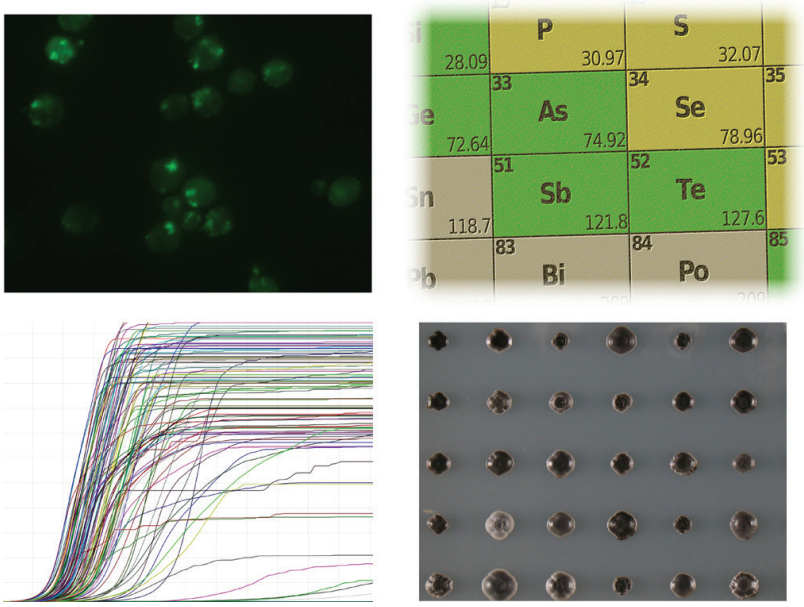
Adaptation and Protein Quality Control Under Metalloid Stress



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Ph.D. thesis

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Adaptation and Protein Quality Control Under Metalloid Stress

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Adaptation and Protein Quality Control Under Metalloid Stress

Doctoral thesis

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Cover
Top left: Hsp104-GFP foci in *S. cerevisiae* under arsenite stress
Top right: Periodic table
Bottom left: Growth curves of *S. cerevisiae* under arsenite stress
Bottom right: Tellurite exposed *S. cerevisiae* colonies

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Tillägnad mamma,
pappa & Rebecca

"I actually do
not believe that there
are any collisions between
what I believe as a Christian,
and what I know and have learned
about as a scientist. I think there's
a broad perception that that's the
case, and that's what scares many
scientists away from a serious
consideration of faith."

"The God of the Bible is also
the God of the genome.
He can be worshipped in the
cathedral or in the laboratory.
His creation is majestic, awesome,
intricate and beautiful."

Francis Collins

Populärvetenskaplig sammanfattning

Exponering av tungmetaller och halvmetaller kan ha skadliga effekter på både hälsa och miljö. Naturliga föroreningar av arsenik i grundvatten är ett folkhälsoproblem i delar av världen med miljontals drabbade, och en ökad användning av ovanliga metaller inom elektronikindustrin har lett till exponering av tidigare väldigt sällsynta ämnen. Det är därför viktigt att förstå de cellulära mekanismerna bakom tungmetallers och halvmetallers giftighet.

I detta arbete har jag undersökt två halvmetaller, arsenik och tellur. *Arsenik* är ett välkänt gift som kan påverka den tredimensionella strukturen och funktionen hos vissa proteiner. Detta har kopplats till ett antal neurodegenerativa sjukdomar hos människor. Jag har använt jäst som modellsystem för att undersöka motsvarande proteiner. Vad gör proteinerna känsliga för arsenik och hur svarar cellen? Mina studier visar att proteiner som är särskilt beroende av andra proteiner (chaperoner) för att bilda rätt struktur, som är högt uttryckta och som har många interaktioner med andra proteiner är särskilt känsliga. När cellen utsätts för arsenik svarar den genom att nedreglera dessa proteiner och aktivera speciella nedbrytningssystem (proteasom). Resultaten kan bidra till att öka förståelsen för arsenikförgiftning även hos andra organismer samt vissa sjukdomsassocierade proteinstrukturförändringar.

Tellur är ett giftigt ämne för de flesta organismer men mekanismerna bakom dess toxicitet är i princip helt okända och förgiftning är svårbehandlat. Jag har kartlagt de cellulära mekanismer som bidrar till giftighet i jäst. Resultaten visar att tellur utövar toxicitet via mitokondriella funktioner och vissa metabola reaktioner i cellen. Resultaten kan ligga till grund för att bättre förstå och på sikt behandla tellurförgiftning..

För att få en mer komplett bild av de processer som format jäst har jag också undersökt vilken roll naturligt urval och slumpmässig genetisk drift har spelat under dess evolutionära historia. Att skilja dessa processer från varandra är ofta mycket svårt, men genom att analysera korrelationen av anpassning i distinkta livsstadier och kartlägga den genetiska grunden för variationen mellan olika stammar, går det att finna ett karaktäristiskt spår av naturligt urval. Metoden kan bidra till att förstå vilka evolutionära processer som har varit verksamma även i andra miljöer och organismer.

Abstract

Toxic metals and metalloids are emerging as major environmental pollutants, having ecological consequences as well as being linked to a broad range of degenerative conditions in animals, plants and humans. While the toxicity of several metalloids is well established, the underlying molecular mechanisms are often not clear.

Several human degenerative diseases are linked to misfolding and aggregation of specific proteins. I have shown that many of these proteins have yeast homologs that are particularly prone to misfolding and aggregation during *arsenite* exposure. The yeast proteins are highly dependent on chaperones for proper folding, whereas arsenite is capable of inhibiting chaperone function as well as causing additional aggregation through a propagating effect. Computational analyses further revealed that aggregation-prone proteins are abundant and have a high translation rate, but are down-regulated when the cell encounters arsenite.

The mechanisms behind *tellurite* toxicity have eluded scientists for over a century. By using a genome-wide phenotypic screen, it was found that tellurite toxicity is linked to accumulation of elemental tellurium. Sulfate metabolism and mitochondrial respiration were found to mediate toxicity.

An understanding of cellular function requires knowledge of the evolutionary processes that have formed it. However, distinguishing between adaptive and non-adaptive differentiation remains an extraordinary challenge within evolutionary biology. The last part of this thesis tests a method for exposing the role of natural selection in evolution of stress tolerance. Analysis of concerted optimization of performance in distinct fitness components followed by mapping of the genetic basis for the optimizations, compellingly suggests that the method is able to detect natural selection.

The results presented here are likely to be relevant in gaining a better understanding of the mechanisms behind arsenite and tellurite poisoning and cellular defense, and may form a basis for elucidating evolutionary adaptations in other environments and organisms.

List of papers

I Lars-Göran Ottosson, Katarina Logg, Sebastian Ibstedt, Per Sunnerhagen, Mikael Käll, Anders Blomberg, Jonas Warringer
"Sulfate assimilation mediates tellurite reduction and toxicity in *Saccharomyces cerevisiae*"
Eukaryotic Cell, 2010, 9(10): 1635–1647
DOI: 10.1128/EC.00078-10

II Therese Jacobson, Clara Navarrete, Sandeep K. Sharma, Theodora C. Sideri, Sebastian Ibstedt, Smriti Priya, Chris M. Grant, Philipp Christen, Pierre Goloubinoff, Markus J. Tamás
"Arsenite interferes with protein folding and triggers formation of protein aggregates in yeast"
Journal of Cell Science, 2012, 125(21): 5073–5083
DOI: 10.1242/jcs.107029

III Sebastian Ibstedt, Theodora C. Sideri, Chris M. Grant, Markus J. Tamás
"Global analysis of protein aggregation in yeast during physiological conditions and arsenite stress"
Biology Open, 2014, 3(10): 913–923
DOI: 10.1242/bio.20148938

IV Sebastian Ibstedt, Simon Stenberg (equal contribution), Sara Bagés, Arne B. Gjuvsland, Francisco Salinas, Olga Kourtchenko, Jeevan Karloss, Anders Blomberg, Stig W. Omholt, Gianni Liti, Gemma Beltran, Jonas Warringer
"Concerted evolution of life stage performances signals recent selection on yeast nitrogen use"
Molecular Biology and Evolution, 2015, 32(1): 153–161
DOI: 10.1093/molbev/msu285

Paper contributions

Paper I

I performed follow-up phenotyping and quantification of tellurite, selenite and selenomethionine stress.

Paper II & III

I performed the computational and statistical analyses.

Paper IV

I performed the calculations (quantification of phenotypes, correlations, linkage analysis and statistics).

Papers not included

Francisco Cubillos, Leopold Parts, Francisco Salinas, Anders Bergström, Eugenio Scovacicricchi, Amin Zia, Christopher Illingworth, Ville Mustonen, Sebastian Ibstedt, Jonas Warringer, Edward Louis, Richard Durbin, Gianni Liti
"High-resolution mapping of complex traits with a four-parent advanced intercross yeast population"
Genetics, 2013, 195(3): 1141–1155
doi: 10.1534/genetics.113.155515

Markus J. Tamás, Sandeep K. Sharma, Sebastian Ibstedt, Therese Jacobson, Philipp Christen
"Heavy metals and metalloids as a cause for protein misfolding and aggregation"
Biomolecules, 2014, 4(1): 252–267
doi: 10.3390/biom4010252

Contents

1 Introduction

During the course of evolution it has been imperative for life to deal with a large number of toxic compounds, many with metallic toxicophores. Different metals can cause harm to organisms in distinct ways due to processes like protein dysfunction, membrane damage and oxidative stress. Various strategies have been developed to deal with toxic metals and metalloids, such as efflux pumps, chelation, compartmental sequestration and metabolic adaptations with increased production of expendable peptides and thiolates with high-affinity binding sites. The ability of organisms to adapt to a shifting environment is vital for survival, but the processes through which this occurs is often not well understood.

1.1 Definitions and scope

This thesis aims to shed some light on how metalloid stress affects cells and the adaptive responses that cells mount during exposure, using the yeast *Saccharomyces cerevisiae* as a model organism. Before this is done, the terms in title, "Adaptation and Protein Quality Control Under Metalloid Stress", deserve some clarification.

The word "***adaptation***" can be used in different ways. Sometimes it is used to refer to the capacity of an organism to acclimatize to environmental changes within the organism's lifetime, so called phenotypic plasticity. Examples include upregulation of membrane export proteins during metalloid stress or activation of heat shock proteins during heat stress. Sometimes it is used to describe the evolutionary acquisition of heritable phenotype differences, driven by natural selection.

In this thesis, I will consistently use it in the evolutionary sense, referring to the process of acquiring heritable phenotypic change through selection (verb) or the acquired change itself (noun). Physiological, biochemical and other changes during an organism's life cycle will be referred to as acclimatizations. This distinction might seem somewhat arbitrary since the capacity for individuals to improve their fitness during stress must ultimately have a genetic basis that has been acquired through evolutionary differentiation. However,

evolutionary differentiation is not necessarily adaptive, since there are also non-adaptive processes that can result in evolutionary change over time. Chapter 5 in this thesis concerns the possibility of distinguishing between adaptive and non-adaptive evolutionary processes.

Distinguishing adaptive from non-adaptive differentiation is not sufficient for answering why cells behave like they do however – we also need knowledge of the physiological processes. Chapter 3 in this thesis deals with the mechanisms behind tellurite toxicity in yeast and chapter 4 concerns arsenite-induced protein aggregation. Why do certain proteins aggregate and how does the cell handle this?

The second part of the title of this thesis, "***protein quality control***", refers to the ability of cells to maintain protein homeostasis. The cellular proteome is controlled by a network of chaperonal and degradative components that together limit the detrimental influence of misfolded proteins.

Several molecular chaperones act co-translationally on proteins to facilitate their folding into a native, functional state. Despite this, some proteins are prone to misfolding and may acquire a nonfunctional state with potentially detrimental consequences. Environmental stress such as metalloids, high temperature or oxidative stress can further aggravate this process. Misfolded proteins have a tendency to form aggregates which might potentially participate in harmful cellular interactions or induce accumulation of further aggregates. This is counteracted by cellular protein quality control mechanisms which work to maintain proteome homeostasis [13].

The word "***metalloid***" has been used in different ways. Originally it referred to alkali metals but was later used as a synonym for non-metals. Today it usually refers to semi-metals – elements that posses metallic properties to some extent. I use the word in this context and include the commonly accepted elements B, Si, Ge, As, Sb, Se and Te (see figure 1 on page 10). The main focus in this thesis will be on As and Te.

1.2 *Saccharomyces cerevisiae* as a model organism

One question should be answered right from the beginning: Why use the budding yeast (*Saccharomyces cerevisiae*) as a model organism? There are

several reasons why it is a suitable ***laboratory*** model organism for investigating adaptations (in both the physiological and evolutionary sense of the word) to metalloid stress: (1) It has a well characterized genome, (2) it is easy to cultivate and analyze phenotypically and genetically, (3) collections of knockout strains are available and (4) it can easily be genetically manipulated.

With respect to studying fundamental ***evolutionary*** processes, it is also a well suited model organism: (1) It is present over an enormous geographic range and a wide range of habitats. (2) Mutations rarely spread horizontally between populations, allowing for investigations of population-specific effects. (3) Its population dynamics is characterized by bursts of rapid expansion from small initial population sizes followed by massive cell death. This facilitates studying the effects of bottlenecks and genetic drift on evolution. (4) It has a mainly haploid life cycle and propagates mainly through mitosis or meiotic self-fertilizations, ensuring that deleterious alleles are frequently exposed to the forces of natural selection [52]. Perhaps most important to my PhD project: The ease of cultivation of *S. cerevisiae* cells allows massive amounts of data to be retrieved with relatively little effort, making it an ideal organism for large-scale studies.

