug containing arsenic to treat syphilis and trypanosomiasis (Sörgel, 2004). Although the involvement of arsenic in drugs is not as much as in earlier times due to its side effects on patients, in recent years, arsenic trioxide (ATO) has been used as a therapeutic reagent against acute promyelocytic leukemia (APL) and multiple myeloma (Litwin, Bocer, Dziadkowiec, & Wysocki, 2013; Tamás et al., 2006; Thorsen et al., 2007; Vujcic, Shroff, & Singh, 2007).

APL is the disease developed as a consequence of the fusion of retinoic acid receptor (RAR-alpha) and PML genes. The protein product of this fusion prevents the differentiation of promyelocytes and expression of differentiation-associated genes, since it gathers the repressors of these genes (Dilda & Hogg, 2007). Retinoic acid is able to stimulate the degradation of this fusion protein and can be used in the treatment of APL. However, it requires elevated doses of retinoic acid and is also associated with a high degree of resistance (Bouganim, David, Wysocki, & Ramotar, 2001). ATO (As_2O_3) is known to target specifically human APL cells but the underlying mechanism has not yet been completely revealed (Dilda et al., 2008). This arsenic form can be used instead of retinoic acid to induce degradation. The separation of PML and RAR-alpha provides the differentiation of the promyelocyte. Additionally, ATO is able to stimulate the phosphorylation of the repressors and their removal, hence the genes for differentiation can be transcribed. If the dose of ATO is increased to the values of 0.5–2.0 M, it results in the apoptosis of promyelocytes (Dilda & Hogg, 2007). It was recorded that eleven patients among twelve were successfully treated with ATO, but then three of the eleven patients did not respond to the therapy (Ghosh, Shen, Rosen, & Kaback, 1999), so it is

easily understood that there is a need to comprehend how these cells can gain resistance to these highly effective and toxic drugs.

Additionally, the arsenic-containing drug Melarsoprol is used in the treatment of sleeping sickness disease derived from a parasitic protozoan (Litwin et al., 2013; Maciaszczyk, Wysocki, Golik, Lazowska, & Ulaszewski, 2004; Maciaszczyk-Dziubinska, Wawrzycka, & Wysocki, 2012).

1.6. Studies on Molecular Mechanisms of Arsenic

Arsenic's abundance in the environment, toxic effects upon exposure and usage as a therapeutic reagent have led scientists to question the molecular mechanisms of arsenic toxicity and resistance. Initial knowledge about the metabolism of arsenicals was derived from studies using bacteria as a model organism starting from the study identifying the bacterial arsenic resistance operon, ars, in *Escherichia coli* (Rosen & Tamás 2010; Rosen 1999; Xu et al. 1998). However, these findings were not compatible with eukaryotic systems.

1.6.1. Yeast as a Model Organism in Arsenic Research

The primary results related to arsenical mechanisms in eukaryotes were gained from Bobrowicz's and his colleagues' studies revealing the arsenic tolerance genes in *Saccharomyces cerevisiae*, budding yeast (Bobrowicz, Wysocki, Owsianik, Goffeau, & Ułaszewski, 1997). This study was first to relate the findings with higher eukaryotes, because many of the yeast genes have homologues in higher eukaryotes and the toxicity, tolerance and metabolism mechanisms show similarity with higher eukaryotes (Bánfalvi, 2011; dos Santos, Teixeira, Cabrito, & Sá-Correia, 2012).

Considering its short life cycle, easy manipulation of the genome, and availability of molecular and genome-wide analysis tools, yeast is a preferred model organism in a wide range of studies. Additionally, it is not expensive to study this non-pathogenic, completely established and unicellular eukaryote (Bánfalvi, 2011; dos Santos et al., 2012; Rosen & Tamás, 2010; Robert Wysocki & Tamás, 2010).

Thanks to further studies using yeast, the molecular mechanisms of arsenical uptake, toxicity and detoxification are beginning to be enlightened, as described below. Furthermore, powerful yeast genetics provided identification of mammalian and plant transporters for metalloid uptake (Bienert, Thorsen, et al., 2008; Bienert, Sch??ssler, et al., 2008; Z. Liu et al., 2002). However, there is a need to fully comprehend the direct targets of arsenic and molecular mechanisms for resistance and toxicity. How yeast cells sense the presence of arsenic and regulate their response is still a question that needs to be answered.

1.7. Arsenic Uptake into Yeast Cells

Organisms encounter arsenic upon direct exposure in contaminated areas or indirectly through the food and water chain (Thorsen et al., 2009, 2012). There are many transporters in the cell membrane for the uptake of nutrients, molecules or elements needed for cell survival and arsenicals utilize these transporters to enter the cell (Figure 1.2). This strategy is also used by other toxic compounds.

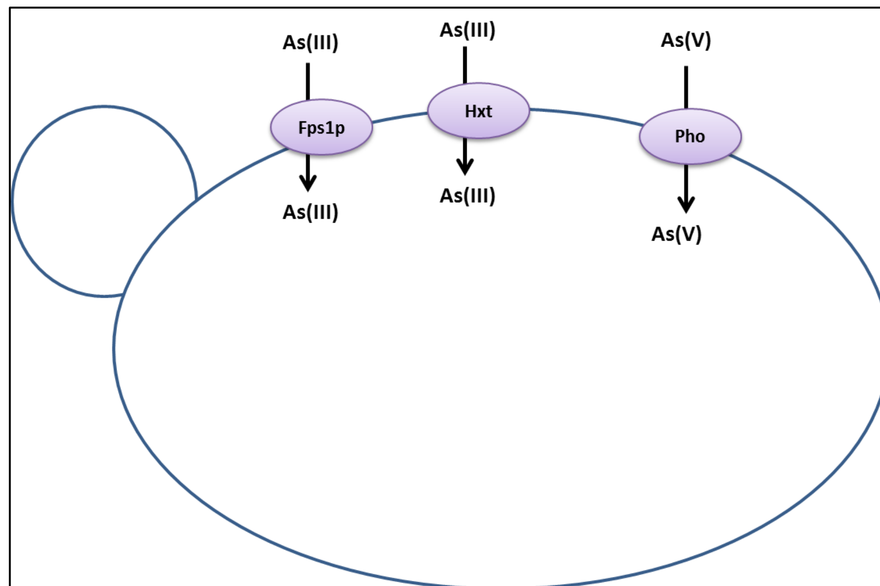


Figure 1.2. Aquaglyceroporins and hexose transporters are utilized by arsenite and phosphate transporters are used by arsenate to enter into the yeast cells

1.7.1. Phosphate Transporters

When arsenate is found in aqueous solution, the structure of arsenate oxyanion resembles phosphate. Arsenate benefits from phosphate transporters (Figure 1.2) which allow aresenate to be taken up by the cells thanks to its structural homology (Rosen & Tamás, 2010; Robert Wysocki & Tamás, 2010). In yeast cells, there are two kinds of phosphate transporters: low and high affinity ones. Pho84p and Pho89p are high-affinity phosphate permeases, while Pho87p and Pho90p are low affinity transporters. Deletion mutants of PHO84 and PHO87 gained resistance to arsenate suggesting their role in arsenate influx (Bun-ya et al., 1996; Bun-Ya, Nishimura, Harashima, & Oshima, 1991; Yompakdee, Bun-Ya, et al., 1996; Yompakdee, Ogawa, Harashima, & Oshima, 1996).

1.7.2. Aquaglyceroporins

Arsenite in aqueous solution, $As(OH)_3$, has a structure which looks like glycerol. The aquaglyceroporin protein transporting glycerol in yeast cells is encoded by the FPS1 gene. While the deletion of the FPS1 gene decreased arsenite uptake and provided arsenite resistance, its higher expression accumulated more arsenite causing arsenite-sensitivity (Robert Wysocki et al., 2001). These results pointed out that yeast aquaglyceroporin is the means of arsenite uptake thanks to this structural similarity (Figure 1.2). In the light of this conclusion from yeast genetics, the mammalian and other eukaryotic aquaglyceroporins responsible for arsenite uptake were identified (Bienert, Thorsen, et al., 2008; Z. Liu et al., 2002; Robert Wysocki & Tamás, 2010).

1.7.3. Hexose Transporters

The general natural habitat of yeast cells is composed of glucose, so they do not require the expression of hexose transporters. However, when they are starved for glucose, the hexose transporters are expressed to fulfill the energy need of yeast cells (Z. Liu, Boles, & Rosen, 2004; Robert Wysocki & Tamás, 2011).

The observation that yeast cells lacking the FPS1 gene were able to accumulate arsenic in the absence of glucose pointed out the role of hexose transporters in arsenic

accumulation. The binding of three $As(OH)_3$ to each other can construct a molecule with a ring structure of six members which resembles the hexose sugars (Figure 1.3). Therefore, arsenite can utilize the hexose transporters for entry into the yeast cells (Figure 1.2) thanks to its crystal structure (Z. Liu et al., 2004).

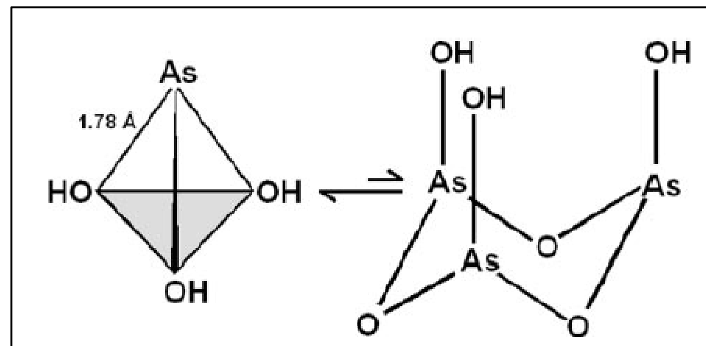


Figure 1.3. The structure constructed by the polymerization of three $As(OH)_3$ molecules resulting in the similarity to hexose sugars
(Source: Liu et al. 2004)

1.8. Toxic Effects of Arsenicals in Yeast Cells

After entering into yeast cells thanks to their structural resemblance to essential compounds, arsenicals interfere with a wide range of molecular mechanisms, again generally benefiting from this similarity.

Arsenate, the non-functional structural analogue of inorganic phosphate, disrupts phosphate transport, so this effects several mechanisms dependent on phosphate or phosphorylation. The most significant effects are observed in energy metabolism due to impairments in ATP synthesis in the mitochondria of yeast cells (Bienert, Sch??ssler, et al., 2008; Talemi et al., 2014; Thorsen et al., 2009; Vujcic et al., 2007; Robert Wysocki & Tamás, 2011).

The high toxicity of arsenite originates from its effects on a broader range of pathways. Arsenite has high affinity for the side chains of certain amino acids. Imidazole nitrogen of histidine and sulfydryl groups on cysteine are the targets of arsenite and its interaction with these amino acids change the redox signaling and cause oxidative stress in yeast cells. Additionally, its binding to the proteins through these residues affect the

structure, function or activity of the proteins and their cellular mechanisms. For instance, growth, cell cycle progression, and apoptosis are all negatively affected by aresenite (Bienert, Sch??ssler, et al., 2008; Talemi et al., 2014; Robert Wysocki & Tamás, 2011). Arsenite interferes with the cytoskeleton by preventing the polymerization of tubulin upon binding to beta-tubulin (Robert Wysocki & Tamás, 2010) and by inhibiting the de novo synthesis of actin and tubulin monomers due to disruption of the GIM complex needed in this process (Thorsen et al., 2009).

Arsenite also affects proteins which require metals as a cofactor. It binds to the metalloproteins and inhibits the binding of the actual metal, thus the functioning of the protein is ceased (Jacobson et al., 2012; Tamás, Sharma, Ibstedt, Jacobson, & Christen, 2014).

In addition to this interference with protein function through preventing metal binding or direct interaction with the functional side chains, it was indicated that arsenite also disrupts the folding of proteins both *in vivo* and *in vitro*. Due to this inhibition of protein folding, cells face the problem of unfolded proteins which accumulate and aggregate in the cell. Therefore, protein homeostasis is aberrant and the viability of the cells decreases. This explains the increase in expression levels of genes related to the chaperones and proteasomes when cells are exposed to arsenite (Jacobson et al., 2012; Tamás et al., 2014).

To sum up, arsenicals exert their toxic effects on yeast cells generally by inhibiting the activity of proteins which may be a part of essential signaling or metabolic pathways.

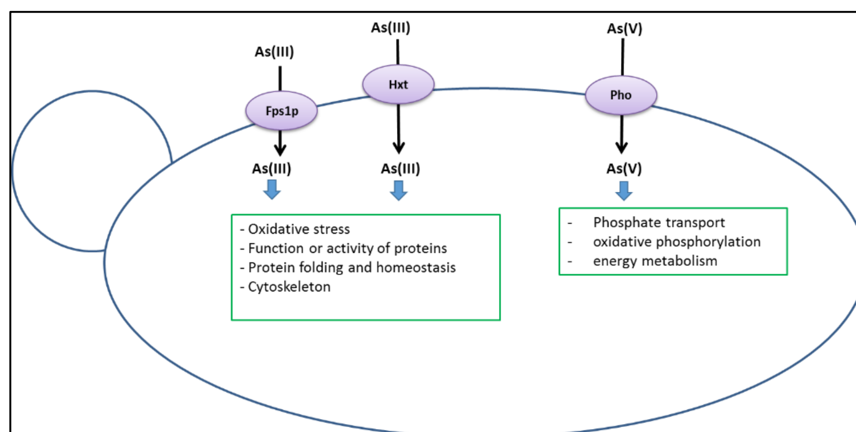


Figure 1.4. After entrance into the cell, arsenite and arsenate target a wide range of cellular mechanisms as exemplified in the figure

1.9. Detoxification of Arsenicals from Yeast Cells

Organisms encounter toxic compounds in their natural habitats or consume contaminated food or water, therefore, there is a demand for the detoxification of toxic compounds for their survival. The strategies for detoxification include down-regulation of transporters responsible for uptake and up-regulation of transporters providing of the toxic compound. Another way is the sequestration of the agent into the vacuole for its removal from the cytosol or the chelation of the agent to metalloproteins.

Yeast cells follow similar strategies to get rid of arsenic. The main determinant in the detoxification of arsenic is efflux through Acr3p regulated by Acr1p. The other means is sequestration into the vacuole after conjugation to glutathione (GSH) through Ycf1p under the control of Yap1p. In addition to these two proteins, Fps1p has a working principle based on a concentration gradient and may transport arsenic out of the cell when the conditions are met.

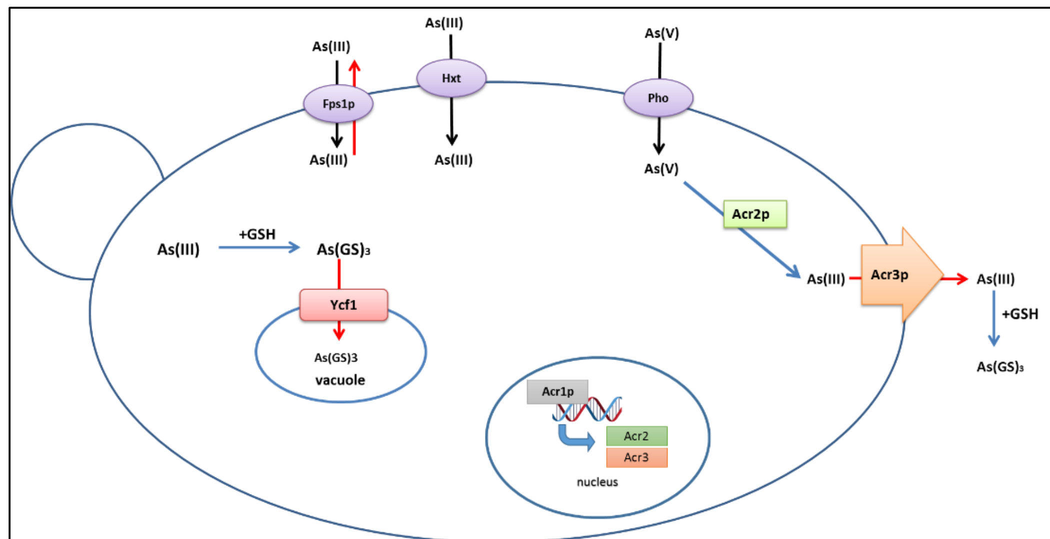


Figure 1.5. The main routes for the removal of arsenic from the cytosol are indicated with red arrows

1.9.1. Arsenic Efflux through Acr3p

Screening of a yeast genomic library revealed a arsenicals resistance gene cluster containing three genes: ACR1, ACR2 and ACR3 (Bobrowicz et al., 1997).

The ACR1 or YAP8 gene encodes a transcription factor in the family of fungal AP1-like transcription factors which are regulators of gene expression related to several stress conditions. Acr1p is responsible for the induction of expression of Acr2p and Acr3p by binding to their common promoter which contains an extended pseudopalindromic sequence recognized by Acr3p, when yeast cells are exposed to arsenic (Figure 1.5). This arsenic-specific induction of the proteins are not regulated at the transcriptional level or a change in the location of the protein (Di & Tamás, 2007; Robert Wysocki & Tamás, 2010). A recent study carried out *in vivo* and *in vitro* indicated that arsenite directly binds to Acr1p converting it to its active form for the induction of target gene expression (Kumar et al., 2016).

Activation of Acr1p upon sensing arsenic in the cell by direct binding induces the expression of Acr3p. Acr3p is a cell membrane-localized efflux pump specifically exporting arsenite out of the cell (Figure 1.5). Its deletion caused hypersensitivity to arsenicals and hyperaccumulation of arsenic inside the cell, while its overexpression significantly decreased the level of arsenic and conferred tolerance to yeast cells (Ghosh et al., 1999; Maciaszczyk-Dziubinska, Wawrzycka, Sloma, Migocka, & Wysocki, 2010; R Wysocki, Bobrowicz, & Ulaszewski, 1997).