1.3 Questions at issue and main findings

The ultimate goal of my PhD project has been to better understand the "whys" and "hows" relating to metalloids and biological organisms. This is a very broad goal, so some delimitations have been necessary. I have chosen to focus on the effects of tellurite and arsenite on yeast, and on the processes that have shaped evolutionary differentiations. This has led to three distinct, although interconnected, research topics. Before they are discussed in detail, it will be helpful to get an overview of the aim, method, main findings and implications of each one.

1. Mechanisms of tellurite toxicity

Aim. Investigate the mechanistic basis of tellurite toxicity.

Method. A genome-wide screen of tellurite sensitive mutants was performed. Phenotypic changes in fitness and accumulation of elemental tellurium were quantified.

Main findings. Toxicity of tellurite is linked to accumulation of tellurium. Mechanisms overlap with selenite toxicity and involves mitochondrial respiration and sulfate assimilation.

Implications. Results may help in understanding the effects of tellurite on eukaryotic organisms.

2. Arsenite induces protein aggregation

Aim. Arsenite exposure induces formation of protein aggregates. Three questions will be considered: (1) Why does arsenite induce protein aggregation? (2) What are the physiological consequences? (3) How does the cell respond in order to maintain protein homeostasis during arsenite stress?

Method. Biochemical assays and fluorescent microscopy were used to study cellular processes. Aggregation-prone proteins were isolated and characterized structurally and functionally through computational analyses.

Main findings. (1) Highly translated and chaperone-dependent peptides are susceptible to misfolding and aggregation by arsenite, either through direct interactions with arsenite on unfolded peptides or because arsenite interferes with chaperone activity. (2) The physiological effects are likely to involve aberrant protein-protein interactions. (3) The cell responds by down-regulating aggregation-prone proteins.

Implications. Results may help in understanding the role of arsenic poisoning in the pathogenesis of aggregation-related disorders.

3. Differentiating adaptive from non-adaptive evolution

Aim. Distinguishing between adaptive and non-adaptive evolutionary differentiation is an extraordinary challenge. I test a method for exposing natural selection, based on concerted optimization of performance in genetically distinct life stages.

Method. Performance was measured in distinct fitness components and the genetic basis for variation was mapped through coinheritance of trait variants and genetic markers.

Main findings. Fitness components showed concerted optimization and QTL effects tend to be unique to a single fitness component. This is a strong indication that evolutionary differentiation has been formed by adaptive processes.

Implications. Results suggest that the proposed method can be used for detecting natural selection also under other conditions.

The evolutionary pilot study does not concern metalloids directly, but the methodology that was test is intended to be applied in a future study that encompasses several metals and metalloids. Hence, metalloids are an overarching theme in this thesis. The next chapter will give an overview of the biological fates of metals and metalloids, followed by an introduction to the two central metalloids that I have focused on: tellurite and arsenite.

2 Metals and metalloids in biological systems

2.1 Metals: First contact

Metals and metalloids are ubiquitous in nature. Having been present since life first emerged, cells have learned to rely on their unique chemical properties [79]. In all domains of life, several metals have become essential for cellular function and structure. Other metals have beneficial but not essential roles, while some are highly toxic. Many metals and metalloids are common in nature, others are present in concentrations that usually have no physiological consequences whatsoever. Even for rare elements, local contaminations caused by human actions or natural disasters might have dramatic consequences for environment and organisms.

Metal and metalloid metabolism has been shaped by historical events. The Great Oxygenation Event (GOE) is believed to have happened around 2.3 billion years ago when the dissolved iron became saturated with oxygen, produced by cyanobacteria. Most organisms were likely unable to adapt, becoming victims of a mass extinction event. Several factors have been suggested to have contributed to the extinction: The increase in reactive oxygen had a directly toxic effect on anaerobic organisms through the production of reactive oxygen species (ROS). Depletion of methane by reactions with oxygen might have led to decreased temperatures and triggered the Huronian glaciation – one of the longest ice-ages in earth's history.

Another consequence of the rising oxygen levels might have been the oxidation of insoluble metal sulfides into more soluble metal sulfates. This would have exposed biological life to increased levels of metal compounds and novel mineral complexes [17]. How much this contributed to the extinction event is difficult to know, but the development of protective systems against metals appear to have coincided with those against oxygen. In retrospect, perhaps it is not so surprising that many protective mechanisms that are used against oxygen are also used against metals.

It is believed that metals that were abundant in the Archean ocean before GOE received essential biological roles early on. According to this model, life

originally utilized a very small number of transition metals as cofactors, but the release of previously rare elements, combined with the ability of metals with similar chemical properties to partially replace each other, allowed life to utilize a larger number of elements in more specialized enzymes. However, although metals might be superficially similar, replacing an essential element with a non-essential can have detrimental consequences. For example, arsenate [As(IV)] can partly mimic phosphate during glycolysis, but results in uncoupling of ATP production from carbon metabolism. It is believed that many metals that were solubilized during or after GOE are the ones that today are toxic to life at low or moderate concentrations [17, 27].

2.2 Beneficial and detrimental roles of metals and metalloids

The toxicity of a metal or metalloid depends on several factors, such as the coordination complex or molecular species, oxidation state, dose and mode of exposure, ligand preferences and intracellular interactions [104].

What elements are essential is dependent on the physiological state of the organism or even individual disposition and therefore it is difficult to give a complete list [47]. The elements that are currently known to be essential to humans are encircled in figure 1, but this is likely to be an underestimate. Essential metals can have very diverse roles, for example structural functions (e.g. as components of bones or in DNA stabilization), be involved in information transfer (e.g. in neural electric impulses), or facilitate chemical reactions as cofactors [47]. The proportion of metalloproteins – proteins that require metal ions as cofactors – varies between life's domains (Archaea, Bacteria and Eukarya) as well as within kingdoms, but is usually estimated to approximately one third [97, 31] (or in some studies half [60]) of all structurally characterized proteins.

Metals are important not only for biological function but also in industry and medicine. As, Te, Ag, Hg and other metals and metalloids that are toxic to microorganisms have long been used as antimicrobial and antiparasitic agents. Ancient cultures like the Persian, Phoenician, Greek, Roman and Egyptian all used vessels made of Cu and Ag for water disinfection and food

preservation. Over the past two centuries, physicians have used As, Te, Mg, Cu and Hg to treat diseases such as tuberculosis, gonorrhoea, syphilis and leprosy [97].

After the discovery of antibiotics by Alexander Fleming, the medical usage of metals diminished, but today, in the era of increasing multidrug resistance, the antimicrobial properties of metals are gaining renewed interest [97, 105]. Furthermore, the occupational and environmental exposure to metals and metalloids is increasing. The electronics industry has introduced some metals and combinations of metals that are novel from an evolutionary perspective, e.g. GaAs, GaAlAs and CdTe that are used in semiconductors or solar cells. Their role for environmental and human health – positive and negative – is therefore of special concern [17].

The physiological range within which a metal or metalloid is beneficial might be very narrow and intake levels outside of these can result in either deficiency or toxicity. Even elements that are considered essential can be highly toxic upon acute or chronic exposure at too high concentrations. Hence, being able to regulate the intracellular pool of metals and metalloids is fundamental to survival. Organisms therefore employ a wide variety of mechanisms to control intracellular levels of both essential trace minerals and non-essential substances which pose threats to the prosperity of the organism. It is likely that the abundant elements, like arsenic, have triggered the evolution of specific defense mechanisms during the history of life, whereas others, such as tellurium, that are rare in the biosphere might have forced organisms to rely on more general defense mechanisms.

While organic toxins are broken down by organisms after an environmental contamination, metals do not disappear but continue to exert their toxic effects, unless physically removed or chemically altered. The varied redox properties and coordination chemistry of many metals may allow them to escape homeostatic control mechanisms and affect the cell in different ways through inappropriate interactions – often covalent bonding or oxidation – with molecules, which might have detrimental consequences to the organism. This calls for clarification of their mechanisms of toxicity.

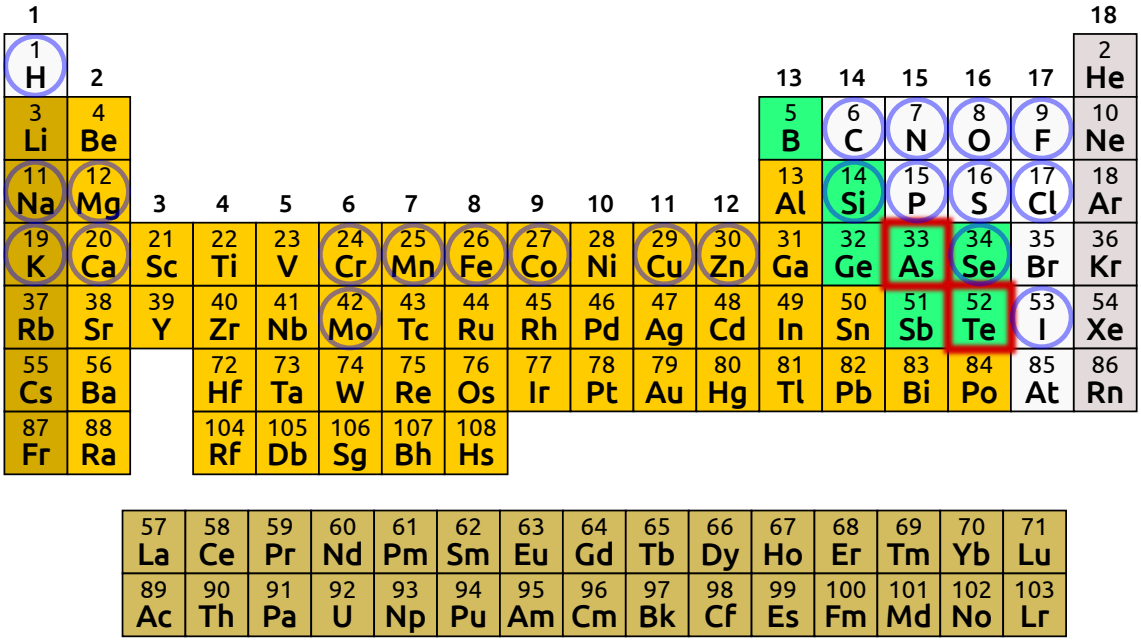

Figure 1: Periodic table of the elements. Metals are shown in orange/brown, metalloids in green and non-metals in gray. Arsenic and tellurium are highlighted. Circles indicate elements that are recognized as essential to humans. This is likely to be an underestimation and different authors have suggested that other elements, like B, V and As, are also essential [47].

2.3 An overview of cellular metal/metalloid processes

2.3.1 Toxicity mechanisms of metals and metalloids

An overview of some ways in which metals and metalloids may affect cells is given in table 1, together with different cellular responses. Metal and metalloid exposure commonly results in ***oxidative stress*** through either the production of reactive oxygen and nitrogen species (ROS/RNS) which can attack all cellular macromolecules, or indirectly through inactivation or depletion of redox regulation components.

Oxidative and nitrosative stress produced by metals and metalloids might have secondary effects such as DNA modifications, lipid peroxidation and

Table 1: Mechanisms of metal/metalloid toxicity and cellular responses.

Toxicity mechanisms	Cellular responses
Oxidative stress	Cell cycle suspension
Depletion of protective systems	Export
Mutagenesis and impaired DNA repair	Sequestration
Perturbed protein structure and activity	Chelation
	Reducing agents
	Diminished import

perturbed sulfhydryl and calcium homeostasis. Lipid peroxides can further react with metals, generating additional mutagenic compounds [96]. ROS are highly reactive and can attack DNA by inducing ***mutations***. Some metalloids, like As, are able to directly induce mutations or inhibit major DNA repair systems, resulting in further genomic instability.

The reactivity of ROS/RNS and of metal complexes can affect ***protein structure*** and induce misfolding. Many metals and metalloids form ligands with sulfur or hydrogen. As a consequence, they readily interact with sulfhydryl groups. If these are part of peptidic cysteines, the result can be devastating with respect to protein structure. This can lead to changes in the catalytic activity of enzymes, altered cytoskeletal organization and impaired cell signal transduction. The interactivity with sulfhydryl groups might also lead to ***depletion*** of glutathione (GSH) pools and other antioxidants [53, 96, 40]. This affects the oxidative stress response and might impair the maturation of iron-sulfur cluster (ISC) proteins [54].

Given the abundance of metalloproteins and the complexity of metal chemistry, it is not surprising that metals sometimes partake in erroneous interactions and ***replace essential elements***. Toxicity that arises due to competition or mimicking is a consequence of the partial similarity between elements, where they can replace each other in some situations but not in others. If the elements could fully replace each other, or not at all, no detrimental competition would arise. As mentioned previously, one reason for arsenate toxicity is because it can mimic phosphate during glycolysis. Instead of producing ATP, the result is an arsenoorganic compound which readily decomposes, thus uncoupling energy production from glycolysis.

Cd is sufficiently similar to Zn to replace it in some situations [59] and the radii of the potassium ion (K^+) and the highly toxic thallium ion (Tl^+) are closely similar, allowing Tl to be imported in the place of K with severe results to the cell [29, 17]. Lead ions (Pb^{2+}) are sufficiently similar to calcium ions (Ca^{2+}) to activate protein kinase C, possibly resulting in neural damage in higher organisms [51, 17]. Many metalloproteins have a much higher affinity for Cu than for their normal metal partner, necessitating the cell to maintain an extremely low level of free Cu. In this thesis I also present results that indicate that Te is able to replace sulfur to some extent.

2.3.2 Cellular responses to metal and metalloid stress

The cell can mount various responses when exposed to metal and metalloid stress, often by inducing ***cell cycle arrest*** until the problem is solved. Cytosolic clearance of reactive metallic species is generally accomplished by three mechanisms: (1) Cellular ***export*** through membrane transporters (e.g. Arr3 (alias Acr3) for arsenite and Pca1 for cadmium in *S. cerevisiae*) [104]; (2) cellular clearance by compartmental ***sequestration***; (3) reduction of reactivity by ***chelation*** with GSH, phytochelatin or sulfhydryl-rich metallothioneins (e.g. Cup1).

While the high metal and metalloid affinity with sulfhydryls is a cause for toxicity, the cell may also take advantage of this property by increasing production of expendable sulfhydryl-containing molecules. This commonly involves the sulfate assimilation pathway (see figure 3 on page 21). Exposure to some toxic elements, like arsenite and cadmium, can stimulate transcriptional changes that directly affect sulfate assimilation. The pathway is upregulated and the sulfur flux is rerouted so that less sulfur is incorporated into proteins and more is utilized for GSH production [104, 95].

GSH is important for all forms of life as a ***reducing agent***. Upon exposure to metals and metalloids, GSH can counteract oxidative effects of metal ions and ROS by donating a reducing equivalent ($H^+ + e^-$). It then becomes reactive itself but combines with another GSH molecule to form glutathione disulfide (GSSG), which can be recycled back to two GSH molecules at the expense of two NADPH. GSH can also participate in post-translational modifications (glutathionylation reactions) by forming disulfide bonds with sulfhydryl

groups in proteins, whereby reactive cysteines are shielded from metal binding and oxidative damage [38].

GSH also serves other roles in the response to metal/metalloid exposure. It is able to chelate ions (e.g. to form $As(GS)_3$), leading to decreased reactivity, facilitated export and sequestration. Finally, GSH can be exported for binding of metals/metalloids extracellularly, thereby ***inhibiting cellular import*** in the first place [96, 94]

2.4 Occurrence and toxicity of tellurium compounds

As a member of group 16 in the periodic table, tellurium shares many chemical and physical properties with oxygen, sulfur and selenium. Together with selenium, tellurium is an abundant element in the cosmos but rare in the earth's crust. They are believed to have been depleted since Precambrian time, when the earth presumably had a reducing atmosphere before the Great Oxygenation Event. It has been suggested that the reductive properties of free hydrogen would lead to formation of volatile hydrides (H_2Te and H_2Se), which would evaporate from the crust to space during the hot formation of the earth. Today, tellurium is one of the rarest elements in the crust with an occurrence of ~0.5 µg/kg and ~0.05 g/kg for selenium. This can be compared to cadmium with an abundance at 0.15 g/kg and arsenic at 2 g/kg (see figure 2) [92]. In general, tellurium compounds are therefore likely to have exerted a much lower selective pressure for the evolution of detoxification mechanisms compared to more abundant elements like arsenic.

Although tellurium is scarce in the crust, its association with sulfur, gold and copper ores, combined with the useful thermoelectric and photoconductive properties of tellurium compounds have recently lead to increased usage in electronic equipment and in the nanotechnology industry [3]. As a consequence, tellurium is increasingly being accumulated in the vicinities of waste dumps and metallurgical plants. There is an increasing interest in the therapeutic effects of tellurium compounds in treatments against AIDS, cancer, stroke and different immune-related diseases [75, 2]. Hence, the effects of tellurium on environment and health are of great concern. Although

it is chemophysically similar to selenium, tellurium is not known to be an essential micronutrient but can induce both acute and chronic toxicity in a variety of species.

The metabolic processes of tellurium have received much less attention compared to those of selenium, most likely because of less frequent human contact with this element and its low solubility [50]. Given the chemical and physical similarities of selenium and tellurium, there is expected to be a certain amount of overlap in the cellular pathways.

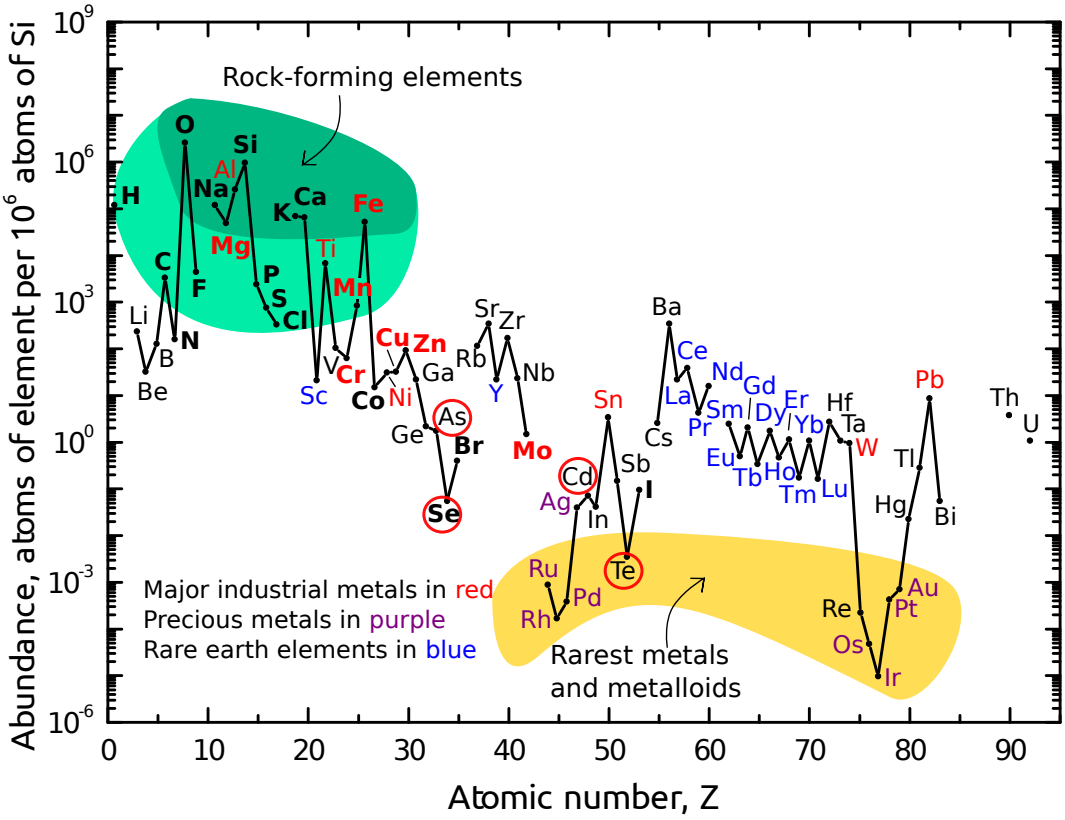

Figure 2: Abundance of the elements in earth's crust, sorted on atomic number. Major industrial metals in red, precious metals in purple, lanthanids in blue, essential or presumably essential to human health in **bold**. Arsenic, selenium, cadmium and tellurium (from left to right) are encircled. Noble gases, artificial and heavy elements are excluded. Data from United States Geological Survey; image is public domain.

Tellurium was discovered in 1782, but its effects on biological systems are still largely unknown [12]. It has been suggested that it might replace sulfur or selenium in proteins, thereby rendering them non-functional. It has also been shown that tellurite [Te(IV)] can oxidize GSH and other thiol groups, indicating that tellurium toxicity might stem from its oxidation of cellular components or through generation of reactive oxygen species [3].

2.5 The effects of arsenite on living organisms

Arsenic is a naturally common element, found in combination with organic and inorganic substances in soils and groundwater. It has gained use in the electronics industry for manufacturing of semiconductors and is routinely used in the production of pesticides.

Arsenic has been used in medicine since ancient times. Recently, arsenite [As(III)] has been found to attack sulfhydryl groups in an oncogene that is responsible for acute promyelocytic leukemia (APL) in humans and is today routinely used in treatments against relapsed APL [106]. *Trypanosoma* infections are regularly treated with arsenic compounds and also in this case arsenic binds to enzymatic sulfhydryl groups, although with serious side effects since human cells are also attacked [26, 46]. Also from a microbial perspective, arsenic can be beneficial. Antimonial compounds are regularly used to treat *Leishmania* infections, but due to the chemical similarity between antimony and arsenic, arsenic contamination in groundwater is believed to have contributed to local resistance against antimony in *Leishmania* parasites [80, 81, 1].

Natural occurrence of arsenic in some parts of the world has led to substantial health problems with millions of people exposed. For example, contamination of groundwater by arsenic in Bangladesh has been described as the largest poisoning of a population in history [87]. Long-term exposure of arsenic is linked to several diseases like stroke, cardiovascular diseases, diabetes and several forms of human cancer [86]. Despite this, the molecular mechanisms behind arsenic toxicity are largely unknown. Possible mechanisms include chromosomal damage and inhibited DNA repair [44], competition with phosphate or alterations in cellular redox levels with increased mutation rate [49].

My studies have focused on the effects of arsenite on protein folding. The discussion in chapter 4 on toxicity relating to arsenite will therefore be limited to this aspect of toxicity and the consequences of protein misfolding and aggregation is discussed in this context.

The next chapter discusses the findings on tellurite toxicity. Main findings are highlighted in the margin.

3 The biological mechanisms of tellurite toxicity

3.1 Tellurite toxicity is linked to tellurium accumulation

3.1.1 Tellurite is reduced to tellurium

Tellurite [Te(IV)] is toxic to most organisms although some bacteria are able to thrive at high Te(IV) concentrations. This bacterial resistance is correlated with a high tolerance against oxidative stress [3]. One mechanism that has been proposed to mediate Te(IV) resistance is the observed reduction from Te(IV) to the less toxic elemental form tellurium [Te(0)].

Coupled to this process is the production of nanometer-sized Te-crystals. These have different sizes and shapes depending on synthesis conditions [3, 62]. Analyses of crystals from bacteria have revealed species-specific variations, with Te(0) crystals in some cases shaped like 200 nm long rods that aggregate to form larger structures, while other species produce small irregularly shaped nanospheres [4]. Common to the accumulation of Te(0) is a characteristic darkening of cells.

Te(0) accumulates intracellularly upon Te(IV) exposure.

In agreement with these observations, we observed a substantial darkening of *S. cerevisiae* and *Schizosaccharomyces pombe* cells when they were exposed to Te(IV) – 2-fold and 8-fold increases in optical density, respectively (paper I, figure 1A). Time-lapse microscopy shows that Te(0) precipitates form proximally to the vacuolar membrane (paper I, figure 1E), agreeing with earlier observations that plaques are associated with membrane structures [65]. Prolonged exposure results in vacuolar disintegration and cell shrinkage, also in agreement with previous reports of organelle degeneration and cell wall deficiency [65].

3.1.2 Exposure to tellurite is toxic

Most studies on Te(IV) toxicity so far have been performed in bacteria. Whereas bacteria show a correlation between Te(IV) tolerance and Te(0)

Te(IV) reduction is linked to toxicity.

accumulation, the situation in *S. cerevisiae* is diametrically opposite. We screened 4311 viable deletion mutants in the presence of Te(IV) and scored colonies for Te(IV) tolerance (size) and Te(0) accumulation (color) (paper I, figure 2B). It is striking that strains that accumulate increased levels of Te(0) (red in the heat map) tend to be sensitive (green) and vice versa. Hence, whereas reduction of Te(IV) to Te(0) is linked to tolerance in bacteria, it is linked to toxicity in yeast. It remains to be investigated whether the link between Te(IV) toxicity and Te(0) accumulation is specific to *S. cerevisiae* or common to eukaryotes.

3.2 Functions involved in toxicity

3.2.1 Tellurite interacts with extracellular GSH

Extracellular GSH increases tolerance to Te(IV).

We found that addition of extracellular GSH ameliorated growth in Te(IV) in both *S. cerevisiae* and *S. pombe*, and led to extracellular reduction of Te(IV) to Te(0) (paper I, figure 3C). However, deletion of the glutathione synthetase *GSH2* in *S. cerevisiae* led to only a minor increase in Te(IV) toxicity at low concentrations (paper I, figure 5). Extracellular GSH therefore seems to be more important than intracellular in the tolerance to Te(IV). Additionally, the *opt1*Δ mutant, which is able to export but not import GSH across the plasma membrane, has higher extracellular GSH levels than the wild-type [94] and is more tolerant to Te(IV) (Table S1).

Other studies have shown that yeast uses extracellular GSH to chelate and prevent import of As(III) [94]. It is possible that a similar protective system is effective in the case of Te(IV), considering its strong affinity with sulfhydryl groups, but this remains to be investigated.