Acr3p can only recognize arsenic in the trivalent state and Acr2p is required for the hyperresistance of yeast cells to arsenate when Acr3p is overexpressed. The ACR2 gene encodes an arsenate reductase turning arsenate into arsenite (figure 1.5). Its deletion did not affect the resistance level to arsenite, but decreased the tolerance to arsenate (Bobrowicz et al., 1997; Rosen & Tamás, 2010; Tamás et al., 2006; Robert Wysocki & Tamás, 2010). Additionally, it was shown *in vitro* that purified Acr2p could reduce arsenate to arsenite (Mukhopadhyay, Shi, & Rosen, 2000).

1.9.2. Sequestration into the Vacuole

Glutathione (gamma-L-glutamyl-L-cysteinylglycine, GSH), which is a thiol molecule with low molecular weight, functions as a redox buffer in the cells to prevent them from oxidative stress and the toxicity originated from metals or metalloids (Thorsen et al., 2007, 2012). Conjugation of glutathione to toxic compounds determines their fate to be sequestered into the vacuole, as in the case of GSH-conjugated arsenite targeting into the vacuole through Ycf1p (Yeast Cadmium Factor 1) (Ghosh et al., 1999; Szczypkas, Wemmiep, Moye-rowley, & Thielesn, 1994; Thorsen et al., 2012). Ycf1p is an ABC (ATP-binding Cassette) transporter localized to the vacuolar membrane and its deletion mutant sensitized yeast cells for arsenic (Ghosh et al., 1999; Robert Wysocki et al., 2001). It was shown *in vitro* that $As(GS)_3$ conjugates were actively pumped into the vacuoles (Ghosh et al., 1999; Robert Wysocki & Tamás, 2011)

1.9.3. Role of Fps1p as an Arsenite Efflux

Fps1p is the transporter taking up arsenite from the extracellular space, but this protein functions as a bidirectional channel protein based on the concentration gradient. Therefore, Fps1p is able to transport arsenite out of the cell when the cellular concentration of arsenite is higher than that in the extracellular space (ECS). At the beginning of arsenite exposure, the uptake of arsenite through Fps1p is decreased by controlling its transcription and also activity. The formation of conjugate of arsenite and glutathione out of the cell upon the release of glutathione to the ECS reduces the concentration of free arsenite at the ECS. At the same time, if there is arsenate in the environment, it is converted into arsenite by Acr2p resulting in higher arsenite concentration inside the cell. Those two mechanisms result in a change in the concentration gradient. Fps1p allows the passage of arsenite down this concentration gradient and is transcribed at higher rates (Maciaszczyk-Dziubinska, Migdal, Migocka, Bocer, & Wysocki, 2010; Maciaszczyk-Dziubinska et al., 2012; Robert Wysocki & Tamás, 2011).

1.10. Aim of the Current Study

It was aimed to carry out a yeast genome library screen on two different yeast strains, W303-1A and GK4, in order to reveal the genes whose overexpression confers resistance to budding yeast against arsenic.

Genome-wide phenotypic screens in yeast cells are commonly applied to elucidate molecular mechanisms related to tolerance, as in the case of previous screens on arsenic (Dilda et al., 2008; Haugen et al., 2004; Jin et al., 2008; Ruotolo, Marchini, & Ottonello, 2008). Nevertheless, these studies benefited from yeast deletion collections which are associated with some drawbacks in terms of, for example, essential genes and genetic redundancy (dos Santos et al., 2012). Yeast genome library screening eliminates these deficiencies. Additionally, those studies had some overlapping results or pointed out common genes or molecular mechanisms affected by arsenicals. Conducting genome library screening will strengthen these findings and give more reliable data to confirm that these genes are direct targets of arsenic, since the phenotypic effect observed in a deletion library may result from the synthetic effects of the deletion (Thorsen et al., 2009).

Elucidation of tolerance genes will provide ideas about the tolerance mechanisms of arsenicals in yeast cells. This understanding may pave the way to determine the mechanisms in higher eukaryotes. Additionally, it may be possible to apply the knowledge gained from this study in biotechnology to phytoremediate contaminated areas or in medicine to improve treatments to cure arsenic toxicity or to overcome resistance associated with arsenic-containing drugs.

CHAPTER 2

MATERIALS AND METHODS

2.1. Approach Used in the Current Study

In this study, genomic DNA library was screened in W303-1A and GK4 cell strains (Table 2.1) to determine the genomic regions for which overexpression provided resistance against arsenite or arsenate. The approach followed throughout the study is summarized in Figure 2.1.

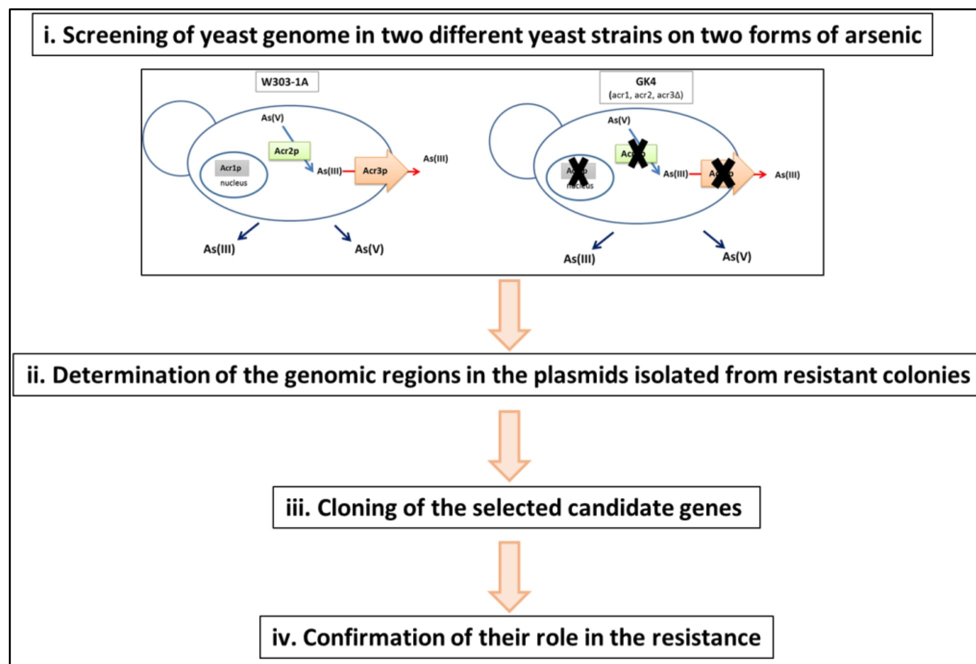


Figure 2.1. The figure indicating the workflow followed throughout the current project

2.1.1. Reproducibility

The results obtained throughout this study were obtained using nearly the same quantity of yeast cells at the same growth phase. The presented spotting assays on the plates are representative results of at least three independent experiments giving consistent qualitative results.

2.2. Yeast Strains and Their Growth Conditions

The strains of *S. cerevisiae* used throughout this study are presented in Table 2.1. The deletion mutants used in sensitivity assay were purchased from EUROSCARF. Haploid (n) and diploid (2n) deletion mutants are in BY4741 and BY4743 backgrounds, respectively, lacking the corresponding gene but carrying kanamycin resistance gene instead.

Table 2.1. The strains of *S.cerevisiae* used in this study

Strain	Genotype
BY4741	MATa, his3Δ1 leu2Δ0 met15Δ0 ura3Δ0
BY4743	MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15 ura3Δ0/ura3Δ0
(n) Δgene	BY4741 ΔDeleted gene:KAN^R
(2n) Δgene	BY4743 ΔDeleted gene:KAN^R
W303-1A	MATa ura3-1 leu2-3/112 trp1-1 his3-11/15 ade2-1 can1-100 GAL SUC2 mal0
GK4	W303-1A acr1, acr2, acr3Δ::loxP-kanMX-loxP

The media for regular cultivation of those strains were YPAD (1% yeast extract, 2% peptone, 0.04 % adenine, 2% glucose) as a rich medium and SD (synthetic dextrose with 0.67% yeast nitrogen base without amino acids, 2% glucose and supplemented with auxotrophic requirements) as a selective medium.

The metalloids used in this study were sodium arsenate, sodium arsenite, potassium antimonyl tartrate and boric acid. Those metalloids obtained from Sigma were added to the growth media based on the concentration of interest.

2.3. Transformation of Plasmids into Yeast Cells

Transformation of genomic DNA library (AB320 genomic DNA library in YEp13 plasmid in *E.coli* obtained from ATCC (No: 37323)), amplified plasmids for yeast-back analysis or overexpression plasmids into the yeast cells was carried out by standard LiAc method (Yeast Genetic Techniques-Cold Spring Harbour). The transformants incubated in the transformation mix at 30°C and heat-shocked at 42°C were plated onto the corresponding selective plates, either with or without arsenic based on the experiment. The transformant colonies were streaked onto selective plates for the expansion of the colonies for the further experiments.

2.4. Bacterial Transformation, Plasmid Isolation and Sequencing

The plasmids in the resistant colonies obtained in the genomic library screening were isolated by commercial miniprep kits (Thermo-Molecular Biology-GeneJET Plasmid Miniprep Kit) following the incubation of yeast cells in lyticase for 30 minutes. Then, those plasmids which were not concentrated enough for further experiments were transformed into *JM109 E.coli* cells by heat shock method for the amplification of the plasmids. Plasmids were isolated from bacteria culture by miniprep kit and then used for the yeast-back transformation to validate that these plasmids were the source of the resistance in the resistant colonies. Plasmids which were validated in yeast-back test were sequenced with vector-specific sequencing primers using DNA sequencer ABI3130xl with ABI PRISM sequencing analysis v5.1 program. The results obtained from the sequencing were analyzed in nucleotide-nucleotide BLAST application of NCBI (National Center for Biological Information) and SGD (*Saccharomyces* Genome Database) to assess the genomic regions in the cassettes of resistant colonies. Consequently, a list of the genes in the genomic cassettes was constructed.

2.5. Cloning of the Selected Candidate Genes

After conducting sensitivity assays and also analyzing the function of the genes in the cassettes, the candidate genes for primary cloning were identified. Among those candidate genes, only PHO86 and VBA3 genes are not available in the yeast Open Reading Frame (ORF) collection in *E.coli* host cells (YSC3868). All cloning experiments were carried out by Gateway Cloning System.

BP reactions for the genes available in the ORF collection were set up by using the plasmids isolated from ORF host to insert the gene of interest into the donor plasmid which was pDONR221. BP reactions for PHO86 and VBA3 genes were conducted after amplification of the genes by PCR (Polymerase Chain Reaction) with Gateway-compatible primers. Then, the reaction product was transformed into OMNI max competent cells and the plasmids isolated from the colonies were restricted with BsrGI enzyme for confirmation of insertion.

The genes that were inserted into the pDONR were used as an entry clone in LR reaction to clone into the destination vector which was pAG425GPD-ccdB. Subsequently, LR products were transformed into OMNI max competent cells and the success of the cloning was assessed by both restriction reaction and sequencing with vector-specific primers.

2.6. Spotting Assay

Spotting assay was utilized in this study for different purposes using different yeast cells and the plates with different metalloids as a qualitative method for the assessment of the resistant or sensitive phenotype.

2.6.1. MIC Determination

Spotting assay analysis of W303-1A, GK4, BY4741 and BY4743 cells for MIC (Minimum Inhibitory Concentration) determination was carried out in YPAD agar plates containing different concentrations of arsenicals. Overnight culture five times diluted

were grown to the logarithmic phase in YPD broth and then, 5 ul of their serial dilutions of OD600= 0.2, 0.02, 0.002, and 0.0002 were spotted onto given concentration of arsenicals. The plates were incubated at 30°C for 3-5 days and their photographs were recorded.

2.6.2. Sensitivity Assay

Diploid deletion mutants of each gene in the identified genomic cassettes were spotted onto the SD all gradient plates containing highest concentration of the arsenical at one end of the plate, while the other end has the lowest value. Therefore, the plate contains a concentration gradient between two ends of the plate. Overnight culture of the mutant cells in the SD all broth were diluted and then grown to the logarithmic phase. Then, 5 ul of the cells with the OD600 value of 0.02 were spotted onto the gradient plates starting from one end of the plate to the other end. The plates were incubated at 30°C for 3-5 days and their photographs were recorded.

2.6.3. Overexpression Analysis

Yeast cells transformed with the expression vectors carrying the corresponding gene were spotted onto the selective gradient plates. Overnight culture of the transformant cells with the overexpressing-plasmids in the selective broth were diluted and then grown to the logarithmic phase. Then, 5 ul of the cells with the OD600 value of 0.02 were spotted onto the gradient plates starting from one end of the plate to the other end. The plates were incubated at 30°C for 3-5 days and their photographs were recorded.

2.6.4. Cross-resistance Analysis

The candidate genes shown to provide resistance to one type of arsenic were tested on the other form of arsenic and two other metalloids, antimonite and boric acid. Yeast cells transformed with the expression vectors carrying the corresponding gene were spotted onto the selective gradient plates containing different metalloids. Overnight culture of the transformant cells with the overexpressing-plasmids in the selective broth

were diluted and then grown to the logarithmic phase. Then, 5 ul of the cells with the OD600 value of 0.02 were spotted onto the gradient plates starting from one end of the plate to the other end. The plates were incubated at 30°C for 3-5 days and their photographs were recorded.

2.7. Growth Curve

Growth curves were used in this study to confirm the MIC determined by spotting and the resistance obtained by the overexpression of the candidate gene.

2.7.1. MIC Determination

Growth curves of W303-1A and GK4 cells in treatment with arsenite or arsenate were constructed. The overnight culture of the cells was diluted to the same OD600 value and their growth in different concentrations of arsenicals was recorded by OD600 measurements every 2 hours. The graph of OD600 versus time was plotted based on the OD600 values obtained in each measurement.

2.7.2. Overexpression Analysis

The cells overexpressing the corresponding gene were grown overnight and those cultures were diluted to same OD600 value and inoculated into the selective broth media containing different concentrations of arsenicals. Those cultures were incubated at 30oC for 24 hours and the final OD600 values were recorded. The graph of concentration versus OD600 was plotted on based on OD600 values at 24 hour.

CHAPTER 3

RESULTS

3.1. Identification of Minimum Inhibitory Concentration of Arsenicals

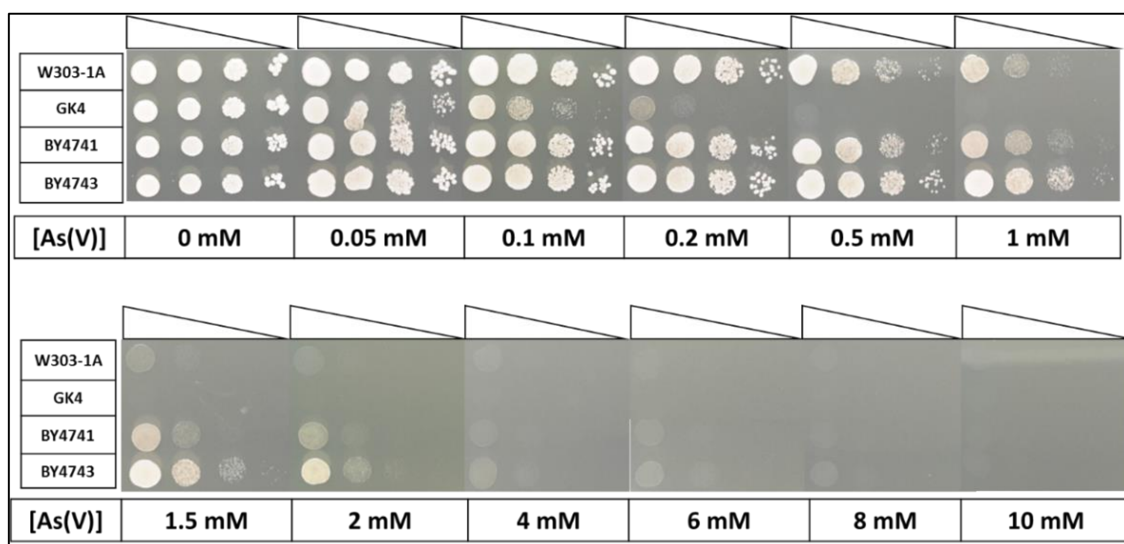


Figure 3.1. Spotting assay analysis for W303-1A, GK4, BY4741 and BY4743 in YPD plates containing given concentrations of arsenate.

Yeast cells with four different backgrounds, which are BY4741, BY4743, W303-1A and GK4 as indicated in Table 2.1, were used throughout the study. Initially, minimum inhibitory concentration (MIC) values of arsenite and arsenate were assessed for all yeast strains by conducting both spotting assay (Figure 3.1 and 3.2) and growth curve analysis (Figure 3.3 and 3.4). The tested concentrations of arsenicals were selected based on the literature (Bobrowicz et al., 1997; Robert Wysocki et al., 2001).