3.2.2 Tellurium accumulation is linked to respiration

Accumulation of Te(0) occurs mainly during the stationary phase, as is seen in paper I, figure 1A. This indicates that reduction takes place primarily after the shift from fermentative to respiratory growth. When cells were cultivated in respiratory medium with ethanol and glycerol as carbon sources, growth was

completely inhibited in the presence of Te(IV) (paper I, figure 1B). This is in agreement with earlier studies that have shown a much higher sensitivity of yeast to Te(IV) when is grown on non-fermentable carbon sources [65].

Cells are sensitive to Te(IV) during respiratory growth.

Several knockouts of genes with mitochondrial localization show reduced Te(0) accumulation (paper I, figure 2B). Perturbations of cytochrome c/c_1 functions, ubiquinone synthesis or mitochondrial import lead to reduced Te(0) accumulation and in some cases to improved tolerance.

Te, like S and Se, has been reported to act as a terminal electron acceptor during anaerobic growth of several bacterial species [4, 19]. If Te(IV) can compete with oxygen as electron acceptor in yeast, this provides a reductive mechanism and a source of the identified mitochondrial Te(0) grains.

3.2.3 Mutants in sulfate assimilation are tolerant

Due to its importance in GSH synthesis, functional sulfate assimilation is usually essential for maintaining homeostasis of the intracellular metal and metalloid pool. In contrast, deletion of several genes in the early steps of sulfate assimilation pathway made cells more tolerant to Te(IV) and led to less accumulation of Te(0) (paper I, figure 3A). Increased tolerance was seen when genes upstream from *MET17* were deleted, whereas deletion of *MET17* made cells more sensitive (paper I, figures 1H & 3A). This suggests that the substrate of *MET17* might confer toxicity under Te(IV) stress.

Sulfate assimilation mediates Te(IV) toxicity.

A common cause of toxicity for metals and metalloids is competition with other elements. Under physiological conditions, Met17 acts on hydrogen sulfide (H_2S) to input sulfur into the methyl cycle (see figure 3 on page 21 in this thesis). If Te is able to replace S, hydrogen telluride (H_2Te) might accumulate in the *met17*Δ mutants. The corresponding substrate under Se(IV) stress, hydrogen selenide (H_2Se), results in mutagenic and oxidative stress, so it is a reasonable assumption that the potential production of the analogous H_2Te might also have toxic effects.

Removal of the downstream Met1 and Met8 likewise resulted in reduced Te(0) accumulation and increased Te(IV) tolerance (paper I, figures 2B, 3A & 3B), likely due to inability to synthesize siroheme. Siroheme is a cofactor that is necessary for production of H_2S (or H_2Te, presumably). Further

evidence for the involvement of sulfate assimilation in Te(IV) toxicity was given by deletion of *MUP1* which encodes a Met transporter. The mutant has an increased activity of the sulfate assimilation pathway and show a high sensitivity to Te(IV) (paper I, figures 3A & 4A).

3.2.4 Tellurite and selenite might share toxicity mechanisms

There is correlation between Te(IV), Se(IV) and SeMet toxicity and between Te(0) and Se(0) accumulation.

Since Te and Se are both found in the chalcogen group in the periodic table, we suspected that the similar chemical properties might result in a phenotypic overlap between the two stresses. Inside the cell, Se(IV) is reduced to its elemental red form, which allows for quantification of Se(IV) toxicity and Se(0) accumulation. We analyzed Se(IV) toxicity and Se(0) accumulation, as well as selenomethionine (SeMet) toxicity, on strains with deviative Te phenotypes. This revealed an intermediately high correlation between deletion mutants with regard to accumulation and toxicity (r = 0.4–0.6), suggesting that Te(IV), Se(IV) and SeMet partially share toxicity mechanisms (paper I, figure 4).

3.3 Speculations about toxicity mechanisms

3.3.1 Formation of telluroproteins is an unlikely cause of toxicity

Based on the results presented here, we can speculate on some possible mechanisms behind Te(IV) toxicity. It is possible that Te is bioassimilated to generate telluro–amino acids. If the bulkier, more reactive and easily hydrolyzed telluromethionine (TeMet) and tellurocysteine (TeCys) are incorporated into proteins in the place of Met and Cys, this could potentially affect protein structure and lead to pathological protein aggregation. Unfortunately, very few experiments with telluropeptides in yeast have been performed, so the consequences of Te incorporation are largely unknown. My preliminary studies indicate that Te(IV) exposure might actually induce protein aggregation, but the reasons for this are not yet known and might not be related to bioassimilation (data not published).

SeMet competes with Met uptake in yeast, bacteria, plants and animals and is indiscriminately incorporated into proteins in the place of Met [74].

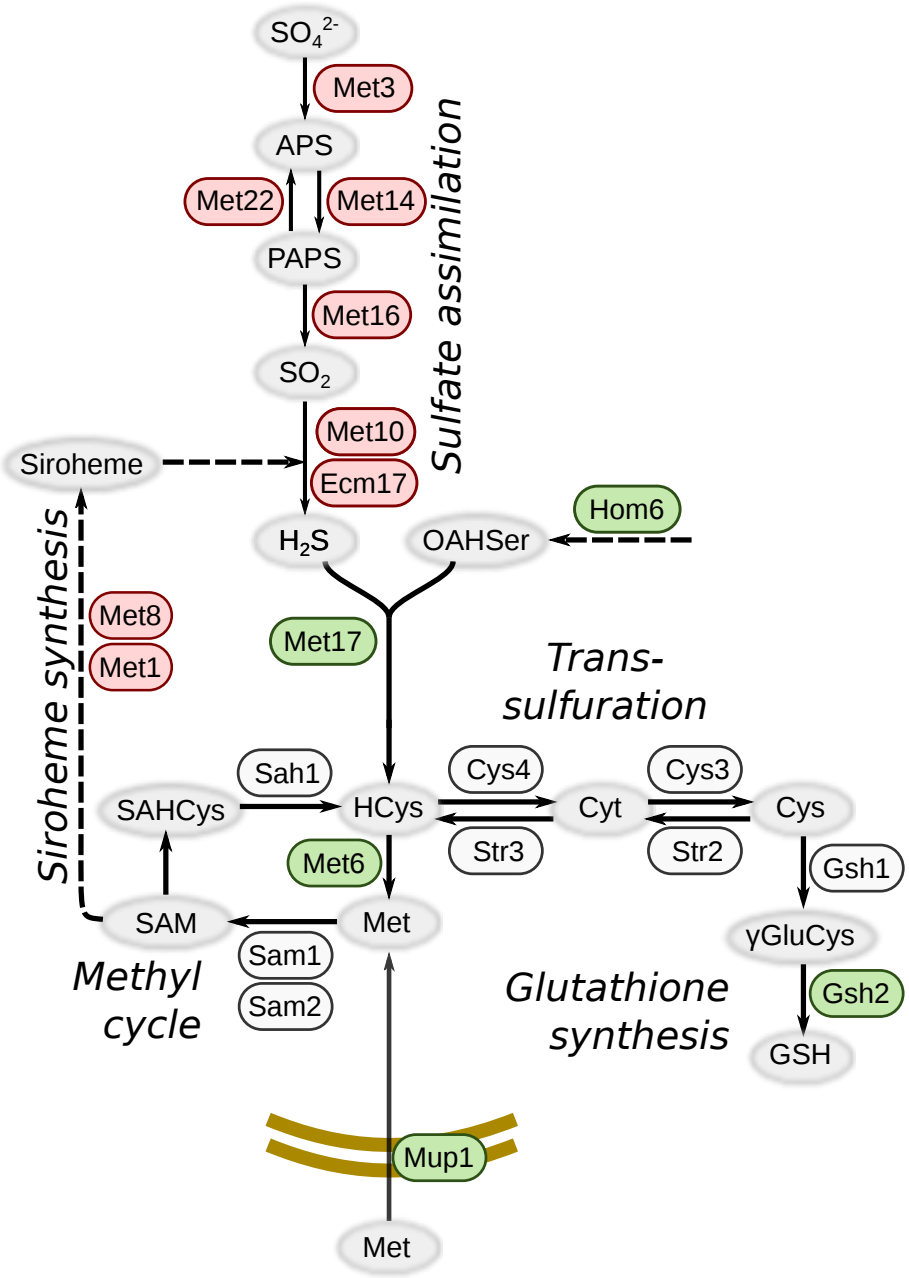

Figure 3: Pathways of sulfur incorporation. During Se(IV) exposure, selenium analogs are produced [58]. Red enzymes = mutant is tolerant to Te(IV); green enzymes = mutant is sensitive; gray enzymes = mutant is neutral. In all cases, sensitivity correlates with increased Te(0) accumulation and vice versa, except for *met6*Δ which shows increased tolerance and increased accumulation of Te(0). APS = adenosine-phosphosulfate, PAPS = phosphoadenosine-phosphosulfate, OAHSer = *O*-acetyl-homoserine, HCys = Homocysteine, SAM = *S*-Ade-Met, SAHCys = *S*-Ade-homocysteine, Cyt = cystathionine.

We therefore screened the sensitivity to SeMet in mutants that showed a deviating Te(IV) phenotype. We found a substantial covariation between Te(IV), Se(IV) and SeMet toxicity (paper I, figure 4A, 4B, 5). However, several lines of evidence make it unlikely that incorporation of TeMet into proteins is a major toxicity mechanism.

First, the reason why we investigated SeMet rather than TeMet is because TeMet is unstable and readily decomposes. *In vitro* translation experiments with SeMet and TeMet have shown that TeMet is incorporated to a smaller extent than SeMet and instead decomposes to Te(0) [74, 7].

Second, the toxicity of SeMet stems from metabolic products rather than from incorporation of SeMet into proteins. In fact, virtually all Met can be replaced by SeMet in yeast without seriously affecting cellular growth [58]. The same might very well be true for TeMet. Experimental studies on telluroproteins in yeast are few, but artificial glutathione peroxidase with telluro–amino acids have not shown toxic effects. [67].

Third, the Met auxotrophic *met17*Δ mutant is more sensitive to Te(IV) than the wild-type. Assuming that Te(IV) is bioassimilated via the sulfate assimilation pathway, the *met17*Δ mutant would be unable to produce any TeMet at all, but instead produce the H_2Te precursor.

Fourth, deletion of components in the transsulfuration pathway that leads to formation of Cys does not ameliorate growth during Te(IV) exposure, indicating that production of TeCys does not contribute to toxicity.

3.3.2 Depletion of GSH might contribute to toxicity

The above observations together make it unlikely that formation of telluroproteins is a major cause of toxicity. Probably, inorganic Te compounds are more toxic than organic [67]. If TeMet is produced and if it contributes to toxicity, it is likely toxic because it decomposes to Te(0). Likewise, the high Te(IV) sensitivity and Te(0) accumulation of the *met17*Δ mutant is likely due to production of H_2Te, which readily decomposes to Te(0) [23]. Such a scenario might partially explain the strong correlation between Te(0) accumulation and Te(IV) toxicity.

We observed a minor increase in tolerance when the glutathione synthetase *GSH2* was deleted. This mutant is unable to produce GSH. It is possible that in the wild-type, Te is incorporated into GSH to produce the tellurol derivative of glutathione (telluroglutathione, GTeH) and that *gsh2*Δ has better performance because GTeH production is inhibited. Tellurol compounds are the R-Te-H analogs of selenols and thiols, although much more unstable. Tellurols have a very low pK_a (~3), so such compounds would exist as tellurate ions ($R\text{-}Te^-$) at physiological pH and react rapidly with intracellular thiols (pK_a 8.3 for Cys), which could possibly lead to depletion of intracellular GSH pools or oxidation of protein sulfhydryls.

We have seen that deletion of all components upstream of H_2S in the sulfate assimilation pathway is beneficial during Te(IV) stress and leads to less Te(0) accumulation, whereas removal of *MET17* which consumes H_2S is detrimental and leads to more accumulation. Assuming that Te is capable of replacing S in the sulfate assimilation pathway, this would imply that production of H_2Te confers toxicity. Under Se(IV) stress, the corresponding compound is the highly toxic H_2Se [58]. This suggests another possible mechanism of GSH depletion.

It is known that under certain conditions, H_2Se is able to react with oxygen to generate colloidal Se(0). Se(0) might in turn catalyze the aerobic oxidation of GSH to regenerate H_2Se and water, leading to a catalytic cycle with an increased rate of sulfhydryl oxidation and GSH depletion [73]. H_2Te is even more unstable than H_2Se and readily decomposes in the presence of oxygen to give elemental Te(0) and water [23]. Assuming that bioassimilation of Te(IV) through the sulfate assimilation pathway generates H_2Te, this suggests two possible toxicity mechanisms: First, if the produced Te(0) oxidizes GSH (analogously to Se(0)) and regenerates oxygen-reactive H_2Te, this could potentially lead to a catalytic cycle and subsequent depletion of the reduced GSH pool in the presence of oxygen. Second, while incorporation of TeMet into proteins does not appear to be toxic, decomposition of the tellurium intermediate H_2Te into Te(0) and water might potentially uncouple downstream reactions from the sulfate assimilation pathway and inhibit production of otherwise functional S/Te-containing compounds (i.e. a loss of function).

Finally, if Te(IV) is able to act as terminal electron acceptor, this might explain

why Te(0) precipitates are found associated to the mitochondrial intermembrane [65]. It is possible that these precipitates could interfere with the structure and function of membrane proteins, thereby affecting mitochondrial respiration. This might affect other processes that are heavily dependent on NADH/NADPH, for example sulfate assimilation (Met10/Ecm17) and GSH production (Gsh2).

Taken together, more studies have to be made on the role of the sulfate assimilation pathway in Te(IV) toxicity and the effects of bioaccumulation of Te(0), whether it leads to production of mutagenic and oxidative tellurium compounds, depletion of reductive molecules like GSH, incorporation into proteins or modification of protein structure due to its reactivity with sulfhydryl groups. It seems clear at least that assimilation of Te(IV) into organic form is strongly correlated with toxicity and production of elemental tellurium.

4 Arsenite induces protein misfolding and aggregation

This part of the thesis will investigate the link between As(III) stress and protein aggregation. Specifically, I will try to answer three questions: (1) Why does As(III) induce protein aggregation? (2) What are the physiological consequences of misfolding and aggregation? (3) How does the cell respond in order to maintain protein homeostasis during As(III)-stress?

The first section will give an overview of protein aggregation in general, then of the protein quality control (PQC) systems that cells employ in order to keep protein misfolding and aggregation at a minimum. The following sections cover different aspects of As(III)-induced protein aggregation such as temporal dynamics and structural properties of aggregation-prone proteins. Together it mounts up to a picture that will help answering the three questions.

4.1 Protein aggregation: actions and counteractions

4.1.1 Causes and consequences of protein aggregation

The functions of most proteins depend on a well-defined three-dimensional structure with a stable equilibrium of anisotropic angles, which in turn is collateral to the amino acid sequence. Folding into these structures is often co-translational: as the polypeptide is being synthesized, it starts to fold, goes through intermediate states and then finally attains a native, functional structure. All proteins have a finite tendency to misfold however; a tendency that depends on the difference in free energy between the native and the intermediary stages.

Although the native state of a protein is energetically favored, it is often a narrow equilibrium and stochastic fluctuations might induce misfolding of a protein. Certain conditions might put an extra load on the folding machinery or lead to destabilization of the native conformation. Examples include increased concentration of the protein, environmental stresses such as certain electropositive metals and other chemicals, temperature changes,

mutations, salinity or pH. Certain metabolic challenges such as aging or cancer can also lead to perturbed protein structures [15, 91, 13].

Misfolded proteins often expose hydrophobic or self-complementary surfaces that are normally shielded from the outside. This can lead to inappropriate interactions and induce formation of insoluble protein aggregates. Such protein aggregates are typically classified as either (1) heterologous ill-defined amorphous granules or (2) semi-crystalline compact amyloid fibrils. The first group, consisting of ***amorphous protein aggregates***, are unordered structures that often contain multiple types of proteins. Amorphous protein aggregates are typically not linked to individual diseases.

The second group, ***amyloid protein aggregates***, are structured, insoluble and usually repetitive fibrils, often consisting of a single type of protein. They are believed to play a role in the etiology of several human diseases, for example Alzheimer's disease (Aβ1-42), Parkinson's disease (α-synuclein) and Huntington's disease (huntingtin). Neuronal cells in particular are thought to be sensitive to amyloid fibrils [63].

In humans, oxidative stress and protein aggregation increases with age. There is also an association between age and increased risk of neurodegenerative disorders [24]. While protein aggregates may have very different structures, aggregation is considered to be a common defining feature of neurodegenerative diseases. For example, unfolded tau or β-amyloid form aggregates in Alzheimer's disease, whereas globular proteins aggregate in different types of systemic amyloidosis [21].

Despite the link between protein aggregation and toxicity, it has been observed that certain aggregation-prone regions in proteins are evolutionary conserved, raising the question whether the potential of a protein to form aggregates might in some cases have a cytoprotective role [39]. For example, certain protein interactions in tumor suppressor pathways in mice and humans involve formation of amyloid-like structures [85], and several human and mouse secretory peptide hormones are stored in an amyloid-like structure ("secretory granule") that is formed by self-association. The toxicity of these granules is dependent on the type of proteins involved and is believed to be diminished by membrane encapsulation [63]. Hence, it is necessary for the cell to balance detrimental and beneficial protein aggregation. The next section will discuss how protein quality and aggregation is controlled by the

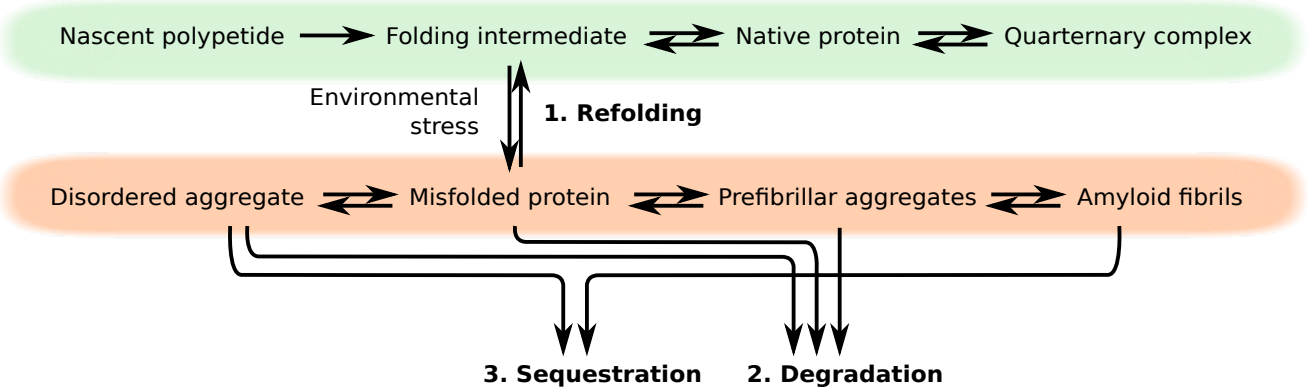

Figure 4: A newly synthesized protein goes through intermediate stages before it reaches its native, three-dimensional state. During the intermediate stages, surfaces that are normally buried inside the protein are exposed, making it vulnerable to misfolding. Environmental stress, mutations and mistranslations can induce misfolding if the result is a lower free energy in the alternative conformation. Misfolded proteins have an increased tendency to form insoluble aggregates that are either disordered, amorphous structures or ordered, β-sheet-rich amyloid fibrils. Protein quality control systems work to maintain protein homeostasis through different mechanisms: Refolding, degradation or sequestration.

cell.

4.1.2 Protein quality control is essential for homeostasis

Cells from all kingdoms of life have evolved complex multifaceted quality control mechanisms to maintain the integrity of the proteome, usually referred to as protein quality control (PQC) systems. These are complementary strategies that work together to maintain cellular proteome homeostasis. An overview of the strategies is provided in figure 4.

When a polypeptide is misfolded, it might be targeted for either (1) refolding, (2) degradation or (3) sequestration. Each method has its advantages and disadvantages.

If a protein misfolds, it might be subject to ***refolding***. This is a fast response

and has the advantage that the protein is recovered. The disadvantage is that this can lead to renewed misfolding and potentially to chaperone overload.

Molecular chaperones are central to both *de novo* folding of newly synthesized peptides and refolding of misfolded peptides. An important class of chaperones is the highly conserved Hsp70, a heat shock protein family that is involved in tolerance against several stresses [18, 8, 41]. Another important class is the Hsp104 chaperone, a disaggregase that is able to extract misfolded proteins from aggregates, remodel them and deliver them to Hsp40 and Hsp70 for refolding [35].

Degradation is a way of purging misfolded proteins from the cell. It is not as energy-efficient as refolding since the protein is not recovered, but the amino acid constituents are recycled. Extensive misfolding might potentially result in proteasome overload. The major degradative pathway in eukaryotes is the ubiquitin-proteasome system (UPS), in which ubiquitin-tagged proteins are degraded by the 26S proteasome. If UPS is impaired, aggregates might in some cases be targeted to the lysosome and degraded through autophagy instead [78].

If the Hsp104 disaggregase or UPS become overwhelmed upon stress, the third arm of PQC can act as a last resort. In this case, the cell will ***sequester*** aggregated proteins into inclusion bodies to protect the intracellular environment from damage and prevent further aggregation [103]. If these inclusion bodies cannot be solubilized before the yeast cell divides, they will be retained in the mother cell while leaving the daughter cell free from protein aggregates, so-called spatial quality control [103, 88].

The selective pressure to maintain proteome homeostasis is likely to have been an important constraint during protein evolution. The cell is constantly exposed to different external stress factors that could potentially destabilize the precarious equilibrium of native protein conformations. Efficient PQC systems are therefore absolutely essential for fitness. Nevertheless, certain stress conditions are able to put an increased load on the PQC systems and increase the aggregation propensity of proteins that under normal circumstances would fold correctly.

The following sections will go through the main findings pertaining to As(III)-

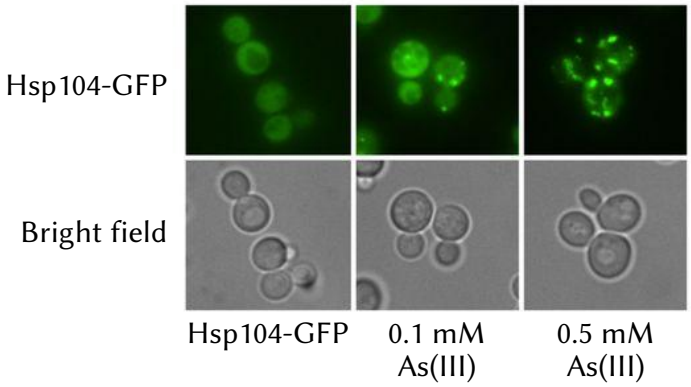

Figure 5: Cells exposed to As(III) show foci of Hsp104-GFP, while there is an even distribution in unstressed cells. This shows that As(III) induces protein aggregation. Adapted from paper II, figure 1A.

induced protein aggregation. What factors contribute to aggregation and why are some proteins more aggregation-prone than others? What are the physiological consequences of misfolding and aggregation, and how does the cell handle this?

4.2 Arsenite induces misfolding of newly synthesized proteins

As(III) exposure induces protein aggregation.

The connection between As(III) stress and protein aggregation was established in two ways with Hsp104 as a marker of protein aggregates: with Western blot (paper II, figure 1C) and with imaging of Hsp104-GFP (paper II, figure 1A). Fluorescent imaging shows that Hsp104-GFP is evenly distributed in the cell without As(III), while As(III)-exposed cells show clear GFP-foci. This is indicative of the presence of protein aggregates, as shown in figure 5 above.

Newly synthesized proteins that undergo *de novo* folding appear to be more sensitive to As(III)-induced protein misfolding and aggregation than already folded, native, proteins. Several lines of evidence support this hypothesis:

1. Inhibition of translation by cycloheximide (CHX) suppresses formation

Aggregation is linked to translation.

of As(III)-induced aggregates (paper II, figure 4D). This strongly links aggregation to translation.

2. Using a [^{35}S]-Met labeling assay, we showed that newly synthesized proteins are primarily targeted by As(III) for aggregation (paper II, figure 4B).

3. As(III) was shown to inhibit spontaneous and chaperone-mediated refolding of denatured firefly luciferase *in vivo* (paper II, figure 5).

In order to better understand the processes behind protein aggregation, we isolated aggregated proteins. Cells were exposed to 1.5 mM As(III) for one hour after which the cell membrane was disrupted. The lysate was centrifuged to isolate aggregated and membrane proteins and the latter was removed by washing with a detergent. Aggregated proteins were purified from an SDS-PAGE gel, digested with trypsin and identified with LC-MS. As control, physiological aggregates from unstressed cells were identified in the same way.

In total, 257 proteins were found to aggregate during As(III)-stress, of which 143 aggregated exclusively during As(III) stress and 114 aggregated also without stress. The former set of proteins will henceforth be referred to as the stress-dependent arsenite set (As-set), and the latter will be referred to as the stress-independent physiological set (P-set). To avoid bias by comparison to genomic proteins that are difficult to detect by mass spectrometry, the identified proteins in the As- and P-sets were compared to a set of proteins that can be detected by this method under physiological conditions [101]. This background set is henceforth called the MS proteome.

Analyses of the proteins in the As- and P-sets gave additional support for the hypothesis that As(III) mainly acts on newly translated proteins:

As-set is enriched in protein synthesis components.

4. The As-set is over-represented in proteins that are involved in protein synthesis and folding (paper III, figure 1). These proteins might have been recruited for refolding and cosedimented with their clients or they might have misfolded and aggregated. In either case, this overrepresentation supports the theory that aggregation is cotranslational.

5. Components of the SSB-RAC and NAC chaperone systems, which assist nascent polypeptides with folding, were identified among the

aggregated proteins (paper III, table S3).

6. Proteins in both the As-set and P-set a) have high translation rates, b) are clients to SSB (Ssb1/2) and c) show increased aggregation in *ssb1*Δ *ssb2*Δ (paper III, figure 4).
7. Proteins in both the As-set and P-set are highly stable under physiological conditions, indicating that misfolding occurs before the native state is reached, (paper III, figure 2H).

We conclude from these observations that As(III)-induced protein aggregation primarily affects newly synthesized proteins. That is no logical necessity, since 42°C heat stress leads to unfolding of previously folded, native peptides (paper II, figure 4A). Hence, As(III)-induced and heat-induced protein aggregates appear to form by different mechanisms.

4.3 Aggregation-prone proteins are dependent on chaperones

4.3.1 The ubiquitous Hsp70 chaperone class

As mentioned previously, chaperones are important to all aspects of the PQC systems, whether it is *de novo* folding, disaggregation and refolding of aggregated and misfolded proteins, targeting of misfolded proteins for degradation, or delivery to inclusion bodies for sequestration.