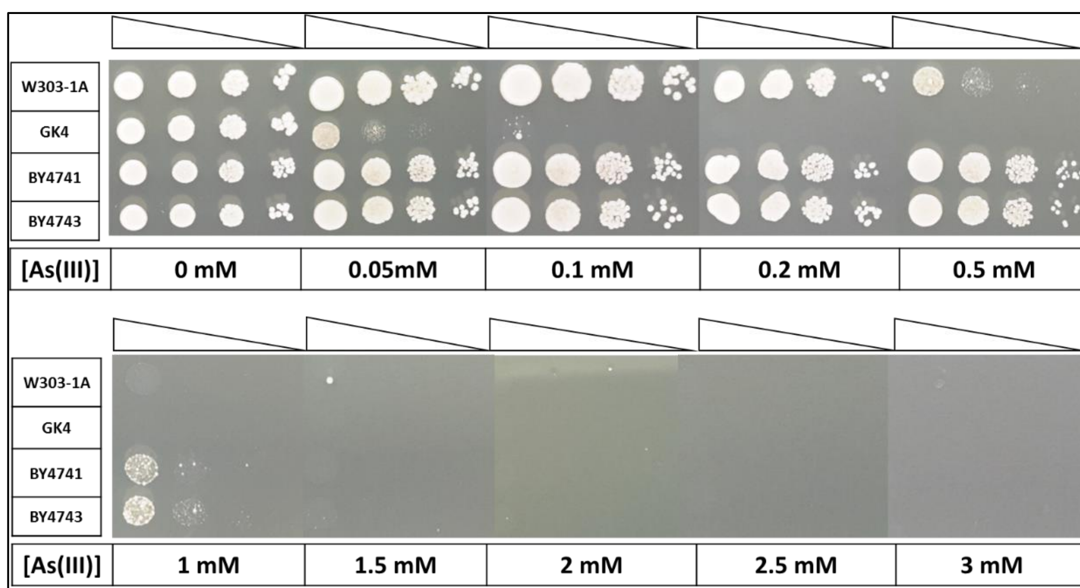
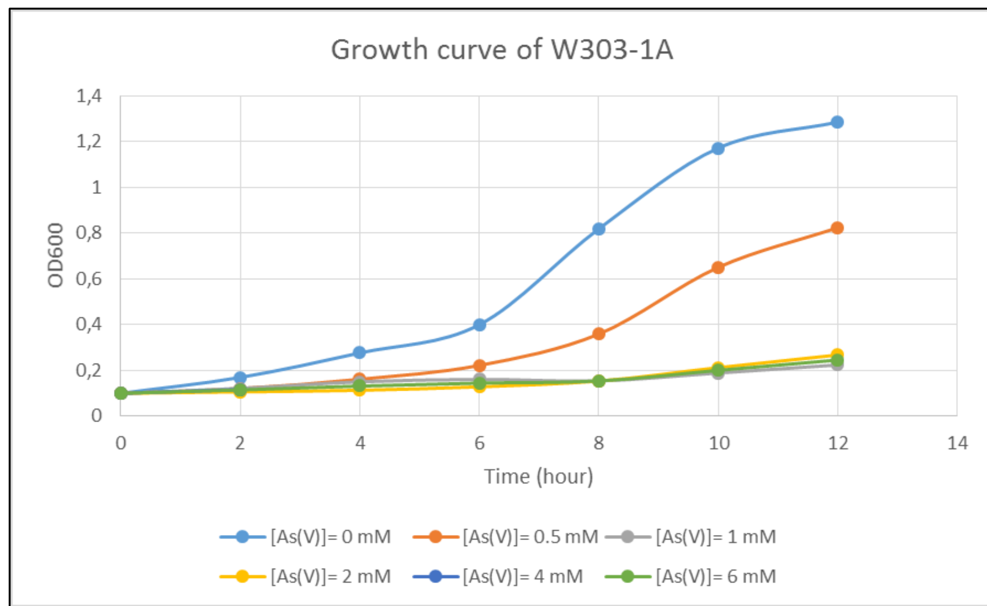


Figure 3.2. Spotting assay analysis for W303-1A, GK4, BY4741 and BY4743 in YPD plates containing given concentrations of arsenite.

Genomic DNA library screening was planned to be conducted in W303-1A and GK4 cells. The concentrations of arsenicals to be used in the screen were selected based on the spotting assay and growth curve results. W303-1A cells did not show significant growth in the 1.5 mM arsenite-containing rich medium, while GK4 cells, which are hyper-sensitive for arsenicals, could resist only up to 0.2 mM arsenate. Arsenite did not allow the growth of W303-1A and GK4 cells when its concentration was more than 1 mM and 0.1 mM, respectively, in the plates. These concentration values were selected as the threshold values for the library screening.

A)



B)

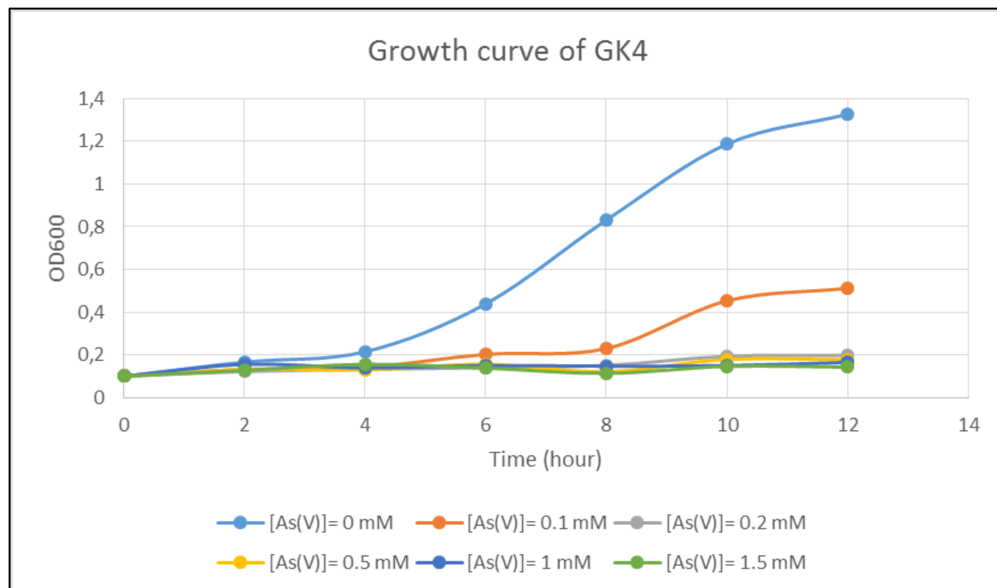
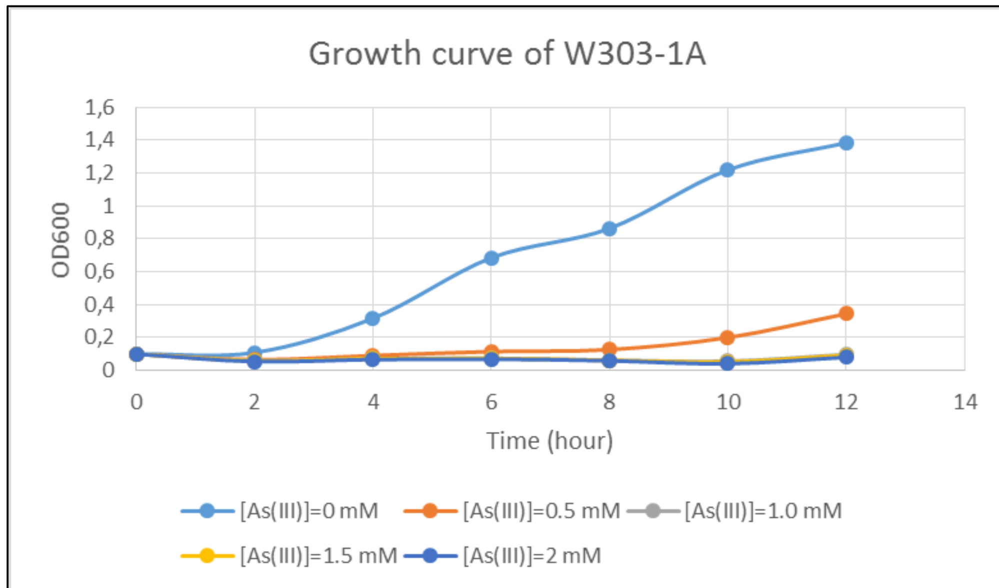


Figure 3.3. Growth curve analysis of A) W303-1A and B) GK4 cells in treatment with given concentrations of arsenate, As(V).

A)



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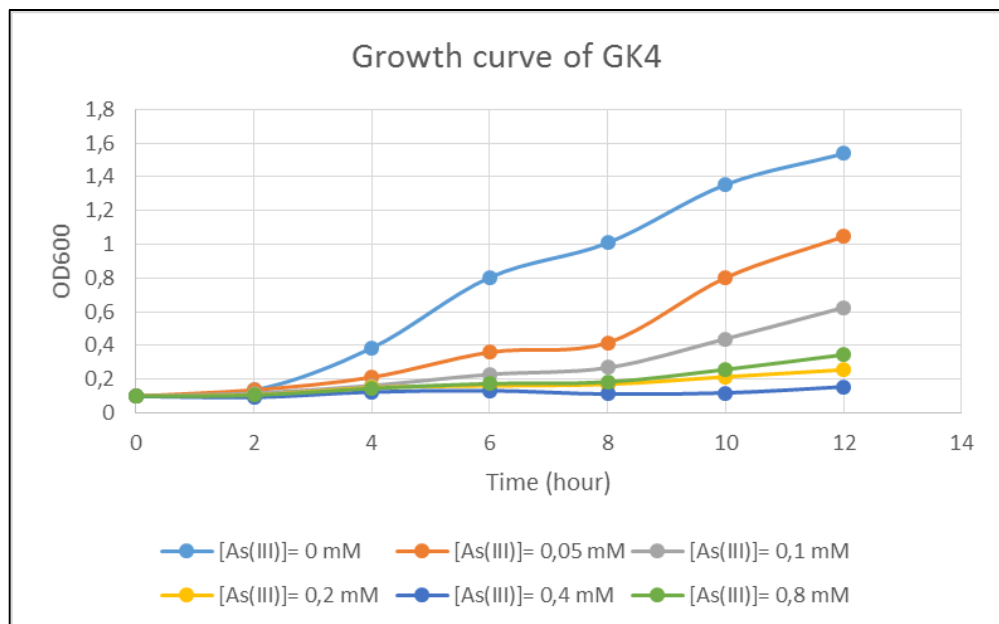


Figure 3.4. Growth curve analysis of A) W303-1A and B) GK4 cells in treatment with given concentrations of arsenite, As(III)

3.2. Genomic Library Screening on Arsenic-containing Selective Plates

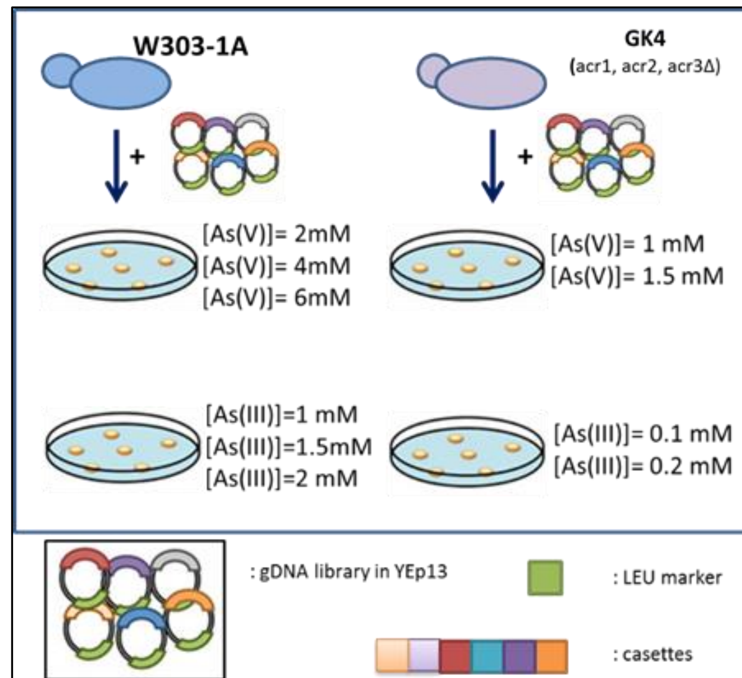


Figure 3.5. Genomic DNA library screening

After determination of MICs of the two arsenic forms for W303-1A and GK4 strains, the yeast genomic library was transformed into both strains. The transformants of W303-1A and GK4 cells were screened on selective plates containing arsenate or arsenite at the concentrations indicated in Figure 3.5. The colonies that were obtained after incubation at 30ºC for 72 hours (Table 3.1) were selected from the plates and expanded on the selective plates for further experiments. Except for a single colony from [As(V)]= 4 mM plate and two colonies from [As(III)]=0.1 mM plate, all colonies could grow on these selective plates. The reason why those three colonies were not able to grow may have been resulted from the loss of the plasmids or false positive colonies.

Table 3.1. The number of colonies which were obtained on the given concentrations of arsenite or arsenate within 72 hours

Strain	Arsenate, As(V)		Arsenite, As(III)	
	[As(V)], mM	# of colonies	[As(III)], mM	# of colonies
W303-1A	2	8	1	-
	4	16	1.5	-
	6	-	2	-
GK4	1	4	0.1	13
	1.5	-	0.2	-

3.3. Validation of Tolerance in the Resistant Transformants

The resistance of the expanded colonies from the library screen was tested on selective plates containing different concentrations of arsenicals by spotting assay. For this spotting assay, initially, arsenical-resistant W303-1A or GK4 transformants were grown to logarithmic phase in SD-LEU broth. Serial dilutions of OD600= 0.2, 0.02, 0.002, and 0.0002 were spotted onto given concentrations of the corresponding arsenic form. The plates were incubated at 30°C for 3-5 days.

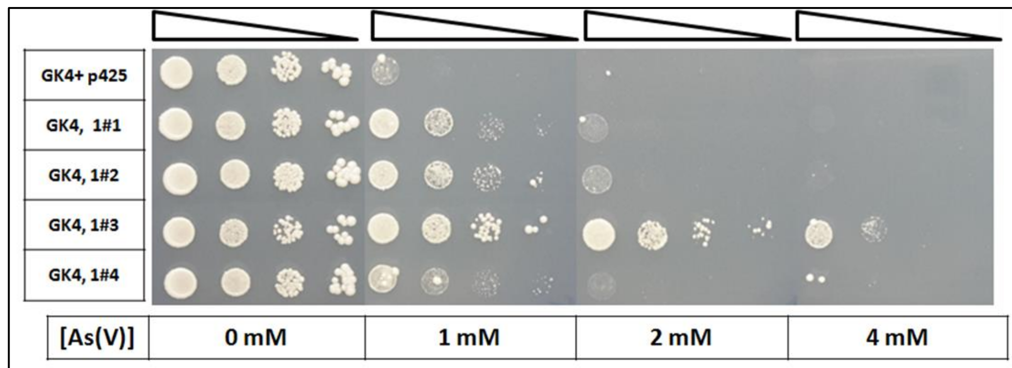


Figure 3.6. Spotting assay analysis for GK4 colonies resistant to [As(V)]=1 mM

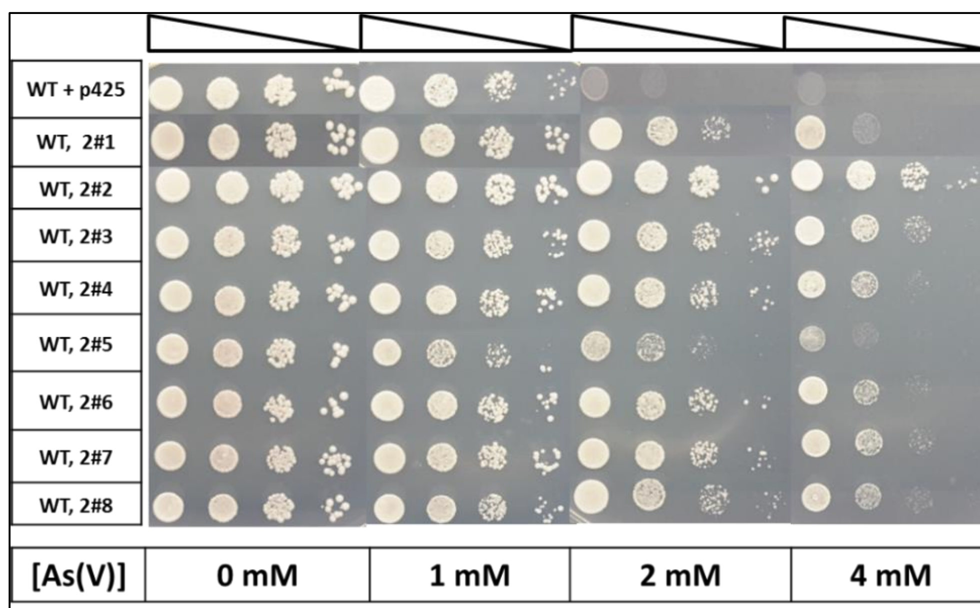


Figure 3.7. Spotting assay analysis for W303-1A colonies resistant to [As(V)]=2 mM

Names of transformants which were able to grow in the arsenic-containing selective media starts with their corresponding strain. W303-1A strain was abbreviated to "WT" and "GK4" abbreviation was used for arsenic hyper-sensitive strain. The number following the strain name indicates the concentration of the plate in which corresponding colony grew. Finally, their names include the number assigned to the corresponding colony in the given concentration of arsenic.

1 mM-arsenate resistant GK4 colonies (GK4, 1#X, Figure 3.6) and 2 mM-arsenate-resistant W303-1A colonies (WT, 2#X, Figure 3.7) were spotted on 1, 2 and 4 mM arsenate containing plates, while 4-mM-arsenate resistant ones (WT, 4#X, Figure 3.8) were tested on arsenate concentrations of 2, 4 and 6 mM arsenate. All colonies were confirmed to be able to grow in the toxic concentrations of arsenate.

GK4 transformants resistant to arsenite were from 0.1 mM arsenite-containing selective plates (GK, 0.1#X) and their resistances were examined on plates with 0.1, 0.2 and 0.4 mM arsenite by spotting analysis (Figure 3.9). All eleven colonies could grow in those hyper-toxic concentrations of arsenite which GK4 cells bearing empty plasmid could not resist.