Several of the 63 chaperones in *S. cerevisiae* belong to the Hsp60/70 class and are involved in folding of nascent polypeptides. The highly conserved chaperone Hsp70 is a major actor in eukaryotic proteostasis and a key player in the protection against protein aggregation due to its binding to early peptides and facilitation of their folding, translocation and association with downstream protein chaperones. Hsp70 chaperones also participate in aggregate disassembly, translocation into ER and mitochondria, regulation of other heat shock proteins and degradation.

There are 9 cytosolic Hsp70 in *S. cerevisiae*: Ssa1, Ssa2, Ssa3, Ssa4, Ssb1, Ssb2, Sse1, Sse2, Ssz1, two ER-associated Hsp70 (Kar2 and Lhs1) and three mitochondrial forms (Ssc1, Ssq1 and Ecm10). Two cytosolic Hsp70, Ssb1

and Ssb2, bind cotranslationally to the growing peptide chain, their ATPase activity being regulated by the Hsp40 Zuo1 and the nuclear exchange factor Sse1. Together with Ssz1, Sse1 forms the ribosome associated complex (RAC) that binds to the ribosome [102].

4.3.2 Proteins in the As- and P-sets are enriched in chaperone interactions

Aggregation-prone proteins are dependent on chaperones.

There are several indications that the aggregation-prone proteins in the As- and P-sets are heavily ***dependent on chaperones***:

1. Several Hsp70 chaperones were identified together with the aggregated proteins and proteins that are involved in translation and protein folding were strongly enriched in the As- and P-sets (paper III, figure 1).

2. Proteins in the As- and P-sets tend to aggregate in the Hsp70 mutant *ssb1*Δ *ssb2*Δ to a higher degree than proteins in the MS proteome (paper III, figure 4C).

3. Both the As-set and P-set are enriched in interactions with the ribosome-associated Ssb2 compared to the MS-proteome (paper III, figure 4B). The P-set contains a higher proportion of Ssb2-binding proteins than the As-set, indicating that dependency on Ssb2 is not specific to As(III)-dependent aggregates.

4. I analyzed the complete chaperone interactome [37] for proteins that are clients to at least one chaperone. Results show that 88% of proteins in the As-set are clients to at least one of the 63 chaperones in yeast, which is significantly more than the P-set (77%) and MS proteome (77%) (paper III, figure S2).

5. Finally, I quantified the number of chaperone interactions per protein. This showed that proteins in the As-set have significantly more chaperone interactions per protein than proteins in the P-set or MS-proteome (paper III, figure 5C).

4.3.3 Arsenite inhibits chaperone function

The dependency on chaperones by proteins especially in the As-set is likely to be an important component in the etiology of As(III)-induced protein aggregation. It should be seen in conjunction with the strong indications that As(III) is capable of direct ***inhibition of chaperones***. This latter conclusion was made by quantifying (1) inhibition of chaperone-mediated refolding of chemically denatured luciferase *in vitro* (paper II, figure 5C); (2) inhibition of chaperone-dependent disaggregation of heat-induced aggregates *in vitro* (paper II, figure 5D); and (3) inhibition of disaggregation of heat-induced aggregates *in vivo* (paper II, figure 4D). In all three cases, As(III) was shown to have a significant negative effect on chaperone activity.

As(III) can inhibit chaperone function.

The ability of As(III) to directly inhibit chaperone activity, together with the heavy dependency on chaperones by proteins in the As-set, suggests a possible cause for aggregation. If As(III) inhibits chaperone activity due to direct binding or aggregation, proteins that require chaperones for correct folding run an increased risk of misfolding. Likewise, if chaperones are overloaded during As(III)-stress due to extensive misfolding, chaperone-dependent proteins are more vulnerable to misfolding and aggregation.

In any case, proteins that are highly dependent on chaperone-interactions would suffer from the extra load on chaperones. These proteins would be particularly prone to aggregation during As(III)-stress. Hence, they would be found in the As-set.

4.4 Physical properties of aggregation-prone proteins

4.4.1 Primary structure

The three-dimensional structure of a protein is ultimately dependent on the amino acid sequence, and some amino acids are more likely to induce aggregation than others. I therefore investigated the amino acid composition of the aggregation-prone proteins (paper III, figure 3).

It was shown that proteins in the As- and P-sets were not enriched in Gln

or Asn (which are common in prions), the As-set was not enriched in Arg or Lys (which are targets of carbonylation) and neither the As-set or P-set were enriched in Pro or Thr (also targets of carbonylation). No set showed an increase in Cys, solitary or vicinal, which was rather surprising since one of the central reactions of As(III) in the cell is with sulfhydryl groups: $As(OH)_3 \overset{GSH}{\rightleftharpoons} As(GS)_3$. On the other hand, both sets have significantly more surface-exposed redox-reactive Cys than the MS proteome but are not statistically different from each other (paper III, figure 5B). Hence, if As(III) binding to protein sulfhydryls have toxic consequences, it is likely to involve mechanisms other than misfolding and aggregation.

Hsp70 is known to have a binding preference for aliphatic (hydrophobic, non-polar) amino acids [83]. *In vitro* experiments have suggested that unfolded stretches that are rich in aliphatic residues might be prone to self-assembly [56]. Likewise, protein aggregates in aging *Caenorhabditis elegans* and in human Alzheimer's disease are enriched in aliphatic or hydrophobic stretches [22, 56, 20]. In agreement with this, proteins in the As- and P-sets were shown to be enriched in aliphatic residues (Gly, Ala, Val) compared to those in the MS proteome (paper III, figure 2E & 3). It is possible that exposure of aliphatic stretches during intermediate stages of the folding process favors aggregation-prone conformational stages due to inappropriate hydrophobic or electrostatic interactions [43, 82]. Proteins in the P-set are further enriched in basic amino acids (Lys and Arg) and underrepresented in acidic amino acids (Asp, Glu and Ser) and accordingly have a lower isoelectric point (paper III, figure 2D & 3), which might make them sensitive to aberrant electrostatic interactions during folding.

Aggregation-prone proteins are enriched in aliphatic amino acids.

4.4.2 Secondary structure

Disease-related and functional aggregates are often enriched in β-sheets [16, 30, 39]. I therefore analyzed the data sets for enrichment of secondary structures. Interestingly, both the As-set and P-set are enriched in both β-sheets and α-helices compared to the MS-proteome. Hence, high β-sheet content does not seem to be a major contributing factor to As(III)- induced aggregation but shows a general, As(III)-independent correlation with aggregation propensity.

Aggregation-prone proteins have more secondary structures.

The enrichment of secondary structures in aggregation-prone proteins might be correlated with more stringent folding requirements and could be a reason why they are more dependent on chaperones for their folding, as well as making them more susceptible to misfolding if interacting chaperones are impaired or depleted. Extensive misfolding during As(III) stress might result in chaperone overload, so that aggregation exceeds the folding capacity. Proteins with extensive secondary structures would be particularly sensitive in such a scenario.

4.5 Arsenite-induced misfolding affects protein interactions

4.5.1 Arsenite affects the protein interaction network

Proteins that misfold can affect the cell in different ways. Misfolding of a protein might be considered as a "loss-of-function" activity if the native conformation and activity are depleted from the cell. This could have potentially toxic consequences. However, analysis showed that there is little overlap between knockout mutants that are particularly sensitive in As(III) stress and proteins in the As-set, suggesting that protein depletion due to aggregation is not a major cause of toxicity (paper III, figure 5D). Nevertheless, depletion or inactivation of particularly important proteins could still have potentially toxic consequences.

Misfolded proteins might also have pernicious "gain-of-function" activities due to their increased propensity to aggregate and participate in inappropriate interactions with other proteins through seeding effects. This might aggravate the collapse of proteome homeostasis and put further load on the PQC systems, leading to a positive feedback. To investigate this possibility, I analyzed the protein interaction network in *S. cerevisiae* for potential covariation in interactivity and aggregation propensity. It turned out that both the As-set and the P-set are overrepresented in protein interactions (43 and 80.5 per protein, respectively) compared to the MS proteome (28 per protein; paper III, figure 5F). Hence, there is a clear correlation between aggregation propensity and interactivity with other proteins.

Aggregation-prone proteins have many interactions.

The increased number of interactions by aggregation-prone proteins can be interpreted in two ways with regard to the physiological effects. (1) Proteins that aggregate have many interaction partners and might therefore be efficient in propagating misfolding (gain-of-function) in a seed-like process. (2) The interactions of a protein that is misfolded or aggregated is likely to be different from the native variant, and since proteins that aggregate under stress are enriched in interaction partners, this leads to wide-spread loss-of-function. Irrespective of whether gain-of-function or loss-of-function (or both) activities contributes most to toxicity, it is likely that protein aggregation by As(III) has substantial physiological consequences and affects more proteins than just those that have been in direct physical contact with the As(III) compound.

4.5.2 Protein aggregates can trigger further aggregation

Often substoichiometric amounts of a fast-aggregating protein can accelerate the conversion of soluble, slow-aggregating proteins into insoluble homo- or heteroaggregates [6]. It has been predicted that the majority of all proteins contain self-complementary sequences. Normally these sequences are buried within a protein, but if a peptide becomes partially unfolded during its synthesis, they can partake in inappropriate interactions that initiates misfolding and aggregation of other proteins. Further, the intermediary states in the folding process typically expose hydrophobic or aliphatic surfaces that are normally buried inside the proteins, making them more sensitive to detrimental perturbations that lead to incorrect hydrophobic interactions and misfolding of other proteins [36, 21, 56].

As(III)-induced aggregates inhibits refolding of unfolded proteins in vitro.

We found *in vitro* experimental support for the hypothesis that As(III)-induced protein aggregation affects protein interactivity through a gain-of-function mechanism. Chemically denatured luciferase on which As(III) had acted, was able to inhibit refolding of unfolded luciferase that had not been exposed to As(III). Although denatured luciferase (without As(III)-exposure) was also capable of inhibiting refolding of unfolded luciferase, As(III) resulted in a four-fold lower IC_{50} (paper II, figure 6C).

The inhibition by substoichiometric amounts of As(III)-aggregated luciferase on refolding of misfolded luciferase is indicative of a gain-of-function seed-

like tendency to induce further inhibition of protein folding *in vitro*. In particular, aggregates that are formed from As(III) stress appear to have a strong inhibitory effect on refolding of misfolded proteins that have not encountered any metalloid. This seeding effect is seen in several aggregation-related disorders and is likely to have major toxic effects on the organism.

4.6 Aggregation-prone proteins are abundant and highly translated

4.6.1 Abundance & half-life

The aggregation tendency of a protein is known to be highly dependent on the protein concentration. High concentrations enhances the chances of intermolecular interactions, including the aggregation of incompletely folded or misfolded proteins [91]. I therefore analyzed the ***abundance*** of aggregation-prone proteins using published data [33, 14]. This data has been generated in a physiological environment, thus representing pre-stress conditions. Results show that aggregation-prone proteins are more abundant than the MS-proteome (paper III, figures 2A & 2B). Proteins that aggregate during physiological conditions are particularly abundant, with proteins in the P-set being several orders of magnitude more abundant than the MS-proteome.

Aggregation-prone proteins are abundant and stable.

The ***half-life*** of the yeast proteome has also been quantified under physiological conditions [5] and shows a correlation with aggregation propensity: Proteins in the As-set are more stable during physiological conditions than the MS-proteome and this is even more pronounced for proteins in the P-set (paper III, figure 2H). Since these proteins are at the same time more aggregation-prone and more stable, this indicates that it is indeed newly synthesized proteins that are sensitive to aggregation and that the mature proteins are relatively stable once they have reached their native states.

4.6.2 Aggregation-prone proteins are highly translated but down-regulated under arsenite stress

The strong indications that As(III) interferes with folding of newly synthesized proteins led us to ask whether translation rate is covariant with aggregation propensity. It is likely that proteins with ***high translation rates*** put a heavy load on the protein folding machinery and that they are extra vulnerable to misfolding if chaperones are inhibited or exhausted. Using published estimations of translation rates, I observed that proteins in both the As-set and P-set have a significantly higher average translation rate than the MS proteome (2-fold and 8-fold, respectively).

Aggregation-prone proteins are highly translated.

One way to look at this is that proteins with very high translation rates (i.e. the P-set) are aggregation-prone under any circumstance, while proteins with only weakly increased translation rates (i.e. the As-set) tend to be folded correctly under physiological conditions but that the extra stress from As(III) compromises their folding process and induces misfolding.

Since high translation appears to make proteins more vulnerable to stress-induced aggregation, it is a reasonable hypothesis that cells respond to stress by ***repressing expression*** of aggregation-sensitive proteins. I therefore compared our data sets to a previous study that identified proteins that are differentially expressed during As(III) stress [95]. 27% of all proteins in the MS-proteome show at least 2-fold decreased expression upon As(III) stress, whereas the corresponding number for the As-set is 40% and for the P-set 69%. 8% of the As-set and the MS-proteome have increased expression at least 2-fold, and 3% of the P-set. Statistical analyses show that both the As-set and P-set contain significantly more proteins with decreased expression compared to the MS proteome.

Aggregation-prone proteins have reduced expression upon As(III) exposure.

Both the As-set and the P-set aggregate upon As(III) stress, with the P-set having some properties that make them particularly aggregation prone (e.g. high translation rate and abundance), so a decreased expression of these proteins, and especially of those in the P-set, might make physiological sense.

In conclusion, it appears that a high translation rate makes proteins increasingly susceptible to aggregation and that As(III) exposure further sen-

sitizes those with a moderately high translation rate. On the other hand, aggregation-prone proteins are not substantially upregulated during As(III) stress, so aggregation is not (to any large extent at least) a consequence of increased protein concentration during As(III) exposure. Hence, it seems like the degree of aggregation is a consequence of either high initial translation rate or absolute expression (or both), but not by increased expression. Rather, yeast's PQC strategy toward As(III) appears to include the down-regulation of aggregation-prone proteins. Decreasing the expression might therefore be a strategy by which the cell strives to ease the burden on chaperones during As(III) exposure, or to keep the concentrations of proteins below the threshold that leads to aggregation.

4.7 Turn-over of protein aggregates

During As(III) stress, chaperone components are upregulated [95] and aggregation-prone proteins are down-regulated (previous section). This is likely to be a way to decrease the load on chaperones during stress, thereby minimizing deleterious misfolding of chaperone-dependent and highly translated proteins. Nevertheless, there is increased protein misfolding and formation of aggregates during As(III) exposure. The cell must have ways to handle this.

As mentioned previously, the PQC system consists of three "arms": refolding, degradation and sequestration (figure 4 on page 27). We have already seen evidence that As(III) can inhibit disaggregation and refolding of aggregated proteins. This section will discuss degradation and sequestration briefly.

4.7.1 Proteasomal clearance of aggregates

The second arm of the cellular PQC systems is the degradation of aggregated proteins. In most wild-type cells, the Hsp104-GFP foci are cleared after 3 hours of 0.1 mM As(III) exposure. We reasoned that this might be due to proteasomal clearance of aggregates, since previous studies have shown an upregulation of proteasomal components in As(III)-exposed cells [95]. Several lines of evidence support this hypothesis:

Protein degradation is an important PQC mechanism during As(III) stress.

1. As(III) stress leads to a 7 to 8-fold increase in 26S activity *in vivo* (paper II, figure 2B).
2. As(III) leads to an increase in proteasomal components (paper II, figure 2C).
3. Cells show an increased sensitivity to As(III) when components of the 19S and 20S subunits are mutated (paper II, figure 2D).
4. Rpn4 is required for upregulation of a majority of proteasomal components and a number of genes related to the ubiquitin-proteasome pathway. The *rpn4*Δ mutant is unable to clear the cytosol from protein aggregates (paper II, figure 2A).
5. The *rpn4*Δ mutant has a much lower upregulation of 26S activity and show reduced amounts of proteasomal components compared to wild-type, in presence and absence of As(III). This mutant is more sensitive to As(III) than the wild-type (paper II, figure 2B & 2D).

These observations together indicate that the strategy to deal with As(III) stress in yeast involves activation of proteasomal activity for aggregate clearance.

4.7.2 Partitioning to inclusion bodies

The third arm of the PQC systems, sequestration, involves partitioning of aggregated proteins to inclusion bodies. Since this has not been investigated in any detail regarding As(III)-induced protein aggregates, it remains an open possibility that aggregated proteins are delivered to JUNQ or IPOD for temporary or irreversible storage.

4.8 Conclusions: Protein aggregation during arsenite exposure

The previous sections have uncovered some clues for answering the following questions: (1) Why does As(III) induce protein aggregation? (2) What are

the physiological consequences? (3) How does the cell respond in order to maintain protein homeostasis during As(III) stress?

4.8.1 Question 1: Why does As(III) induce protein aggregation?

We have seen several lines of evidence that protein aggregation is ***linked to translation***, e.g. reduced aggregation when translation is inhibited, the presence of folding-related proteins together with aggregation-prone proteins, and the dependency of aggregation-prone proteins on ribosome-associated chaperones. The evidence for this is strongest for the As-set, but the P-set does also include proteins that are involved in ribosome-associated protein folding and that have high translation rates.

The increased vulnerability of nascent polypeptides makes biological sense: En route to its native structure, the nascent peptide must pass through several intermediate stages. During these intermediate stages, hydrophobic, aliphatic and self-complementary stretches that are normally buried inside the protein are exposed, increasing the risk for inappropriate interactions that can lead to propagation of misfolding and aggregation. The increased occurrence of ***alipathic amino acids*** might therefore contribute to aggregation [56] and higher ***abundance*** and ***translation rate*** are likely to result in an increased number of inappropriate interactions.

The observations that aggregation-prone proteins in our sets have ***more secondary structures*** and are ***more dependent on chaperones*** suggest that they might require a more meticulous folding process than the average protein. If chaperones are depleted or overloaded so that aggregation overwhelms the PQC capacity, these proteins will be particularly vulnerable.

Taken together, there are several properties that might make the proteins in our sets (As-set and P-set) more aggregation-prone. For most of these properties, proteins in the P-set are more extreme than those in the As-set, while the As-set is intermediate between the P-set and the MS-proteome. All proteins in both the As-set and P-set aggregate in the presence of As(III), but only those in the P-set aggregate in the absence of As(III). One way to interpret this is that the proteins in the As-set are close to the threshold to

misfold, and that As(III) gives them the final "push over the edge".

If the "edge" represent the properties that have just been mentioned, what is the "push"? We have some clues: Proteins in the As-set appear to be significantly more dependent on ***chaperone*** functions for proper folding and function. We have also seen that As(III) is capable of inhibiting chaperone activity with regard to refolding and disaggregation. Hence, if As(III) inhibits chaperones directly or indirectly, this would negatively impact the cell's PQC systems and lead to increased levels of misfolded and aggregated proteins. This might be a consequence of either increased misfolding and aggregation, or reduced disaggregation and refolding.

4.8.2 Question 2: What are the physiological consequences of protein aggregation?

As(III) is known to be toxic and we have seen that As(III) induces formation of protein aggregates. But we do not know what the connection between toxicity and aggregate formation is. It might be that the misfolded or aggregated proteins themselves participate in inappropriate and detrimental interactions (gain-of-function), or it might be that misfolding and aggregation leads to depletion of proteins that perform important cellular functions (loss-of-function).

There is an even more fundamental question however: Are the aggregates toxic or beneficial? There is an increasing number of reports that protein aggregates might not just be functional (in the sense of inducing further aggregation) but also occasionally beneficial for the cell [39, 32, 45]. In the case of As(III), aggregation might possibly be an evolutionary adaptation to prevent misfolded proteins from participating in inappropriate gain-of-function interactions. Most evidence that aggregates might be beneficial appear to apply to amyloid fibrils, and the aggregation-prone proteins in the As- and P-sets lack some properties that are common in amyloid fibrils, such as enrichment of intrinsically disordered regions (paper III, figure 2G). One argument in favor of aggregate toxicity is the *rpn4*Δ mutant which is unable to clear aggregates from the cytosol and have an increased sensitivity.

Irrespective of the role of aggregates in toxicity vs. protection, it is likely that

As(III) exposure can have widespread affects on protein functionality. Several lines of evidence suggest this: (1) As(III) is capable of inhibiting chaperone activity, thereby influencing protein folding and activity of other proteins that require chaperones for proper folding. (2) As(III)-induced protein aggregates can act as seeds and inhibit refolding of misfolded proteins that have not been exposed to As(III). (3) Proteins that are particularly vulnerable to aggregation under As(III) stress are enriched for protein-protein interactions. Empirical evidence indicates that perturbation of proteins with many interactions is usually more detrimental to the cell than perturbations of those with few interactions [42]. If some of these proteins are completely or partially depleted due to misfolding and aggregation, other cellular functions could be affected.

Together, these observations indicate that As(III) exposure can have effects also on proteins that have not been in direct contact with As(III) molecules. This could lead to widespread effects on cellular functions.

4.8.3 Question 3: How does the cell respond in order to maintain protein homeostasis during As(III) stress?

We have seen that *de novo* folding of aggregation-prone proteins is dependent on ribosome-associated Hsp70 chaperones. The importance of PQC for clearance of chaperones is also telling, since all three arms of the PQC system – refolding, degradation, and sequestering – require chaperones for proper function. For example, disaggregation of aggregates and refolding of misfolded proteins is dependent on Hsp104 and Hsp70 chaperones, whereas ubiquitylation of proteins for degradation requires Hsp40 chaperones. Partitioning of ubiquitylated proteins to sequestration or degradation seems to be dependent on Hsp70 components [84].

It has earlier been seen that As(III) stress induces upregulation of chaperones [95] and the results presented here show that As(III) exposure leads to enhanced proteasomal activity. We have also seen evidence that aggregation-prone proteins are down-regulated in response to As(III) exposure, which likely helps preventing that chaperone-mediated disaggregation and refolding or proteasomal degradation are overwhelmed.

Both spontaneous and chaperone-mediated refolding of misfolded proteins appear to be inhibited by As(III) exposure. Perhaps that is why, based on our experiments, degradation seems to be the most important route to clearance of As(III)-induced aggregates. It is currently not known what the role of protein sequestration is during As(III) stress. Perhaps this mechanism is used in parallel with refolding and degradation, or as a resort if the refolding and degradation systems are temporarily overwhelmed. It is also possible that sequestration of As(III)-induced protein aggregates offers a permanent storage that facilitates spatial quality control and allows rejuvenation of the daughter cell, cleared from protein aggregates [28].

4.8.4 What are the medical implications of these results?

As mentioned previously, there is a correlation between protein aggregation and several human neurodegenerative diseases. Protein deposition has been linked to at least 20 different disorders [10], including Alzheimer's, Parkinsons's, Huntington's, amyotrophic lateral sclerosis (ALS), fatal prion diseases such as Creutzfeld-Jakob disease, and non-neurodegenerative disorders such as diabetes, cancer and cystic fibrosis [70].

A great interest has also emerged in the medical sciences on how metals and metalloids affect protein aggregation and the etiology of different pathologies. For example, the β-amyloid plaques that are characteristic of Alzheimer's disease contain high quantities of several metal ions such as Zn, Cu(II), Fe(III) and Al [48, 55, 90], and tau phosphorylation is strongly aggravated by Hg [76], corroborating the correlation between the usage of amalgam in dental applications and Alzheimer's cases compared to controls [69]. Metal ions may induce radical reactions with β-amyloid, resulting in its precipitation and becoming resistant to proteolysis, but neurodegenerative toxicity may also be caused by metal deficiency [64], highlighting the importance of metal homeostasis for proper protein folding and cell viability [90].

Several yeast orthologs to disease-associated aggregation-prone proteins were identified in our sets. The As- and P-sets are strongly enriched in orthologs to (1) β-amyloid-associated aggregates, (2) proteins that co-aggregate with the tau protein in neurofibrillary tangles in Alzheimer's disease and (3) with α-synuclein in Parkinson's disease. (4) The sets are also highly

enriched in orthologs to proteins that aggregate in a mouse familial ALS model. The medical applicability of the results in this thesis will be left for future studies to determine, but overrepresentation of orthologs at least suggests that common mechanisms might exist between protein aggregation in yeast and disease-related aggregation in humans and that the results obtained here might be relevant also for gaining a better understanding of disease-associated aggregation.

4.8.5 What are the evolutionary implications of these results?

Cells live on the verge of a proteopathic catastrophe: an increase in temperature by as little as 4° can destabilize 20% of the proteome [34]. Protein levels, structure and interconnectivity all affect the vulnerability to aggregation and indirectly the fitness of the organism [93]. Maintaining a functional PQC system is therefore essential for proteome homeostasis. The dependency of aggregation propensity on so many diverse aspects of protein structure and function suggests that the evolutionary process has finely tuned the proteome for optimal performance and that proteins have co-evolved with their intracellular environments as the external conditions have changed [93].

The fitness advantage of proteome homeostasis is likely to have exerted significant constraints on protein evolution with regard to several properties that are associated with increased risk of misfolding and aggregation. Structural properties such as hydrophobicity and sulfhydryl content as well as functional properties like protein interactivity and abundance might have been subject to natural selection acting to minimize protein aggregation.

There is however a difference between correlation and causality: That certain metal phenotypes and cellular functions exist does not necessarily mean that they have a beneficial role from the perspective of the organism and evaluating the role of natural selection during evolution is therefore not as easy as it may seem.

For example, if a gene influences more than one trait (so-called pleiotropy), then a gene variant can become fixed in the population due to natural selection, not because a certain trait is beneficial but because it can hitchhike

with another trait that is beneficial. Likewise, genetic drift can drive random changes of variations in a population. This can have a very large effect on small populations and lead to fixation of sub-optimal gene variants. Distinguishing between adaptive and non-adaptive evolutionary changes is therefore critical to understanding why organisms function the way they do. We will turn to this problem in the next chapter.

5 Detecting selection in an evolving population

5.1 Introducing the problem

5.1.1 Why do cells behave the way they do?

Tolerance to metalloid stress can be loosely defined as the property that makes some individuals able to deal with metalloid concentrations that would be inhibitory or lethal to non-tolerant individuals. This property might consist in decreased import or increased export through dedicated transporters, intra- or extracellular chelation of the metalloid, vacuolar sequestration, or shielding or down-regulation of potentially reactive targets.