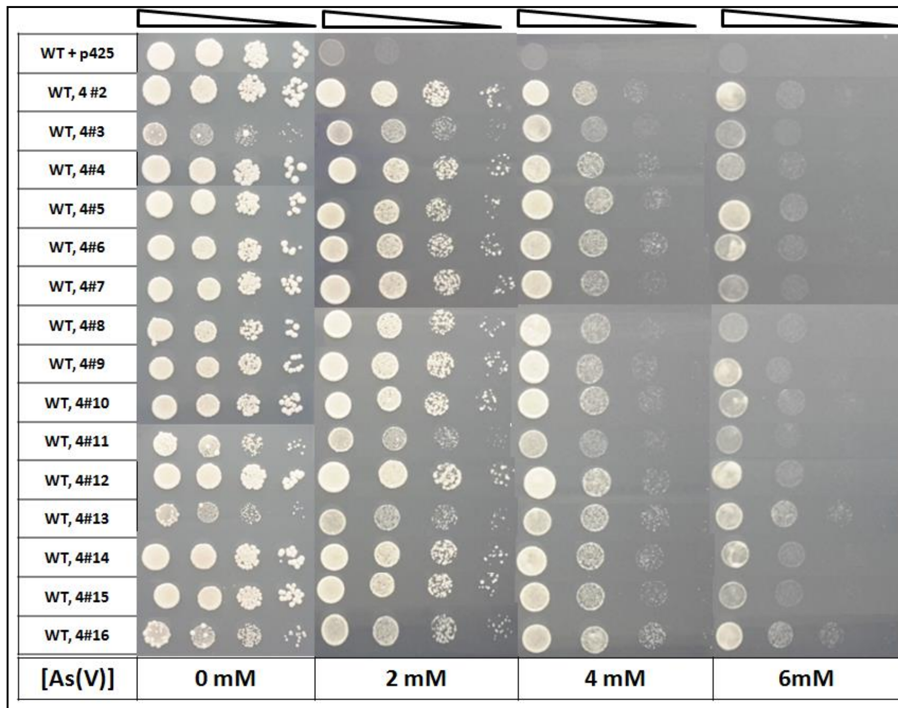


Figure 3.8. Spotting assay analysis for W303-1A colonies resistant to [As(V)]=4 mM

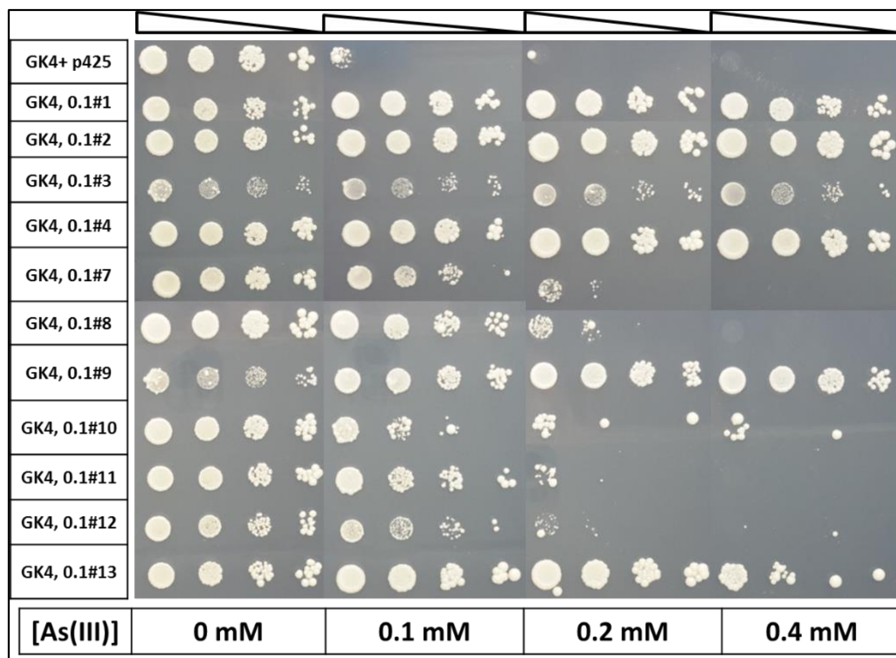
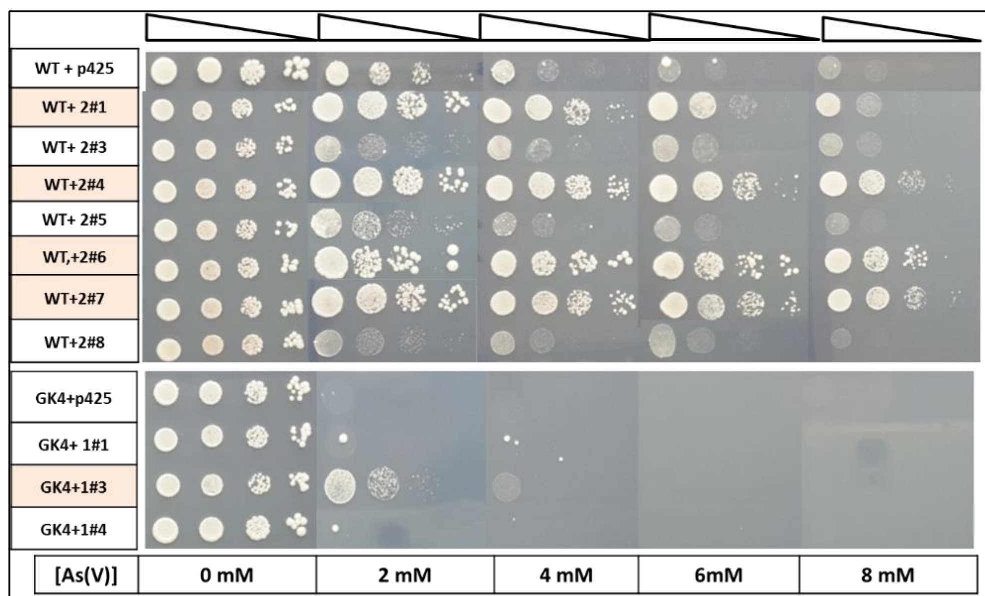


Figure 3.9. Spotting assay analysis for GK4 colonies resistant to [As(III)]=0.1 mM

3.4. Further Confirmation on Yeast-back Transformants

Plasmids were isolated from the colonies for which resistance was validated by spotting assay in the previous step, and were amplified in the JM109 strain of *E. coli*. The amplified plasmids were transformed back into the corresponding yeast strain and spotting assay was carried out with these transformants to indicate that the resistance observed in the transformants was only due to the presence of the plasmids in those colonies.



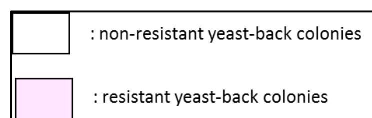


Figure 3.10. Spotting assay analysis for yeast-back transformants of W303-1A colony from [As(V)]=2 mM and GK4 colony from [As(V)]=1 mM

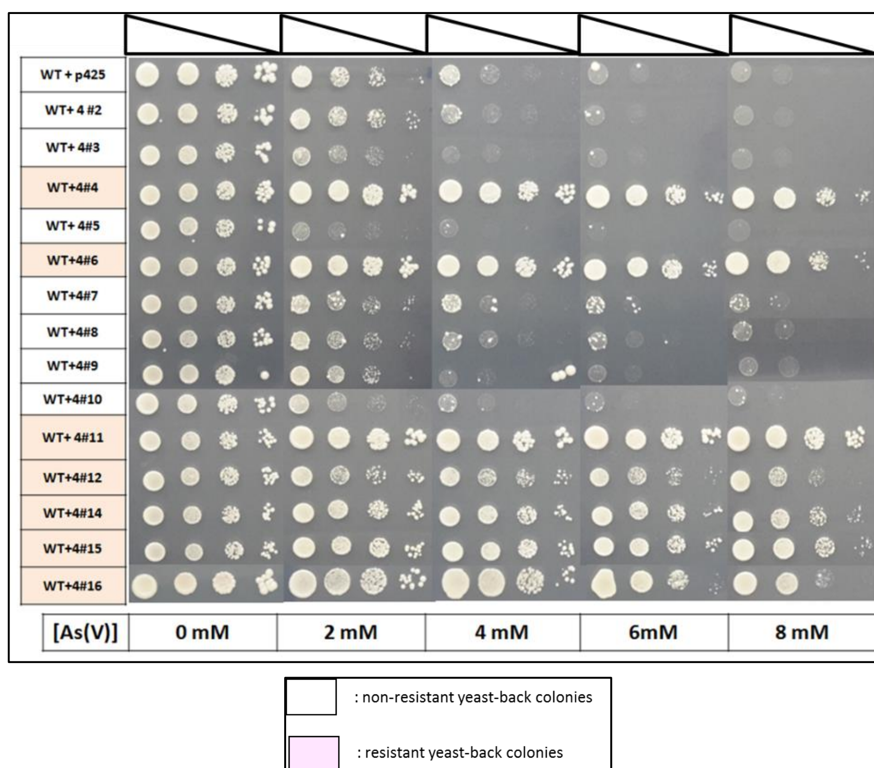
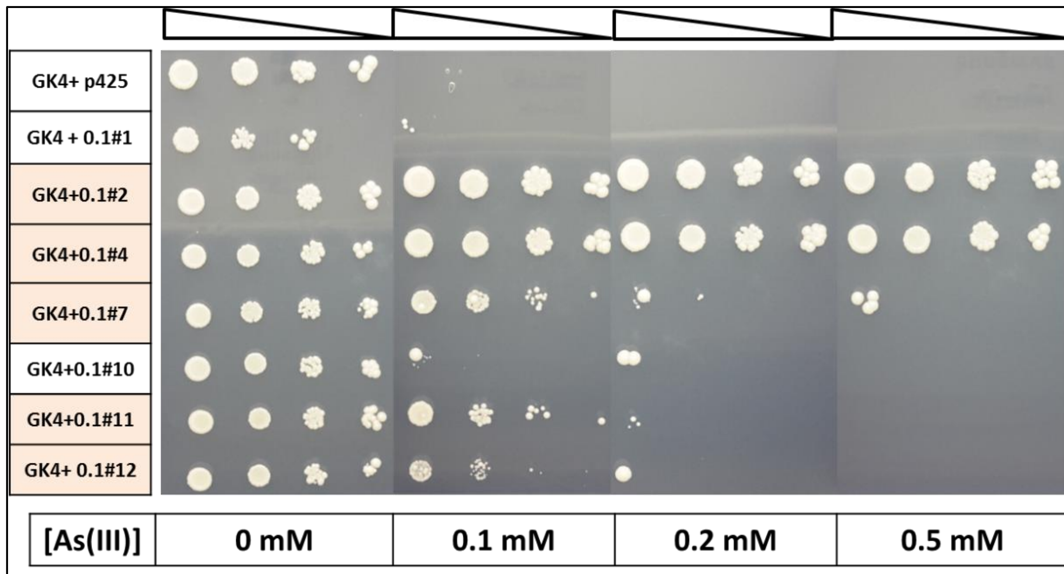


Figure 3.11. Spotting assay analysis for yeast-back transformants of W303-1A colony from [As(V)]=4 mM

Most of the plasmids were successfully isolated from the arsenate- or arsenite-resistant yeast cultures and transformed into bacteria for amplification of the plasmids for sequencing analysis. Nevertheless, no bacterial colonies were obtained after transformation of competent cells with isolated plasmids from the arsenate-resistant colonies coded as 2#2, 1#2 and 4#13 and arsenite-resistant ones coded as 0.1#3, 0.1#8, 0.1#9 and 0.1#13. These colonies were expanded in the selective plates indicating the availability of the plasmids inside the cells. This result may have resulted from failure in breakage of the yeast cell wall during plasmid isolation process and other enzymes or glass-beads could be tried to isolate spheroblasts (Feldmann, 2005).

When the spotting assay figures for yeast-back colonies are analyzed (Figure 3.10, 3.11, and 3.12), the pink-color-coded colonies are the highly-resistant ones for which plasmids were selected for sequencing.



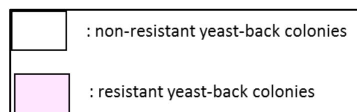


Figure 3.12. Spotting assay for yeast-backs of GK4 colony from [As(III)]=0.1 mM

3.5. Sequencing of the Plasmids for the Identification of Genomic Cassettes

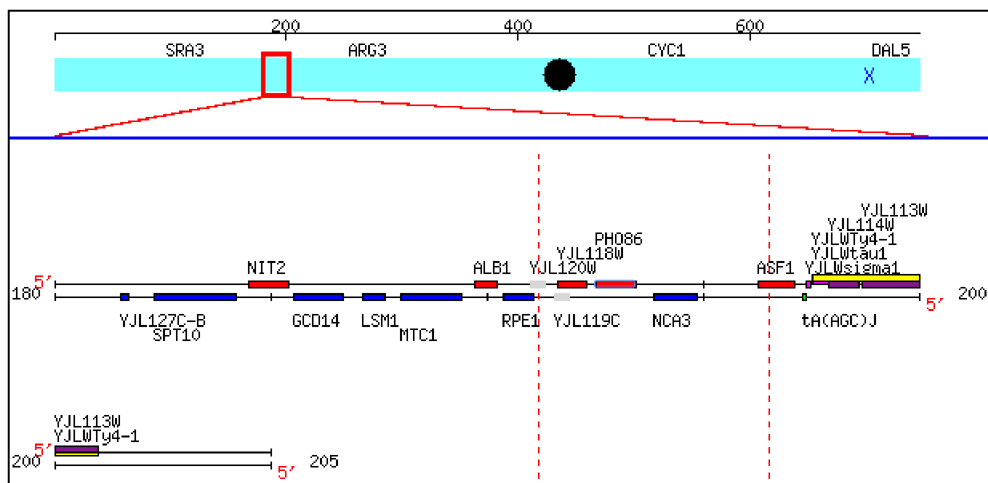


Figure 3.13. The sequence analysis of the plasmid from colony W303-1A, 4#6

A genomic DNA library was constructed by inserting randomly restricted genomic fragments into Yep13 plasmid, so vector-specific primers were used as sequencing primers. The genomic regions in the plasmids isolated from resistant colonies were determined by analysis of the sequencing results in BLAST application of *Saccharomyces* Genome Database (SGD) as exemplified in Figure 3.13. The genomic regions and the genes present in these genomic cassettes are indicated in Table 3.2. However, cassettes of some plasmids could not be identified due to noisy chromatograms or failure in sequencing.

Table 3.2. The genomic regions and the genes present in the cassettes of the plasmids isolated from (A) arsenate-resistant and (B) arsenite-resistant colonies.

A)

[As(V)]=15 mM

Colony	Source of the colony	Region	Candidate genes
W303-1A + p425	-	-	W303-1A + p425
W303-1A + 5.1	As(V)=4#4	Chr X, 188721..195519	NCA3, PHO86, YJL118W, RPE1, ALB1, MTC1
W303-1A + 5.2	As(V)=4#6	Chr X, 191189..196530	ASF1, NCA3, PHO86, YJL118W
W303-1A + 5.3	As(V)=4#11	Chr XVI, 929988...941100	ARR1, ARR2, ARR3, SGE1, YPR196W
W303-1A + 5.4	As(V)=4#12	Chr XI, 368844..373815	TUL1, UGP1, AIM26
W303-1A + 5.5	As(V)=4#14	Chr X, 188958...196530	ASF1, NCA3, PHO86, YJL118W, RPE1, ALB1, MTC1
W303-1A + 5.6	As(V)=4#15	Chr X, 191207..196530	NCA3, PHO86, YJL118W
W303-1A + 5.7	As(V)=4#16	Chr X, 189834..204440	ASF1, NCA3, PHO86, YJL118W, RPE1, ALB1
W303-1A + 5.9	As(V)=2#4	Chr X, 190300..196690	ASF1, NCA3, PHO86, YJL118W, RPE1
W303-1A + 5.11	As(V)=2#7	Chr X, 188958..194280	NCA3, PHO86, YJL118W, RPE1, ALB1

[As(V)]=2 mM

Colony	Source of the colony	Region	Candidate genes
GK4+p425	-	-	GK4+p425
GK4 + 5.12	As(V)=1#3	Chr X,186335..193712	PHO86, YJL118W, RPE1, ALB1, MTC1, LSM1,

B)

colony	Source of the colony	Region	Candidate genes
GK4+p425	GK4+p425	-	GK4+p425
GK4 + 3.2	As(III)=0.1 #4	Chr XVI, 932323..943489	ARR1, ARR2, ARR3, SGE1
GK4 + 3.3	As(III)=0.1 #7	Chr XIII, 250986..256885	GIS4, YAP1, ERG6, MRPL39
GK4 + 3.4	As(III)=0.1 #11	Chr III, 9676..14296	VBA3
GK4 + 3.5	As(III)=0.1 #12	Chr XIII, 250986..256885	GIS4, YAP1, ERG6, MRPL39

[As(III)]=0.2 mM

Color code	The genomic region between bases...
	186335 and 204440 on Chromosome X
	929988and 941100 on Chromosome XVI
	368844 and 373815 on Chromosome XI

Color code	The genomic region between bases...
	932323 and 943489 on Chromosome XVI
	250986 and 256885 on Chromosome XVI
	9676 and 14296 on Chromosome III

3.6. Sensitivity Assays for Diploid Deletion Mutants of Candidate Genes

As Table 3.2 indicates, all of the cassettes contain at least two genes, except for the one coded as 3.4. Sensitivity assays for diploid deletion mutants of the candidate genes were conducted to compare the sensitivity of the mutants with wild type yeast cells in order to determine the genes to be primarily cloned.

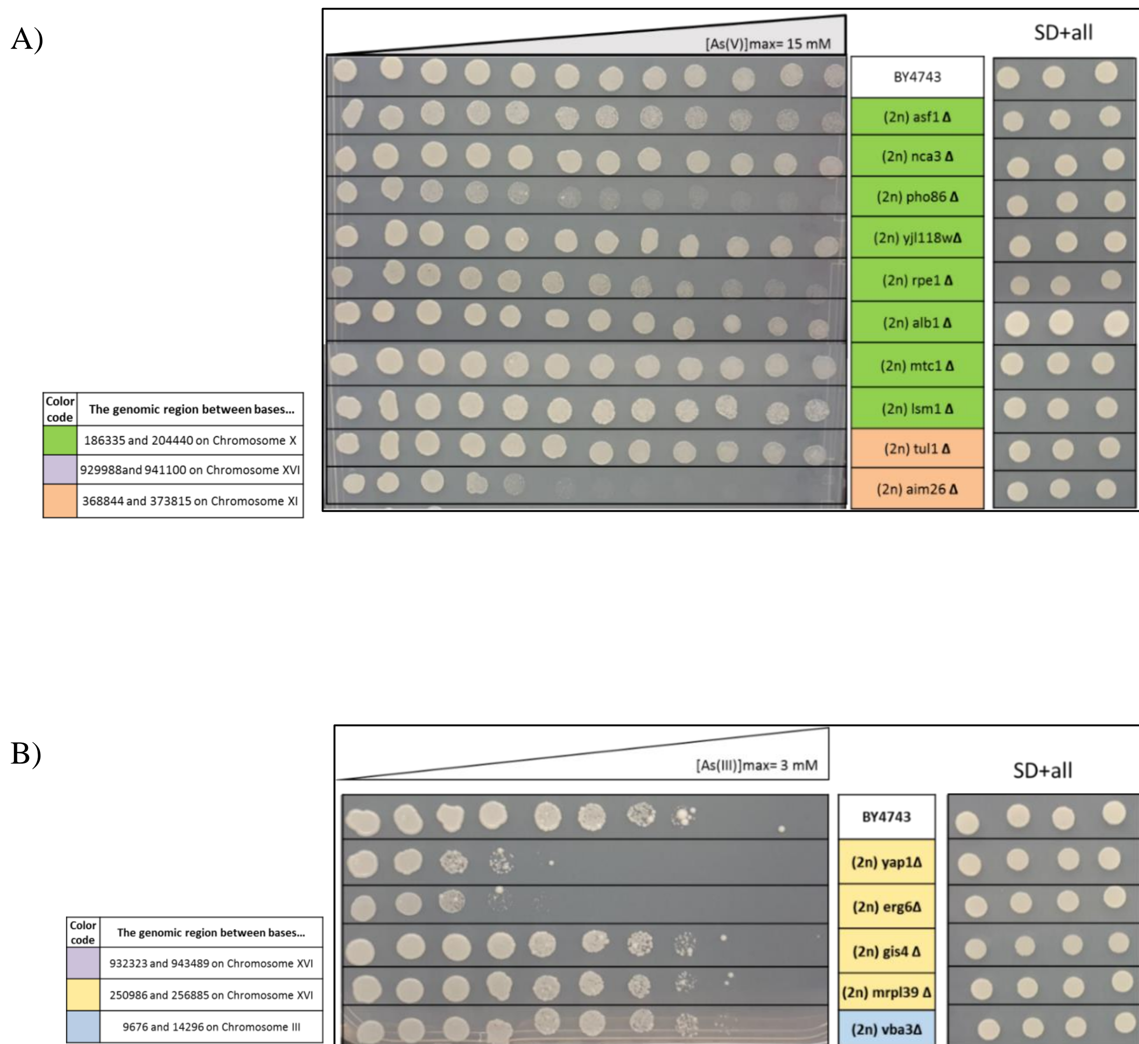


Figure 3.14. Sensitivity assay for diploid deletion mutants of candidate genes in gradient plates (A) with the highest concentration of [As(V)]= 15 mM and (B) with up to 3 mM As(III)

Homozygous deletion of PHO86 and AIM26 genes in diploid yeast cells sensitized them to arsenate, while yap1 and erg6 deletion mutants were sensitive for arsenite. Therefore, those four genes were selected for cloning. Additionally, UGP1 was

also selected for the primarily cloning list, as this essential gene does not have a deletion mutant to give any idea about its sensitivity. Although VBA3 was not sensitive to arsenite, it was also chosen for cloning, because it was the only gene in its cassette.

3.7. Cloning of the Selected Genes into Expression Vectors

The two-step cloning system of Gateway Cloning Technology was carried out to clone selected genes into expression vectors. Except for PHO86 and VBA3 which were amplified with Gateway-compatible primers by PCR from the genomic DNA, all selected genes were available in the yeast Open Reading Frame (ORF) collection which is compatible with Gateway Technology. The success of the cloning into pAG425GPD-ccdB expression vector was assessed by restriction digestion reaction with BsrGI enzyme (Figure 3.15). The removal of the restriction sites from the plasmid upon the insertion of the gene into the vector changes the pattern of the restriction and this can be detected by gel electrophoresis. Additionally, the cloning was confirmed by sequencing.

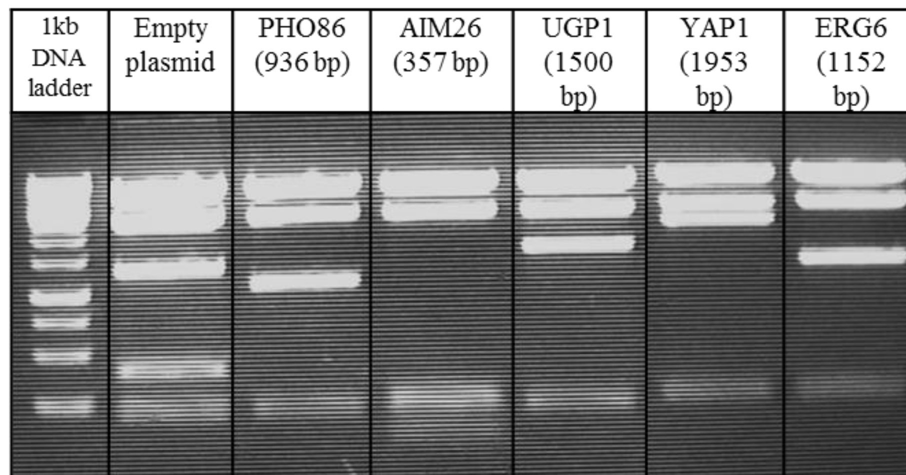


Figure 3.15. Gel electrophoresis result showing the BsrGI restriction products of the cloned genes into expression vector

3.8. Validation of the role of the selected genes in arsenic tolerance

Expression vectors confirmed to bear the candidate genes were transformed into corresponding yeast strains and overexpression analysis on the arsenic-containing gradient plates and growth curve analysis in the arsenic-containing broth culture was performed to verify their roles in arsenical resistance.

3.8.1. Spotting Assay

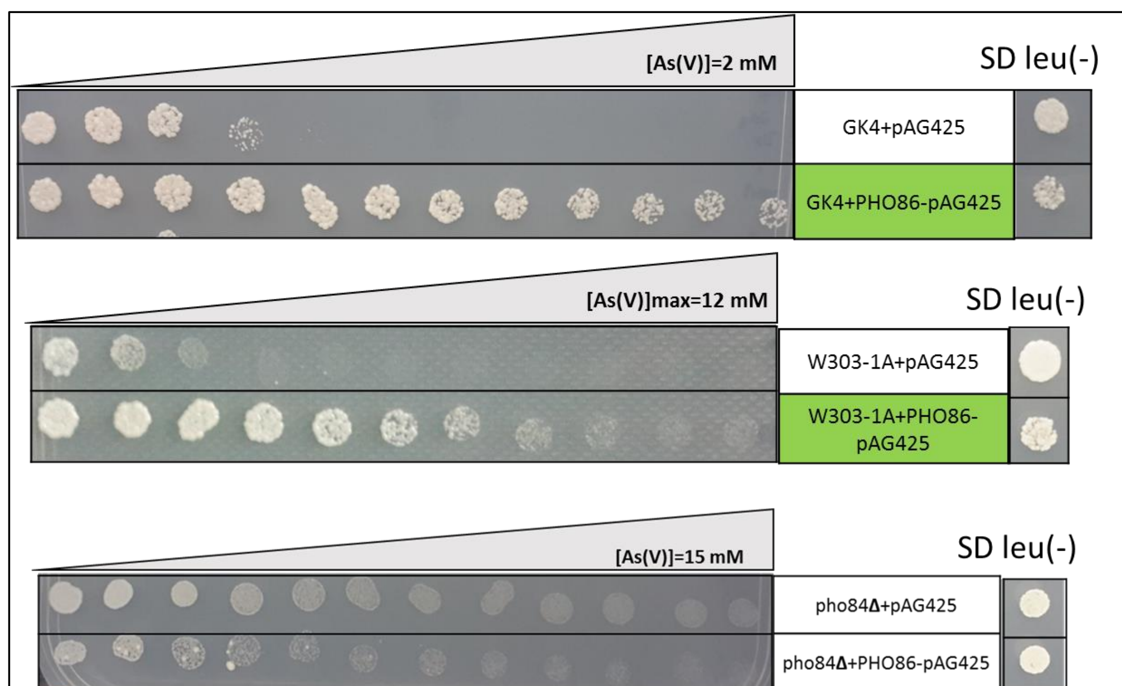


Figure 3.16. Overexpression analysis for W303-1A, GK4 and haploid deletion mutant of PHO84 cells transformed with empty plasmid, pAG425, or PHO86-pAG425 on gradient plates with up to 2, 12 and 15 mM arsenate, respectively. (OD600=0.02 for all spots)

Overexpression of Pho86p in W303-1A and GK4 cells was achieved thanks to high copy expression vectors and provided yeast cells with better growth in arsenate gradient plates in comparison to yeast cells transformed with empty plasmid. However, there was no growth difference in the haploid deletion mutant of the PHO84 overexpressing Pho86p.

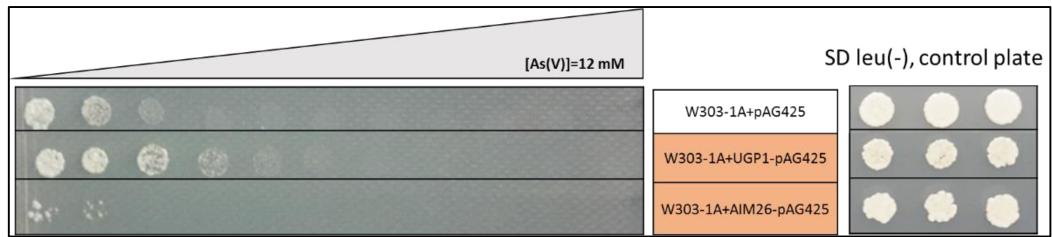


Figure 3.17. Overexpression analysis for W303-1A cells transformed with empty plasmid, pAG425,, AIM26-pAG425 and UGP1-pAG425 in gradient plates with the highest concentration of [As(V)]= 12 mM (OD600=0.02 for all spots)

High copy expression of AIM26 did not confer resistance to W303-1A yeast cells, while overexpression of UGP1 slightly increased the growth of W303-1A cells in the presence of arsenate.

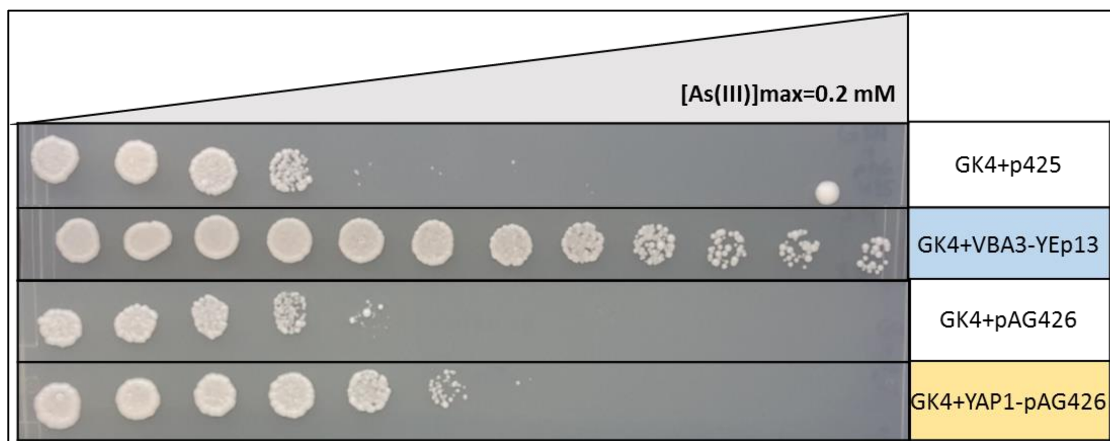


Figure 3.18. Overexpression analysis for GK4 cells transformed with empty plasmid, pAG425, YAP1-pAG426 or VBA3-Yep13 in gradient plates with the highest concentration of [As(III)]= 0.2 mM (OD600=0.02 for all spots)

When Vba3p was overexpressed in the GK4 cells, they could resist even to 0.2 mM arsenite. Although Yap1p- and Erg6p overexpression was not as strong as Vba3p, they grew in higher concentrations than empty plasmid-bearing GK4 cells (Figure 3.18 and Figure 3.19).

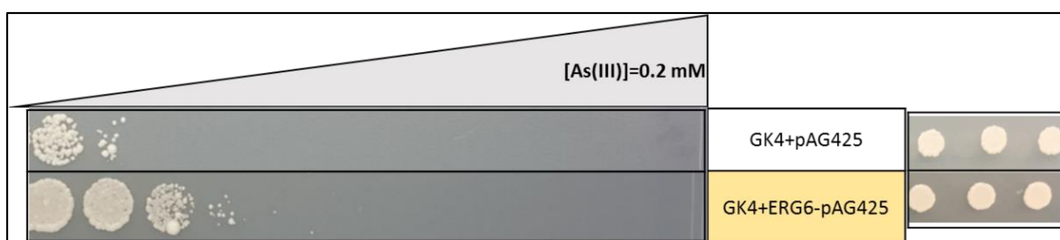


Figure 3.19. Overexpression analysis for GK4 cells transformed with empty plasmid, pAG425, or ERG6-pAG425 in gradient plates with the highest concentration of [As(III)]= 0.2 mM (OD600=0.02 for all spots)

3.8.2. 24-hour Growth Analysis

After validation by spotting assay, the role of the candidate genes in arsenical tolerance was further confirmed by growth analysis in the broth medium containing different concentrations of arsenical.

The initial optical density (OD) at 600 nm of the yeast cells was arranged to be the same value and then they were inoculated into selective broth media with corresponding concentrations of arsenic. While W303-1A cells carrying empty or PHO86-inserted plasmids were tested in arsenate, GK4 cells with empty, ERG6- or VBA3-inserted plasmids were analyzed in arsenite, based on their original strain in the initial screening.

Overexpression of Pho86p resulted in higher OD600 values up to 10 mM arsenate than plasmid-only control group (Figure 3.19). GK4 cells overexpressing Vba3p could resist up to 0.2 mM arsenite, while GK4 cells with empty vector were not able grow in even 0.1 mM arsenite. When Erg6-overexpression was analyzed, it was not as strong as Vba3, but it still grew better than control cells in the culture inoculated into 0.1 mM arsenite-containing broth medium.

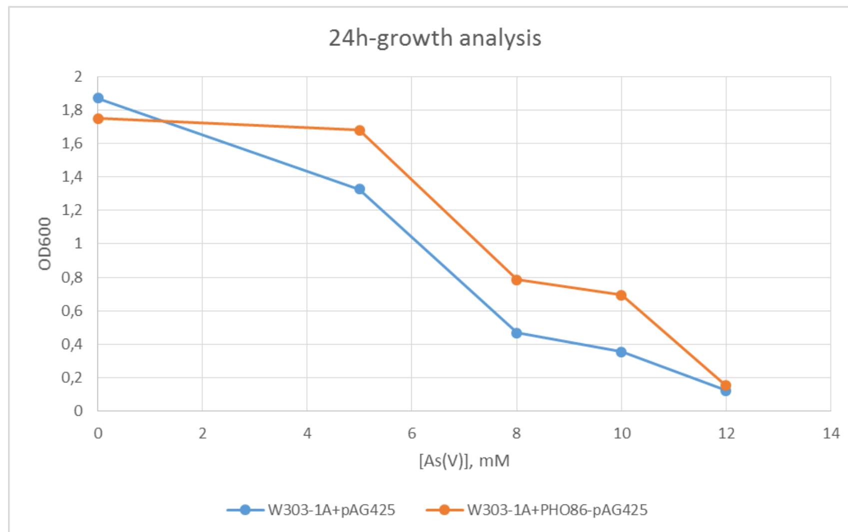


Figure 3.20. 24-hour-growth analysis for W303-1A cells transformed with empty plasmid, pAG425, and PHO86-pAG425 plasmids in given concentrations of arsenate

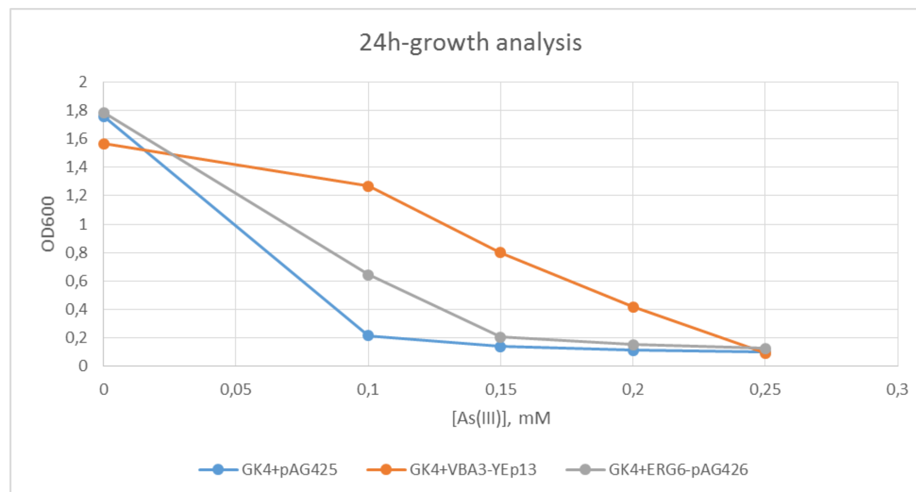


Figure 3.21. 24-hour-growth analysis for GK4 cells transformed with empty plasmid, pAG425, VBA3-Yep13 and ERG6-pAG425 plasmids in given concentrations of arsenite

3.9. Cross-resistance Analysis for the Candidate Genes

While some resistance mechanisms are specific to certain metal or metalloid, some mechanisms can supply resistance to other metal or metalloid species (Thorsen et al., 2009). To elucidate whether the candidate genes obtained in our screen provide tolerance for other metalloids, the growth of the yeast cells overexpressing these candidate genes were tested in arsenate, arsenite, boric acid and antimonite.

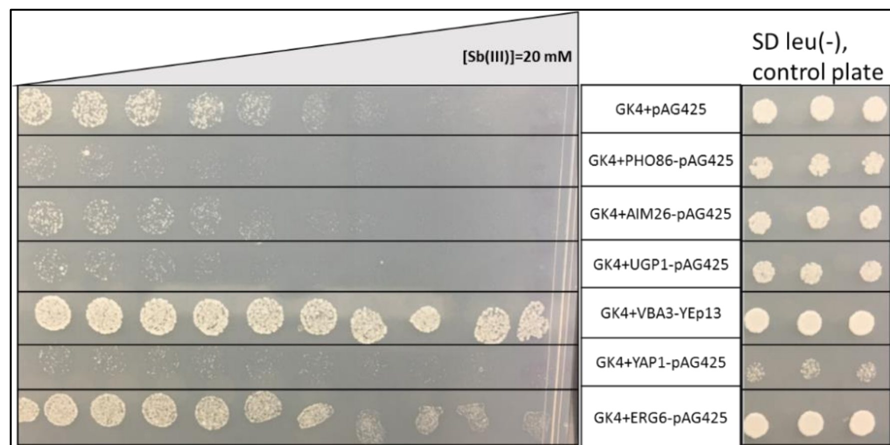


Figure 3.22. Spotting assay for the yeast cells overexpressing candidate genes to test their growth on antimonite

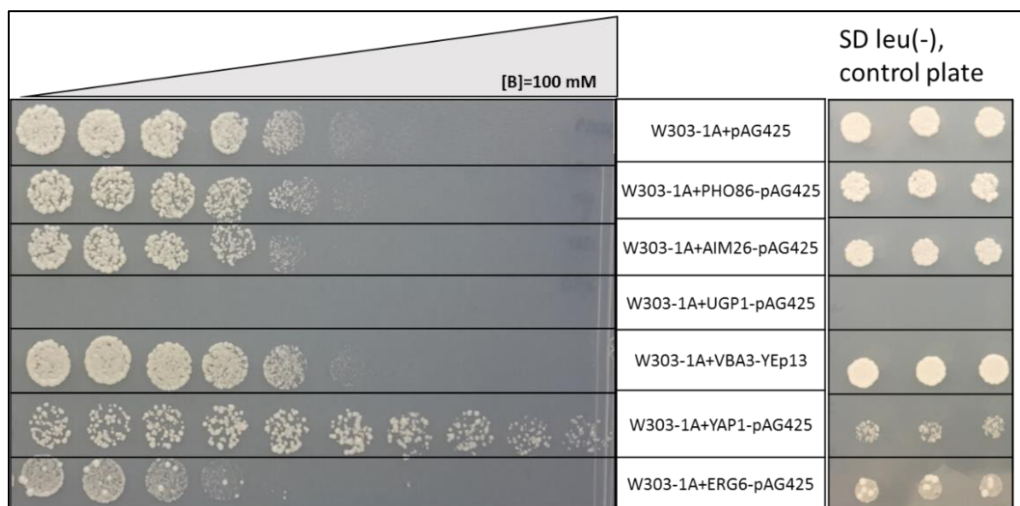


Figure 3.23. Spotting assay for the cross-resistance test on boric acid

VBA3 which was obtained in the arsenite-containing plates in the initial screen showed resistance to both antimonite and arsenate (Figure 3.21 and 3.23, respectively). Only YAP1-overexpression gained resistance to boric acid (Figure 3.22) and none of the arsenate-resistant-associated genes caused better resistance to arsenite indicating that they are specific for arsenate tolerance.

Figure 3.24. Spotting assay for the yeast cells overexpressing candidate genes for arsenite resistance to test their growth on arsenate

Figure 3.25. Spotting assay for the yeast cells overexpressing candidate genes for arsenite resistance to test their growth on arsenate

CHAPTER 4

DISCUSSION

Arsenic is an element classified as a metalloid and is abundant in the environment mainly in two forms which are pentavelant [arsenate, As(V)] and trivalent [arsenite, As(III)] forms. It is highly toxic and causes many diseases including several cancer types such as skin, bladder and liver cancers (Jacobson et al., 2012; Thorsen et al., 2007). On the other hand, arsenic has been used as a therapeutic reagent for many years in the treatment of diseases like acute promyelocytic leukemia, however cancer cells develop resistance against these drugs (Thorsen et al., 2007). Therefore, there is a need to understand the molecular mechanisms related to both arsenical toxicity and tolerance to better handle arsenicals in therapies and protections.

Budding yeast has been an ideal model organism when the available molecular and biological tools and awesome power of yeast genetics are taken into consideration. Furthermore, the similarity in the molecular mechanisms of toxicity, tolerance and adaptation with higher eukaryotic organisms is another advantageous property of yeast as a model organism in toxicogenomic studies (dos Santos et al., 2012).

Two main transporters were identified in yeast cells as exporters of arsenicals from the cytosol. While Ycf1p pumps GSH-conjugated arsenite into the vacuole, Acr3p localized to the cell membrane is responsible for the removal of arsenite from the cytoplasm (Ghosh et al., 1999; R Wysocki et al., 1997). Although the double mutant lacking both transporters was much more sensitive than single deletion mutants of each gene, these double mutants were still able to grow in arsenic-containing medium (Ghosh et al., 1999). This result points out that there are other pathways or regulatory mechanisms for the detoxification of the arsenicals which have not been enlightened yet. At this point, comprehensive genome-wide phenotypic screens are promising approaches to identify cellular systems for tolerance against arsenic and yeast is a suitable system with the biological collections for genome-wide analysis(Dos Santos & Sá-Correia, 2011; Thorsen et al., 2009).

Considering these aspects, this project aimed to screen yeast genomic library to elucidate novel genes of which overexpression provide yeast cells with arsenic tolerance.

Two different yeast strains were screened in two major forms of arsenic which are arsenate and arsenite. The first strain has the wild type background of W303-1A and the other one is the hypersensitive mutant, GK4, which is lack of the arsenical resistance gene cluster in the same background.

Minimum inhibitory concentration (MIC) is defined as the lowest concentration of the substance, chemical or the antimicrobial reagent which prevents the growth of the microorganisms in the defined conditions (Andrews & Andrews, 2001). After determination of MIC values of both arsenicals for both strains by spotting assay and growth curve analysis (Figure 3.1- Figure 3.4), yeast genome library was transformed into W303-1A and GK4 cells to be screened on the arsenic-containing selective plates. The number of the colonies which were able to grow in the given toxic concentrations of arsenite or arsenate are recorded in Table 3.1. Some of the colonies could not be expanded in the selective medium and this may have been because of the false positive colony in the screen or the loss of the plasmids from the yeast cells. Since Yep13, the host vector of the yeast genome library, is an episomal vector and does not have CEN (centromere) element, these plasmids are prone to be lost in the consecutive divisions (Dani & Zakian, 1983; Feldmann, 2005; Sherman, 2002; Whiteway & Ahmed, 1984).

The resistance of the colonies able to be expanded in the selective plates was confirmed by spotting serial dilutions of the colonies on the arsenic-containing plates (Figure 3.6-Figure 3.9). Subsequently, the plasmids available in the resistant colonies were isolated, however, the concentration of the plasmids isolated from the yeast cells were not high enough for transformation and sequencing. Therefore, the amplification of plasmids in *E.coli* is a pre-requisite for the further confirmation of the resistance and for the sequencing analysis of the plasmids with vector-specific primers.

The amplified plasmids were transformed into corresponding yeast strains to prove that the resistance in the colonies is only derived from the plasmids (Figure 3.10-Figure 3.12) and as a result of this step twelve candidates and five candidates which were hyper-resistant to arsenate and arsenite, respectively, were selected. The plasmids belonging to those colonies were sequenced with the primers designed for YEp13 plasmid hosting the genomic library.

The bioinformatic analysis of the sequencing results revealed the genomic regions available in the plasmids of arsenate and arsenite resistant colonies, as can be seen in

Table 3.2. Some of the sequencing data did not give appropriate chromatogram preventing the determination of the genomic cassettes.

When the arsenate-resistant cassettes are analyzed (Table 3.2.A), one of the cassettes belongs to arsenical resistance gene cluster, which was already determined to encode for ACR1, ACR2 and ACR3 genes. Acr3p is the arsenite permease and Acr2p converts arsenite to arsenate, while Acr1p is the transcription factor regulating the expression of both genes (Bobrowicz et al., 1997; R Wysocki et al., 1997). This finding confirms both the previous study based on the genomic DNA library screening and the coverage of the available yeast genome library. Another cassette includes three genes, AIM26, UGP1 and TUL1, which have not been previously associated with arsenical resistance. Remaining eight cassettes coded with green color belong to close genomic regions at the Chromosome X and all eight cassettes has PHO86 and YJL118W genes in common (Figure 4.1 and Table 3.2.A).

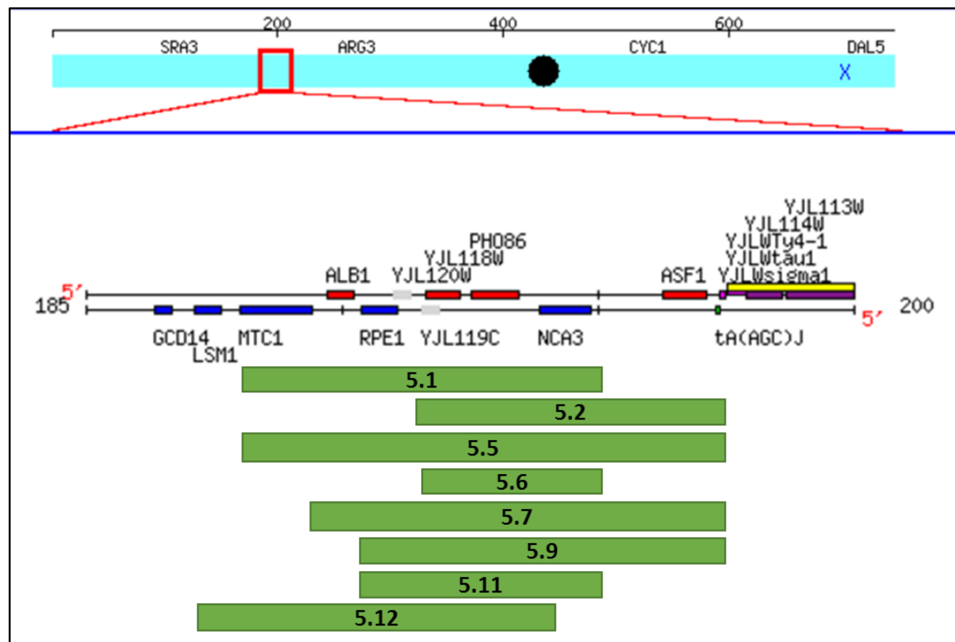


Figure 4.1. The region of chromosome X available in the green-coded cassettes. Each green bar indicates the region covered by the corresponding cassette

The arsenite-resistant colonies are in GK4 background which is hyper-sensitive for arsenicals. The arsenical resistance cluster in colony 3.2 complemented this sensitivity in GK4 cells. The other two cassettes include the same genes which are GIS4, YAP1,

ERG6 and MRPL39. In the final cassette, there is a single gene which is VBA3 (Table 3.2.B).

These results showed that there is more than one gene in all cassettes except for "GK4+3.4" due to the random digestion of the genome by Sau3A restriction enzyme in construction of the library (Nasmyth & Reed, 1980). Sensitivity assay was carried out to select the genes to be initially cloned. In this sensitivity assay, deletion mutants of the candidate genes were spotted on the gradient plates which were prepared to have increasing concentration of arsenicals from one terminus of the plate with the lowest concentration to the other having the highest arsenic concentration. The concentration values at which wild type stains are viable was chosen as the highest concentration of the gradient plate for the sensitivity assay, so that it is possible to differentiate the deletion mutants which cannot grow at the concentrations at which wild type strain can grow. Instead of using a haploid deletion mutant collection, homozygous diploid deletion mutants were used in the sensitivity assay to minimize the effect of mutations in other genes (Hoon et al. 2008).

Green-coded diploid deletion mutants of the arsenate-resistant candidate genes are located in the genomic region between bases 186335 and 204440 on Chromosome X as determined by sequencing analysis. The most sensitive one among the genes available in this region is the homozygous diploid deletion mutant of the PHO86 gene. Additionally, this gene is present in all eight green-coded cassettes. Because of the sensitivity of the deletion mutant and its presence in all cassettes of the close genomic regions, the PHO86 gene seems to be a strong candidate for arsenate resistance. Therefore it was selected as a primary gene to be cloned from those eight green-coded cassettes.

The orange-coded cassette is in the genomic region between bases 368844 and 373815 on Chromosome XI. When the deletion mutants of orange-coded ones were analyzed, diploid deletion of the AIM26 gene is easily distinguishing with its hyper-sensitivity for arsenate making it a member for the cloning list. On the other hand, the growth of the TUL1 deletion mutant was not different from the growth of the wild type strain, hence, it was not included in the cloning list. There is a third gene, UGP1, in the orange-coded cassette, however, this gene is essential for yeast growth and its deletion mutants are not viable (Daran, Dallies, Thines-Sempoux, Paquet, & Francois, 1995). Since it is not possible to have an idea about the sensitivity of ugp1Δ deletion mutant, the UGP1 gene was also included in the cloning list. UGP1 gene is a good example to show

the advantage of genomic library screening over deletion library screening. The disadvantage of deletion library collections is that complete loss of essential genes do not allow the growth of the cells, thus the roles of essential genes in the studied process cannot be examined by deletion library screening (dos Santos et al., 2012; Hoon et al., 2008). If deletion library screening was used as an approach in this study, the likely role of UGP1 would be overlooked.

When all these aspects are considered, PHO86, AIM26, and UGP1 were selected as the initial cloning list for arsenate.

The sensitivity assay for the candidate genes associated to arsenite resistance revealed YAP1, ERG6 and VBA3 as genes to be primarily cloned. Homozygous deletion mutants of YAP1 and ERG6 in the yellow-coded cassette are highly sensitive for arsenite in comparison to wild type cells. VBA3 is the only gene in the blue-coded cassette, so its deletion mutant is expected to be sensitive for arsenite. Nevertheless, the growth of vba3Δ in the presence of arsenite is not different from BY4743 cells. This unexpected finding may point to genetic redundancy. The VBA3 gene has a paralog called VBA5 that resulted from segmental duplication (Katju, Farslow, & Bergthorsson, 2009) Functional compensation by paralog genes is a commonly observed genetic robustness in which the effect of the deletion of the specific gene is not observed thanks to redundant function by the paralog protein (Diss, Ascencio, Deluna, & Landry, 2014). Hence, paralog genes are known to cause paralogous compensation and this may be true for the paralogous couple of Vba3p and Vba5p. Thus, the function of Vba3p may be compensated by Vba5p in vba3Δ deletion mutant cells. VBA3 exemplifies the disadvantage of deletion library screening which is associated with the problem of gene redundancy (Giaever & Nislow, 2014). The double deletion of VBA3 and VBA5 may provide a better idea about the sensitivity of the cells against arsenite.

Gateway Cloning Technology is an advantageous and convenient approach for the cloning of not only PCR products amplified with appropriate adaptor sequences but also ORF plasmids compatible with Gateway plasmids. AIM26, UGP1, TUL1, YAP1 and ERG6 are available in the yeast ORF collection (YSC3868) and these genes were cloned into expression vectors by Gateway Cloning Technology. Confirmation of cloning was achieved by both restriction reaction analysis with BsrGI enzyme (Figure 3.15) and sequencing analysis by vector-specific primers. However, PHO86 and VBA3 are not included in the ORF collection, hence primers were designed for those two genes to

amplify them by PCR for the cloning experiments. The PHO86 gene was successfully cloned into expression vector as confirmed by both sequencing and restriction analysis (Figure 3.15). However, the cloning of VBA3 was not achieved and the plasmid isolated from the resistant colony was used in the further experiments, as VBA3 is the only gene in its cassette. Consequently, those cloned genes were transformed into yeast cells for the validation of their roles in the arsenical resistance by overexpression analysis in spotting assay and growth curves.

As the structure of arsenate resembles inorganic phosphate (Figure 4.2), arsenate can utilize phosphate transporters to enter into the cell and Pho84p is one of these proteins importing arsenate into the yeast cells (Ghosh et al., 1999; Shen, Shah, Chen, & Da Silva, 2012). Pho84p is responsible for the uptake of inorganic phosphate from the extracellular space in phosphate starvation (Bun-Ya et al., 1991). It is a high-affinity phosphate transporter working as a symporter carrying H^+ and PO_4^{3-} into the cell (Mouillon & Persson, 2006; Wykoff & O'Shea, 2001). The levels of inorganic phosphate in the cellular environment induce the localization of Pho84p to the cell membrane (Lau, Howson, Malkus, Schekman, & O'Shea, 2000; Petersson, Pattison, Kruckeberg, Berden, & Persson, 1999). Pho86p is an endoplasmic reticulum (ER)-resident protein which is necessary for the exit of Pho84p from the ER into COPII vesicles (Lau et al. 2000; Yompakdee, Bun-Ya, et al. 1996; Yompakdee, Ogawa, et al. 1996).

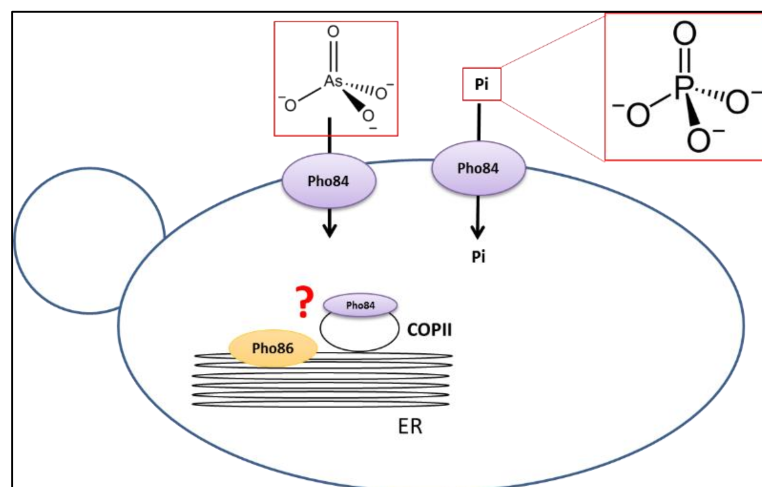


Figure 4.2. ER-resident protein Pho86 is required for the ER exit of Pho84p, which imports inorganic phosphate and arsenate into the cell, through unknown process.

In the current study, it was indicated that deletion of PHO86 gene sensitized the yeast cells for arsenate and also that overexpression of Pho86p in both wild type W303-1A cells and in hypersensitive GK4 cells gained resistance to arsenate as confirmed in both broth and solid media (Figure 3.16.A and B). To investigate if the resistance observed in the overexpression of Pho86p is based on Pho84p or another mechanism independent of Pho84p, PHO86-overexpressing plasmids were transformed into PHO84-lacking cells. There was no growth difference between deletion mutants of PHO84 cells with empty or PHO86-bearing expression vectors (Figure 3.16.C). This shows the requirement of Pho84p for the resistance derived from the overexpression of Pho86p. Nevertheless, these results are in contradiction with the previous study in which it was demonstrated that deletion of the PHO84 gene showed resistance to arsenate and this was also true for the deletion of PHO86 gene (Bun-ya et al., 1996; Yompakdee, Bun-Ya, et al., 1996).

The ER is the site for folding of proteins and is the quality control center for folding (Ellgaard & Helenius, 2003). Proteins with multiple membrane-passing domains, as in the case for Pho84p, need to be inserted into the ER membrane through the Sec61 complex. In this insertion process through the Sec61 complex, there is a likelihood of improper intermolecular or intramolecular interactions which may cause inaccurate folding of the membrane proteins. It was shown that deletion of the PHO86 gene from yeast cells resulted in the cross-linking and aggregation of Pho84p and ultimately prevented the ER exit of Pho84p. Therefore, Pho86p was proposed as a membrane-localized chaperone responsible for the protection of undesired interactions during the translocation or folding of Pho84p (Kota & Ljungdahl, 2005). This idea supports another study in which they concluded that Pho86p is necessary for the specific passage of Pho84p from ER to COPII vesicles, while Pho86 does not leave the ER. However, there was no evidence whether it is required for the recruitment of COPII to the ER or for ER-modifications of Pho84p (Lau et al., 2000). The results from these two studies strengthen the hypothesis that Pho86p acts as an outfitter. Outfitters are ER-resident accessory proteins that are always located in the ER and responsible for the maintenance of the conformation required for ER exit (Herrmann, Malkus, & Schekman, 1999).

When these findings are taken into consideration, the overexpression of Pho86p in yeast cells may change the folding pattern or interaction profile of Pho84p resulting in aberrant modifications, aggregation and failure to exit from the ER. An *in vitro* vesicle

budding assay can be carried out for the cells overexpressing Pho86p to confirm this hypothesis. The determination of Pho84p localization and intracellular arsenic concentration in yeast cells overexpressing Pho86p will determine if the plasma membrane localization of Pho84p is prevented. Moreover, these results may provide a better understanding of the relationship between the Pho86 and Pho84 proteins.

Another possible effect of Pho86p-overexpression may be on the endocytotic down-regulation of Pho84p. The endocytosis and localization of Pho84p into the vacuole for degradation is a way to down-regulate or inactivate Pho84p, but the signal for sorting by endocytosis has not been identified (Petersson et al., 1999). It was shown that overexpression of Rpd3p caused early endocytosis of Pho84p with an unknown mechanism but probably through change in the regulation of the endocytic machinery (Wongwisansri & Laybourn, 2005). Overexpression of Pho86p may affect other proteins regulating the membrane-localization or down-regulation of Pho84p, as in the case of the Rpd3 protein. This also requires further experiments to check the turnover process or speed of the Pho84p in case of overexpression of Pho86p.

Cross-resistance analysis in other metalloids showed that overexpression of Pho86p did not confer resistance to arsenite, boric acid or antimonite. This result is an indicator for the arsenate-specific function of Pho86p in tolerance.

Although yeast cells lacking of AIM26 gene whose function is not known were highly sensitive to arsenate, overexpression of Aim26p did not result in resistance. Sensitive phenotype of AIM26 gene may have been related to synthetic effects of this deletion which is another drawback of a deletion library screening.

Ugp1 overexpression provided better growth than control group. Ugp1p, UDP-glucose pyrophosphorylase (UGPase), is the protein responsible for catalysis of the reaction converting glucose-1-phosphate and UTP into UDP-Glucose by introduction of an uridydyl group to glucose-1-phosphate (Daran et al., 1995). Although there have been no studies relating carbohydrate mechanism to arsenical resistance, previous studies indicated that carbohydrate and sugar mechanisms have roles in resistance against cadmium which is another metalloid. Deletion library screening revealed some sugar metabolism-related genes deletion of which sensitized cells to cadmium and the expression of some genes in carbohydrate mechanisms were induced by cadmium treatment (Thorsen et al., 2009).

Metalloids and metals are known to interact with proteins and prevent their proper functioning, and consequently affect the survival of the cells, as in the case of L-glutamine:D-fructose-6-phosphate amidotransferase protein (GFAT) which is the target of methylmercury. When GFAT was overexpressed in the yeast cells, they gained resistance to methylmercury (NAGANUMA et al., 2000). Ugp1 protein may be a direct target of arsenate, thus the function of the proteins in association with arsenate may be inhibited. This disturbs carbohydrate metabolism altering the survival of yeast cells. Ectopic overexpression would produce much more UGP1 products compensating for this inhibition and providing resistance. Further experiments may include the analysis if arsenate directly binds to Ugp1p or not by As-biotin assays where arsenate is conjugated to biotin for the pull-down experiment (Kumar et al., 2016).

VBA3 was the only gene in its cassette and was overexpressed in the yeast cells thanks to high-copy plasmid YEp13. Both 24-hour growth analysis and spotting assays revealed that overexpression of Vba3p provided yeast cells with growth in even 0.2 mM arsenite (Figure 3.18 and 3.20). On the other hand, it showed cross-resistance to arsenate and antimonite (Figure 3.21 and 3.23). VBA3 is localized to vacuolar membrane and transports basic amino acids into the vacuole which is the organelle for the storage and degradation in yeast cells. This protein benefits from the ATPase at the vacuolar membrane which creates a proton electrochemical gradient to actively take up histidine and lysine. It was predicted that Vba3p may recognize and transport other cationic molecules (Shimazu, Sekito, Akiyama, Ohsumi, & Kakinuma, 2005). Vacuolar sequestration is a strategy for the removal of toxic substances from the cytosol and it was shown that Ycf1p localized to vacuolar membrane detoxifies arsenite and cadmium conjugated to glutathione (Ghosh et al., 1999; Szczypkas et al., 1994). Vba3p may have a novel function to sequester metalloids into the vacuole for their detoxification. The comparison of accumulation of arsenate, arsenite and antimonite between the cells with the empty and VBA3-bearing plasmid may give an idea about the likely function of Vba3p in metalloid removal from the cytosol. *in vitro* and *in vivo* uptake assays through Vba3p for $As(GS)_3$ can be further experiments to understand if those three metalloids are substrates for Vba3p.

YAP1, the yeast AP-1, gene encodes a transcription factor which regulates the transcription of approximately 70 genes related to oxidative stress and also drug and metal resistance (Haugen et al., 2004; Thorsen et al., 2007). The overexpression of Yap1

induced the expression of Ycf1p suggesting that Ycf1p is under the control of Yap1p (Wemmie, Szczypka, Thiele, & Moye-Rowley, 1994). The role of Yap1p in arsenite resistance was assessed in the study indicating that arsenite treatment increased the expression of Yap1p, while the deletion of YAP1 sensitized yeast cells to arsenite (Menezes et al., 2008). These results were obtained in our study, too, as seen in Figure 3.14 and 3.18. When Yap1p is overexpressed, it increases the level of Ycf1 expression and more arsenite can be sequestered into the vacuole from the cytosol through this transporter.

Additionally, Yap1p overexpression increased the tolerance of W303-1A cells to boric acid in cross-resistance test (Figure 3.22) and this finding supports the previous study in which it was proved that yeast cells overexpressing Yap1p were more resistant to boron than empty-plasmid containing ones (Uluisik et al., 2011). Additionally, they revealed that ectopic overexpression of YAP1 induced the main boron transporter which is Atr1p and enhanced boron resistance (Kaya, Karakaya, Fomenko, Gladyshev, & Koc, 2009; Uluisik et al., 2011).

When Erg6p was overexpressed in GK4 cells, both spotting assay and 24-hour growth analysis indicated their slightly better growth in arsenite containing solid and broth media, respectively. However, this growth is not as strong as the overexpression of VBA3 gene. Erg6p has a role in ergosterol biosynthesis and its deletion was found sensitive to arsenite in the deletion library screening (Thorsen et al., 2009). As the alterations in the level of Erg6p change ergosterol content in cellular membrane, this phenotypes in the deletion or overexpression may be related to alterations in the cell membrane content affecting the integrity of the membrane. Fps1p is an aquaglyceroporin and utilized as an entrance route by arsenite and antimonite (Robert Wysocki et al., 2001). The activity of this protein was shown to be dependent on the ergosterol content (Thorsen et al., 2009), hence alterations in the ergosterol levels by overexpression or deletion of Erg6p may affect the uptake rate of arsenite through this aquaglyceroporin. Cross-resistance test in antimonite also strengthens this inference. Erg6p overexpression provided better growth in antimonite as compared to empty plasmid-containing GK4 cells, since Fps1p also carries antimonite into the cell. Further experiments may include the measurements of intracellular arsenite and antimonite levels and of rate of their uptake through Fps1p to understand if the phenotype observed in Erg6p-overexpressing or deleted cells is dependent on Fps1p.

Genome-wide phenotypic screens are advantageous tools for the identification of drug or toxic compound targets and the genes associated with their resistance or toxicity mechanisms by comparing the fitness of yeast cells to these compounds. This study benefited from the yeast genome library which enables the overexpression screening of yeast genome regions and also eliminates the disadvantages of deletion library screening. However, there is still a need for further confirmation to assess the mode of action of the defined genes by conducting follow-up experiments as exemplified for each gene. Results obtained from molecular, biochemical and cellular follow-up experiments will strengthen the results obtained from this genome-wide screening. After this further understanding, the information may be applied in plant biotechnology for the development of strategies to phytoremediate contaminated regions which will reduce the number of people under the risk of arsenic pathogenesis. Additionally, these results may improve therapeutic approaches in arsenic pathogenesis or arsenic-containing drugs associated with resistance.

In summary, genome-wide phenotypic screen using a yeast genome library revealed some genes conferring resistance to yeast cells when overexpressed. The majority of these genes have not been previously proposed to have role in arsenical tolerance mechanisms. Their exact involvement in the tolerance or being a direct target for arsenicals demands further biochemical and molecular experiments for validation.

CHAPTER 5

CONCLUSION

Genome-wide screening approach by overexpression library provided novel genes whose overexpression provided yeast cells with resistance to two major arsenic forms, arsenite or arsenate. In general, tolerance mechanisms are composed of alterations in the activity of transporters or sequestration of arsenicals into the vacuole. Additionally, this study may suggest a toxicity mechanism of arsenic, since Ugp1 may be a direct target of arsenic in yeast cells.

In arsenate-tolerance screen, three candidate genes, PHO86, AIM26 and UGP1, were selected for analysis. Overexpression of Pho86p available in the green-coded genomic region confers arsenate tolerance to both WT and GK4 cells. It was shown that the resistance in the Pho86 overexpression is dependent on the presence of Pho84p whose ER exit is regulated by Pho86p. Further experiments are required to show the relationship between these two proteins. UGP1 and AIM26 genes lies on the orange-coded genomic region. Overexpression of Aim26p had no impact in yeast cells' tolerance to arsenate. UGP1 is an essential gene for the growth of yeast cells and its overexpression in W303-1A cells provided slightly better growth in arsenate-containing medium. Further experiments may include the ones to show if Ugp1 protein is a direct target of arsenic. Since there was no strong tolerance effects of both genes, TUL1 gene from orange-coded cassette may also be cloned in order to assess its impact in resistance.

Genomic library screen for arsenite tolerance revealed three candidate genes, VBA3, YAP1 and ERG6 and Vba3p was shown to confer strongest resistance in arsenite containing media when overexpressed in GK4 cells. Further experiments can be carried out to validate if Vba3p can sequester arsenite into the vacuole. The role of YAP1 gene arsenite resistance was confirmed by assessing the impact of its overexpression and deletion. When ERG6 was overexpressed, GK4 cells grew better than the cells bearing empty plasmid. Follow-up experiments can be conducted to show if the change in lipid composition affects the activity of Fps1p.

Cross-resistance analysis elucidated that VBA3 conferred resistance to other metalloids, arsenate and antimonite. Additionally, previously determined role of YAP1

in boric acid tolerance was confirmed. Other candidate genes seem to be specific in their function for tolerance to their corresponding arsenic form.

Although this study could suggest novel genes for arsenic tolerance, there is need to conduct follow-up experiments to elucidate the mode of action of those genes. Furthermore, it is not known if these mechanisms are physiological response of yeast cell when exposed to arsenic. Therefore, transcriptional analysis by real-time PCR for candidate genes can be carried out to comprehend this question.

REFERENCES

Andrews, J. M., & Andrews, J. M. (2001). Determination of minimum inhibitory concentrations. *The Journal of Antimicrobial Chemotherapy*, *48 Suppl 1*, 5–16. https://doi.org/10.1093/jac/48.suppl_1.5

Bánfalvi, G. (2011). *Heavy metals, trace elements and their cellular effects*. *Cellular Effects of Heavy Metals*. https://doi.org/10.1007/978-94-007-0428-2_1

Bienert, G. P., Sch??ssler, M. D., & Jahn, T. P. (2008). Metalloids: essential, beneficial or toxic? Major intrinsic proteins sort it out. *Trends in Biochemical Sciences*, *33*(1), 20–26. https://doi.org/10.1016/j.tibs.2007.10.004

Bienert, G. P., Thorsen, M., Schüssler, M. D., Nilsson, H. R., Wagner, A., Tamás, M. J., & Jahn, T. P. (2008). A subgroup of plant aquaporins facilitate the bi-directional diffusion of As(OH)3 and Sb(OH)3 across membranes. *BMC Biology*, *6*(Iii), 26. https://doi.org/10.1186/1741-7007-6-26

Bobrowicz, P., Wysocki, R., Owsianik, G., Goffeau, A., & Ułaszewski, S. (1997). Isolation of Three Contiguous Genes, <I>ACR1</I>, <I>ACR2</I> and <I>ACR3</I>, Involved in Resistance to Arsenic Compounds in the Yeast <I>Saccharomyces cerevisiae</I>. *Yeast*, *13*(9), 819–828.

Bouganim, N., David, J., Wysocki, R., & Ramotar, D. (2001). Yap1 overproduction restores arsenite resistance to the ABC transporter deficient mutant ycf1 by activating ACR3 expression. *Biochem Cell Biol*, *79*(4), 441–448. https://doi.org/10.1139/bcb-79-4-441

Bun-Ya, M., Nishimura, M., Harashima, S., & Oshima, Y. (1991). The PHO84 gene of Saccharomyces cerevisiae encodes an inorganic phosphate transporter. *Molecular and Cellular Biology*, *11*(6), 3229–38. https://doi.org/10.1128/MCB.11.6.3229

Bun-ya, M., Shikata, K., Nakade, S., Yompakdee, C., Harashima, S., & Oshima, Y. (1996). Two new genes, PHO86 and PHO87, involved in inorganic phosphate uptake in Saccharomyces cerevisiae. *Current Genetics*, *29*(4), 344–351. https://doi.org/10.1007/s002940050055

Dani, G. M., & Zakian, V. A. (1983). Mitotic and meiotic stability of linear plasmids in yeast. *Proceedings of the National Academy of Sciences of the United States of America*, *80*(11), 3406–10. https://doi.org/10.1073/pnas.80.11.3406

Daran, J. M., Dallies, N., Thines-Sempoux, D., Paquet, V., & Francois, J. (1995). Genetic and biochemical characterization of the UGP1 gene encoding the UDP-glucose pyrophosphorylase from Saccharomyces cerevisiae. *European Journal of Biochemistry*, *233*(2), 520–530. https://doi.org/10.1111/j.1432-1033.1995.520_2.x

Di, Y., & Tamás, M. J. (2007). Regulation of the arsenic-responsive transcription factor Yap8p involves the ubiquitin-proteasome pathway. *Journal of Cell Science*, *120*(V), 256–264. https://doi.org/10.1242/jcs.03346

Dilda, P. J., & Hogg, P. J. (2007). Arsenical-based cancer drugs. *Cancer Treatment Reviews*, *33*(6), 542–564. https://doi.org/10.1016/j.ctrv.2007.05.001

Dilda, P. J., Perrone, G. G., Philp, A., Lock, R. B., Dawes, I. W., & Hogg, P. J. (2008). Insight into the selectivity of arsenic trioxide for acute promyelocytic leukemia cells by characterizing Saccharomyces cerevisiae deletion strains that are sensitive or resistant to the metalloid. *International Journal of Biochemistry and Cell Biology*, *40*(5), 1016–1029. https://doi.org/10.1016/j.biocel.2007.11.002

Diss, G., Ascencio, D., Deluna, A., & Landry, C. R. (2014). Molecular mechanisms of paralogous compensation and the robustness of cellular networks. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, *322*(7), 488–499. https://doi.org/10.1002/jez.b.22555

Dos Santos, S. C., & Sá-Correia, I. (2011). A genome-wide screen identifies yeast genes required for protection against or enhanced cytotoxicity of the antimalarial drug quinine. *Molecular Genetics and Genomics*, *286*(5–6), 333–346. https://doi.org/10.1007/s00438-011-0649-5

dos Santos, S. C., Teixeira, M. C., Cabrito, T. R., & Sá-Correia, I. (2012). Yeast toxicogenomics: Genome-wide responses to chemical stresses with impact in environmental health, pharmacology, and biotechnology. *Frontiers in Genetics*, *3*(APR), 1–17. https://doi.org/10.3389/fgene.2012.00063

Ellgaard, L., & Helenius, A. (2003). Quality control in the endoplasmic reticulum. *Nature reviews.Molecular Cell Biology*, *4*(3), 181–191. https://doi.org/10.1038/nrm1052

Feldmann, D. H. (2005). 04 Yeast Molecular Techniques.doc, 1–13. Retrieved from papers3://publication/uuid/2E03D633-493C-4CD6-AC8B-BE81E0E5A98B

Ghosh, M., Shen, J., Rosen, B. P., & Kaback, H. R. (1999). Pathways of As(III) detoxification in Saccharomyces cerevisiae (ACR3̅YCF1̅Sb(III)̅resistance̅ABC transporters). *Cell Biology*, *96*(April), 5001–5006.

Giaever, G., & Nislow, C. (2014). The yeast deletion collection: A decade of functional genomics. *Genetics*, *197*(2), 451–465. https://doi.org/10.1534/genetics.114.161620

Haugen, A. C., Kelley, R., Collins, J. B., Tucker, C. J., Deng, C., Afshari, C. A., … Van Houten, B. (2004). Integrating phenotypic and expression profiles to map arsenic-response networks. *Genome Biology*, *5*(12), R95. https://doi.org/10.1186/gb-2004-5-12-r95

Herrmann, J. M., Malkus, P., & Schekman, R. (1999). Out of the ER--outfitters, escorts and guides. *Trends in Cell Biology*, *9*(98), 5–7. https://doi.org/10.1016/S0962-8924(98)01414-7

Hoon, S., St.Onge, R. P., Giaever, G., & Nislow, C. (2008). Yeast chemical genomics and drug discovery: an update. *Trends in Pharmacological Sciences*, *29*(10), 499–504. https://doi.org/10.1016/j.tips.2008.07.006

Jacobson, T., Navarrete, C., Sharma, S. K., Sideri, T. C., Ibstedt, S., Priya, S., … Tamás, M. J. (2012). Arsenite interferes with protein folding and triggers formation of protein aggregates in yeast. *Journal of Cell Science*, *125*(Pt 21), 5073–83. https://doi.org/10.1242/jcs.107029

Jin, Y. H., Dunlap, P. E., McBride, S. J., Al-Refai, H., Bushel, P. R., & Freedman, J. H. (2008). Global transcriptome and deletome profiles of yeast exposed to transition metals. *PLoS Genetics*, *4*(4). https://doi.org/10.1371/journal.pgen.1000053

Katju, V., Farslow, J. C., & Bergthorsson, U. (2009). Variation in gene duplicates with low synonymous divergence in Saccharomyces cerevisiae relative to Caenorhabditis elegans. *Genome Biology*, *10*(7), R75. https://doi.org/10.1186/gb-2009-10-7-r75

Kaya, A., Karakaya, H. C., Fomenko, D. E., Gladyshev, V. N., & Koc, A. (2009). Identification of a novel system for boron transport: Atr1 is a main boron exporter in yeast. *Molecular and Cellular Biology*, *29*(13), 3665–3674. https://doi.org/10.1128/MCB.01646-08

Kota, J., & Ljungdahl, P. O. (2005). Specialized membrane-localized chaperones prevent aggregation of polytopic proteins in the ER. *Journal of Cell Biology*, *168*(1), 79–88. https://doi.org/10.1083/jcb.200408106

Kumar, N. V., Yang, J., Pillai, J. K., Rawat, S., Solano, C., Kumar, A., … Tamás, M. J. (2016). Arsenic Directly Binds to and Activates the Yeast AP-1-Like Transcription Factor Yap8. *Molecular and Cellular Biology*, *36*(6), 913–922. https://doi.org/10.1128/MCB.00842-15

Lau, W. T., Howson, R. W., Malkus, P., Schekman, R., & O'Shea, E. K. (2000). Pho86p, an endoplasmic reticulum (ER) resident protein in Saccharomyces cerevisiae, is required for ER exit of the high-affinity phosphate transporter Pho84p. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(3), 1107–1112. https://doi.org/10.1073/pnas.97.3.1107

Litwin, I., Bocer, T., Dziadkowiec, D., & Wysocki, R. (2013). Oxidative Stress and Replication-Independent DNA Breakage Induced by Arsenic in Saccharomyces cerevisiae. *PLoS Genetics*, *9*(7). https://doi.org/10.1371/journal.pgen.1003640

Liu, S. X., Athar, M., Lippai, I., Waldren, C., & Hei, T. K. (2001). Induction of oxyradicals by arsenic: implication for mechanism of genotoxicity. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(4), 1643–1648. https://doi.org/10.1073/pnas.98.4.1643

Liu, Z., Boles, E., & Rosen, B. P. (2004). Arsenic Trioxide Uptake by Hexose Permeases in Saccharomyces cerevisiae. *Journal of Biological Chemistry*, *279*(17), 17312–17318. https://doi.org/10.1074/jbc.M314006200

Liu, Z., Shen, J., Carbrey, J. M., Mukhopadhyay, R., Agre, P., & Rosen, B. P. (2002). Arsenite transport by mammalian aquaglyceroporins AQP7 and AQP9. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(9), 6053–8. https://doi.org/10.1073/pnas.092131899

Maciaszczyk, E., Wysocki, R., Golik, P., Lazowska, J., & Ulaszewski, S. (2004). Arsenical resistance genes in Saccharomyces douglasii and other yeast species undergo rapid evolution involving genomic rearrangements and duplications. *FEMS Yeast Research*, *4*(8), 821–832. https://doi.org/10.1016/j.femsyr.2004.03.002

Maciaszczyk-Dziubinska, E., Migdal, I., Migocka, M., Bocer, T., & Wysocki, R. (2010). The yeast aquaglyceroporin Fps1p is a bidirectional arsenite channel. *FEBS Letters*, *584*(4), 726–732. https://doi.org/10.1016/j.febslet.2009.12.027

Maciaszczyk-Dziubinska, E., Wawrzycka, D., Sloma, E., Migocka, M., & Wysocki, R. (2010). The yeast permease Acr3p is a dual arsenite and antimonite plasma membrane transporter. *Biochimica et Biophysica Acta - Biomembranes*, *1798*(11), 2170–2175. https://doi.org/10.1016/j.bbamem.2010.07.017

Maciaszczyk-Dziubinska, E., Wawrzycka, D., & Wysocki, R. (2012). Arsenic and antimony transporters in eukaryotes. *International Journal of Molecular Sciences*, *13*(3), 3527–3548. https://doi.org/10.3390/ijms13033527

Matia-González, A. M., & Rodríguez-Gabriel, M. A. (2011). Slt2 MAPK pathway is essential for cell integrity in the presence of arsenate. *Yeast*, *28*(1), 9–17. JOUR. https://doi.org/10.1002/yea.1816

Menezes, R. a, Amaral, C., Batista-Nascimento, L., Santos, C., Ferreira, R. B., Devaux, F., … Rodrigues-Pousada, C. (2008). Contribution of Yap1 towards Saccharomyces cerevisiae adaptation to arsenic-mediated oxidative stress. *The Biochemical Journal*, *414*, 301–311. https://doi.org/10.1042/BJ20071537

Mouillon, J. M., & Persson, B. L. (2006). New aspects on phosphate sensing and signalling in Saccharomyces cerevisiae. *FEMS Yeast Research*, *6*(2), 171–176. https://doi.org/10.1111/j.1567-1364.2006.00036.x

Mukhopadhyay, R., Shi, J., & Rosen, B. P. (2000). Purification and characterization of Acr2p, the Saccharomyces cerevisiae arsenate reductase. *Journal of Biological Chemistry*, *275*(28), 21149–21157. https://doi.org/10.1074/jbc.M910401199

NAGANUMA, A., MIURA, N., KANEKO, S., MISHINA, T., HOSOYA, S., MIYAIRI, S., … KUGE, S. (2000). GFAT as a target molecule of methylmercury toxicity in Saccharomyces cerevisiae. *The FASEB Journal* , *14*(7), 968–972. JOUR. Retrieved from http://www.fasebj.org/content/14/7/968.abstract

Nasmyth, K. A., & Reed, S. I. (1980). Isolation of genes by complementation in yeast: molecular cloning of a cell-cycle gene. *Proceedings of the National Academy of Sciences of the United States of America*, *77*(4), 2119–23. https://doi.org/10.1073/pnas.77.4.2119

Petersson, J., Pattison, J., Kruckeberg, A. L., Berden, J. A., & Persson, B. L. (1999). Intracellular localization of an active green fluorescent protein-tagged Pho84 phosphate permease in Saccharomyces cerevisiae. *FEBS Letters*, *462*(1–2), 37–42. https://doi.org/10.1016/S0014-5793(99)01471-4

Ralph H. Petrucci, F. G. H., Jeffry D. Madura, C. B. (2011). *General Chemistry: Principles and Modern Applications*. United States of America: Pearson Canada Inc., Toronto, Ontario.

Rosen, B. P. (2016). Families of arsenic transporters. *Trends in Microbiology*, *7*(5), 207–212. JOUR. https://doi.org/10.1016/S0966-842X(99)01494-8

Rosen, B. P., & Tamás, M. J. (2010). Arsenic transport in prokaryotes and eukaryotic microbes. *Advances in Experimental Medicine and Biology*, *679*(V), 47–55. https://doi.org/10.1007/978-1-4419-6315-4_4

Ruotolo, R., Marchini, G., & Ottonello, S. (2008). Membrane transporters and protein traffic networks differentially affecting metal tolerance: a genomic phenotyping study in yeast. *Genome Biology*, *9*(4), R67. https://doi.org/10.1186/gb-2008-9-4-r67

Shen, M. W. Y., Shah, D., Chen, W., & Da Silva, N. (2012). Enhanced arsenate uptake in Saccharomyces cerevisiae overexpressing the Pho84 phosphate transporter. *Biotechnology Progress*, *28*(3), 654–661. https://doi.org/10.1002/btpr.1531

Sherman, F. (2002). Getting Started with Yeast • Contents •. *Methods in Enzymology*, *350*(2002), 3–41. https://doi.org/http://dx.doi.org/10.1016/S0076-6879(02)50954-X

Shimazu, M., Sekito, T., Akiyama, K., Ohsumi, Y., & Kakinuma, Y. (2005). A family of basic amino acid transporters of the vacuolar membrane from Saccharomyces cerevisiae. *Journal of Biological Chemistry*, *280*(6), 4851–4857. https://doi.org/10.1074/jbc.M412617200

Sörgel, F. (2004). The Return of Ehrlich's "Therapia magna sterilisans" and Other Ehrlich Concepts? *Chemotherapy*, *50*(1), 6–10. JOUR. Retrieved from http://www.karger.com/DOI/10.1159/000077277

Szczypkas, M. S., Wemmiep, J. A., Moye-rowley, W. S., & Thielesn, J. (1994). AYeast Metal Resistance Protein Similar to Human Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) and Multidrug Resistance-associated Protein *, 22853–22857.

Talemi, S. R., Jacobson, T., Garla, V., Navarrete, C., Wagner, A., Tamás, M. J., & Schaber, J. (2014). Mathematical modelling of arsenic transport, distribution and detoxification processes in yeast. *Molecular Microbiology*, *92*(6), 1343–1356. https://doi.org/10.1111/mmi.12631

Tamás, M. J., Labarre, J., Toledano, M. B., & Wysocki, R. (2006). Mechanisms of toxic metal tolerance in yeast. In M. J. Tamas & E. Martinoia (Eds.), *Molecular Biology of Metal Homeostasis and Detoxification: From Microbes to Man* (pp. 395–454). inbook, Berlin, Heidelberg: Springer Berlin Heidelberg. https://doi.org/10.1007/4735_105

Tamás, M. J., Sharma, S. K., Ibstedt, S., Jacobson, T., & Christen, P. (2014). Heavy metals and metalloids as a cause for protein misfolding and aggregation. *Biomolecules*, *4*(1), 252–67. https://doi.org/10.3390/biom4010252

Thorsen, M., Jacobson, T., Vooijs, R., Navarrete, C., Bliek, T., Schat, H., & Tamás, M. J. (2012). Glutathione serves an extracellular defence function to decrease arsenite accumulation and toxicity in yeast. *Molecular Microbiology*, *84*(6), 1177–1188. https://doi.org/10.1111/j.1365-2958.2012.08085.x

Thorsen, M., Lagniel, G., Kristiansson, E., Junot, C., Nerman, O., Labarre, J., & Tamás, M. J. (2007). Quantitative transcriptome, proteome, and sulfur metabolite profiling of the Saccharomyces cerevisiae response to arsenite. *Physiological Genomics*, *30*(February 2007), 35–43. https://doi.org/10.1152/physiolgenomics.00236.2006

Thorsen, M., Perrone, G. G., Kristiansson, E., Traini, M., Ye, T., Dawes, I. W., … Tamás, M. J. (2009). Genetic basis of arsenite and cadmium tolerance in Saccharomyces cerevisiae. *BMC Genomics*, *10*, 105. https://doi.org/10.1186/1471-2164-10-105

Uluisik, I., Kaya, A., Fomenko, D. E., Karakaya, H. C., Carlson, B. A., Gladyshev, V. N., & Koc, A. (2011). Boron stress activates the general amino acid control mechanism and inhibits protein synthesis. *PLoS ONE*, *6*(11). https://doi.org/10.1371/journal.pone.0027772

Vujcic, M., Shroff, M., & Singh, K. K. (2007). Genetic determinants of mitochondrial response to arsenic in yeast Saccharomyces cerevisiae. *Cancer Research*, *67*(20), 9740–9749. https://doi.org/10.1158/0008-5472.CAN-07-1962

Wemmie, J. A., Szczypka, M. S., Thiele, D. J., & Moye-Rowley, W. S. (1994). Cadmium tolerance mediated by the yeast AP-1 protein requires the presence of an ATP-binding cassette transporter-encoding gene, YCF1. *Journal of Biological Chemistry*, *269*(51), 32592–32597.

Whiteway, M. S., & Ahmed, a. (1984). Recombinational instability of a chimeric plasmid in Saccharomyces cerevisiae. *Molecular and Cellular Biology*, *4*(1), 195–8.

Wongwisansri, S., & Laybourn, P. J. (2005). Disruption of histone deacetylase gene RPD3 accelerates PHO5 activation kinetics through inappropriate Pho84p recycling. *Eukaryotic Cell*, *4*(8), 1387–1395. https://doi.org/10.1128/EC.4.8.1387-1395.2005

Wykoff, D. D., & O'Shea, E. K. (2001). Phosphate transport and sensing in Saccharomyces cerevisiae. *Genetics*, *159*(4), 1491–1499.

Wysocki, R., Bobrowicz, P., & Ulaszewski, S. (1997). The Saccharomyces cerevisiaeACR3 gene encodes a putative membrane protein involved in arsenite transport. *J Biol Chem*, *272*(48), 30061–30066.

Wysocki, R., Chéry, C. C., Wawrzycka, D., Van Hulle, M., Cornelis, R., Thevelein, J. M., & Tamás, M. J. (2001). The glycerol channel Fps1p mediates the uptake of arsenite and antimonite in Saccharomyces cerevisiae. *Molecular Microbiology*, *40*(6), 1391–1401. https://doi.org/10.1046/j.1365-2958.2001.02485.x

Wysocki, R., & Tamás, M. J. (2010). How Saccharomyces cerevisiae copes with toxic metals and metalloids. *FEMS Microbiology Reviews*, *34*(6), 925–951. https://doi.org/10.1111/j.1574-6976.2010.00217.x

Wysocki, R., & Tamás, M. J. (2011). Cellular Effects of Heavy Metals. *Springer Science + Business*, 87–112. https://doi.org/10.1007/978-94-007-0428-2

Xu, C., Zhou, T., Kuroda, M., & Rosen, B. P. (1998). Metalloid Resistance Mechanisms in Prokaryotes. *Journal of Biochemistry* , *123*(1), 16–23. JOUR. Retrieved from http://jb.oxfordjournals.org/content/123/1/16.abstract

Yompakdee, C., Bun-Ya, M., Shikata, K., Ogawa, N., Harashima, S., & Oshima, Y. (1996). A putative new membrane protein, Pho86p, in the inorganic phosphate uptake system of Saccharomyces cerevisiae. *Gene*, *171*(1), 41–47. https://doi.org/10.1016/0378-1119(96)00079-0

Yompakdee, C., Ogawa, N., Harashima, S., & Oshima, Y. (1996). A putative membrane protein, Pho88p, involved in inorganic phosphate transport in Saccharomyces cerevisiae. *Molecular and General Genetics*, *251*(5), 580–590. https://doi.org/10.1007/s004380050205