Tolerance that is acquired through acclimatization during low-level exposures of metalloid stress is typically not inherited to offspring and therefore lost when conditions are restored to normal. In contrast, tolerance that is acquired through genetic changes is not lost upon propagation and are therefore retained when environmental conditions improve [68]. The distinction between physiologically and genetically acquired tolerance is to some extent arbitrary, since the capacity for physiological and biochemical acclimatization ultimately must have a genetic basis. It is an important distinction nonetheless, since an evolutionary acquired tolerance function is not necessarily an adaptation in an evolutionary sense. Having in previous chapters discussed the factors that underlie the capacity of organisms to acclimatize physiologically, we will now turn to the mechanisms that allow organisms to adapt in ways that can be inherited by future generations.

Natural sites with metal or metalloid pollution are often poor in biological diversity compared to nearby sites, indicating that many organisms lack the genetic potential for tolerance [68]. Some natural yeast strains live in habitats with high levels of cadmium and have acquired tolerance by increased cellular export of cadmium, but they show reduced performance compared to other yeast strains during low cadmium levels [11]. Such trade-off mechanisms, where a detrimental trait is linked with a beneficial trait, can make it very difficult to determine whether the existence of a trait in a population is due

to adaptation by natural selection or because it is somehow linked to another trait that has been adaptively selected.

Another source of confusion in understanding the factors that underlie fixation of a trait is stochastic processes. A population bottle-neck can lead to the purging of beneficial alleles from the population, leaving behind sub-optimal variants that will constitute founders for the future population ("founder-effect"). Random genetic processes act on populations and can lead to increased or decreased genetic and phenotypic variation. In other words, that certain metal phenotypes and cellular functions exist does not at all imply that they have a beneficial role from the perspective of the organism.

Influences by these non-adaptive processes make it difficult to expose the role of natural selection during evolution. Nevertheless, in order to understand biological function, answering the "why" question is as important as answering the "how" question. Why are some organisms better at handling metalloid stress than others? Is it because of a lucky accident (like a bottle-neck) or because of a selective pressure to cope with the problem? Any proposed answer to these questions that does not include a description of the origin of the cellular systems would not be complete.

Distinguishing between the different processes that have shaped life has been a desirable goal for evolutionary biologists, reflected in the longstanding debate on the neutral vs. non-neutral theories of evolution [71, 66]. This chapter will present a method for doing this, using *S. cerevisiae* as a model. The suitability of this project is discussed in the next session.

5.1.2 *S. cerevisiae* as a model for evolutionary studies

Some advantages of using *S. cerevisiae* as a model for evolutionary studies were mentioned in the introduction:

1. It is present over a large geographic range and a wide range of habitats.
2. Mutations rarely spread horizontally between populations, allowing for investigations of population-specific effects.
3. Its population dynamics is characterized by bursts of rapid expansion from small initial population sizes followed by massive cell death,

leading to repeated bottle necks. Founder effects commonly result in fixation of distinct genetic variants that are sub-optimal. This likely have provided a source of variation on which both natural selection and genetic drift have acted [25, 100].

4. It has a mainly haploid life cycle and propagates mainly through mitosis or meiotic self-fertilizations, ensuring that deleterious alleles are frequently exposed to the forces of natural selection [52].

5. The practicality of *S. cerevisiae*: It is easy to cultivate, making large-scale phenotypic analyses feasible [99].

Natural *S. cerevisiae* strains that are geographically and habitually diverse have been investigated for genetic and phenotypic diversity [61, 98]. These studies show that the genetic variation among strains is clustered into five distinct populations (with some mosaic genomes in between). The different groups are characterized by monomorphism for the majority of segregating sites, strongly suggesting an evolutionary history with a largely independent propagation of populations [61].

The clustering into five distinct populations means that a few strains can be chosen that are representative of a large proportion of the genetic variation in the species. Four strains from different populations were used in this study. These will henceforth be referred to as European, North American, West African and Sake (although the names are somewhat contrived, since population boundaries do not strictly correspond to geographical regions). The fifth population is more or less reproductively isolated from the rest and so were not used. The four strains are separated by millions of generations of evolution, represent different geographical and habitual origins and >50% of the SNP and phenotypic diversity that has been identified outside of China [98].

5.2 Adaptive vs. non-adaptive processes

5.2.1 Adaptive and non-adaptive processes that shape populations

Evolutionary change can occur when Hardy-Weinberg equilibrium (HWE) is violated. HWE states that the genetic variation (allele and genotype frequencies) in a population will remain constant from one generation to the next in the absence of disturbing factors.

There are several factors that can disturb HWE in a population, e.g. mutations, non-random mating, natural selection, meiotic drive, genetic drift and gene flow. ***Mutations*** are the ultimate source of genetic variation. They disturb HWE by introducing new alleles into the population and they can harm or enhance the reproductive success of the organisms that carry them.

The tendency of individuals with a certain genotype to be more likely than individuals with another genotype to survive and reproduce is called ***natural selection***. This process might affect allele frequencies through either positive or negative (purifying) selection, which leads to an increase of certain alleles variants over time at the expense of other variants, or through balancing selection, which maintains polymorphisms in the population.

Key to understanding natural selection is the connection between fitness and phenotype. If the phenotype is genetically based (in contrast to physiologically acquired), there will also be an indirect connection between fitness and genotype. Fitness of an organism has two components on which natural selection acts: survival and fecundity (reproductive capacity). When a selective pressure such as metalloid pollution or nutrient restriction acts on a population, certain individuals might have a genotype that encodes a phenotype that leads to increased probability of survival and reproduction during the stress. These individuals will transmit their genotype to future generations to a larger extent than individuals that are less fit. This leads to an adaptation over time and persistent genetic changes that provide future generations with the possibility to better tolerate the stress condition.

Genetic drift is the random change in allele frequencies over time. In contrast to natural selection, which strives to optimize the phenotypic variant,

genetic drift can lead to fixation of variants that are sub-optimal with respect to fitness. This is a consequence of the sampling error when a finite population transmits a finite number of gametes to the next generation.

Without other processes that affect the gene pool, genetic drift will proceed until the involved alleles are either lost or are the only ones remaining at a locus. The random effects of genetic drift are larger in smaller populations (as sampling errors are larger). Hence, if the population is small, or if the selective pressure is weak, genetic drift may be able to overcome the effects of natural selection, possibly leading to complete loss of a genetic variant. Since *S. cerevisiae* has gone through several bottle-necks during its evolution, non-random sampling of alleles are likely to have played an important role in shaping present populations.

The transfer of genes into or out of populations is called ***gene flow*** and can happen through migration. In natural *S. cerevisiae* populations, it is mediated mainly through insect vectors. A recent source of gene flow in *S. cerevisiae* evolution has likely been the hitchhiking on human traffic [61].

5.2.2 Genetic correlations

As if the inherent difficulty in distinguishing between the evolutionary processes that influence allele frequencies in populations was not enough, certain genetic properties might make the task even more difficult. One such property is ***pleiotropy***, the property that a single gene influences multiple phenotypic traits. If a gene variant encodes a trait variant that confers an increased fitness (i.e. increased survival or fecundity), the selective pressure might be strong enough for natural selection to lead to fixation of the genetic variant. However, if the gene is pleiotropic, other traits might also be fixed due to "hitchhiking". These other traits might be neutral with respect to natural selection, but they have nevertheless been fixed as a result of natural selection.

A gene variant might also have antagonistic pleiotropic effects, meaning that the phenotypes that it influences have both positive and negative impact on fitness. For example, a genetic variant might confer improved survival but reduced fecundity, or improved fitness when a stress is present but reduced

fitness when it is absent.

Another source of genetic correlation is ***physical linkage*** between genes. A shorter chromosomal distance will lead to non-random assortment of alleles and thus to increased linkage disequilibrium. This will result in co-segregation of genes among gametes and statistical association between phenotype variants and gene variants that do not necessarily have any influence on these phenotypes.

5.2.3 Distinguishing between adaptive and non-adaptive evolution

Given that adaptive, in contrast to non-adaptive, changes act to optimize the fitness (survival and fecundity) of an organism, they should leave marks that can be used to distinguish them from non-adaptive changes. Under the assumptions that different parts of an organism's life cycle (e.g. the rate of reproduction or time before reaching reproductive age) are open to optimizations and that these parts occur in the same environmental context (so that the organism does not maturate in one environment and reproduce in another), we can expect natural selection to optimize performance during more than one life stage in response to an environmental challenge. This would be reflected by a correlation of performance during different life stages. Since they influence survival and fecundity and hence can be acted on by natural selection, these life stages are sometimes called "*fitness components*".

In contrast, non-adaptive evolutionary forces like genetic drift or gene flow influence the allele content of a population independently of fitness. Thus, no correlation between performances in different parts of the life cycle would be expected. Hence, the following condition appears to be central in distinguishing adaptive from non-adaptive evolutionary change:

- In a given environmental context, a phenotypic optimization of performance in one fitness component is correlated with a phenotypic optimization of performance in another fitness component.

This would seem to apply to adaptive processes acting in a given environmental context, but not to non-adaptive. Hence, this criterion could be used to distinguish between adaptive and non-adaptive processes. However, as

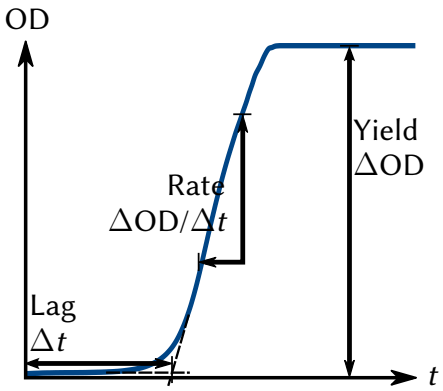

Figure 6: For each measurement, three fitness components were extracted: Time of acclimatization (lag), growth rate and maximum efficiency (yield).

the preceding discussion shows, from this we cannot exclude confounding genetic correlation like pleiotropy and physical linkage between genes. These genetic correlations can lead to correlations between performance in different fitness components independently of natural selection. We therefore need the following additional criterion:

- The genetic loci that influence the performance in the first fitness component must be distinct from the genetic loci that influence the performance in the second fitness component.

If these two criteria are fulfilled, this is strong support for the occurrence of natural selection. Genetic drift might be expected to produce correlations between fitness components if the underlying genetic determinants are pleiotropic or physically linked, but if they are independent, a stochastic process like genetic drift is unlikely to lead to strong correlations between fitness components.

How can fitness be evaluated? As was mentioned previously, the natural life cycle in *S. cerevisiae* is mainly asexual, so natural selection should act primarily on the asexual life cycle. Hence, survival and fecundity in *S. cerevisiae* can be translated into three properties of its growth: (1) The time of acclimatization before entering the reproductive phase (lag), (2) the maximum net growth rate during reproduction (rate), and (3) the maximum population density (yield). Shorter lag time, higher growth rate and higher yield all

contribute to performance in distinct parts of the life cycle. Thus, the three variables constitute three different fitness components. Their relation to the *S. cerevisiae* growth cycle is illustrated in figure 6.

5.2.4 Choice of environmental stress

When we set out to investigate what evolutionary forces that have shaped *S. cerevisiae* populations, our motivation was to test the proposed principle on metal traits. This project is still underway, but in order to establish and test the principle, we wanted to use a set of phenotypes on which we were reasonably sure that strong selection had been acting.

We reasoned that the driving forces during evolutionary differentiation might best be studied by investigating the optimized usage of a limiting essential nutrient. While carbon is commonly available in excess from sugar, nitrogen is obtained from a wide variety of sources, often in limiting amounts. Hence, there is likely to have been a strong selective pressure for utilization of different nitrogen sources, and since nitrogen is necessary for all parts of the life cycle, it is expected to have optimized performance in all three fitness components.

The natural habitats of *S. cerevisiae* – decaying fruits, plant leaves, intestinal tracts et.c. – have one or a few nitrogen sources that dominate. In total, 28 different nitrogen-containing compounds are known to be utilized by *S. cerevisiae*. We evaluated the growth performance of the four natural strains (European, North American, West African, Sake) in the presence of each of these 28 nitrogen sources and extracted values for lag, growth rate and yield according to figure 6.

5.3 Fitness components show concerted optimization

The model for distinguishing between adaptive and non-adaptive evolutionary differentiations is based on the recognition of independent optimization of different parts of the life cycle. I therefore evaluated the correlation between

performance in the three fitness components during nitrogen restriction and found extensive covariation (see also figure 2A in paper IV):

	Generation time	Lag time
Yield	r = -0.85	r = -0.61
Lag time	r = 0.71	

The Pearson correlation coefficient of r = -0.85 between yield and generation time shows that as the efficiency of nitrogen utilization increases, the generation time during the reproductive phase also becomes shorter. We also see a positive correlation between increased efficiency and shorter lag time. Likewise we see a positive correlation between increased generation time and increased lag time and therefore between decreased generation time and decreased lag time. In other words, there is concerted optimization in performance between all three fitness components. Hence, condition number one is fulfilled.

Fitness components show concerted optimization.

More than 98% of the genomes are identical between the four strains, so it is likely that the majority of the correlation between fitness components has arisen in the common ancestor of the strains and is due to genetic variants that are shared between all four strains. Figure S2 in paper IV shows the large phenotypic covariation between the strains, indicating ancestral selection. When the four strains are considered independently, the average correlation between the three components is $|r|$ = 0.30, substantially lower than when considering all strains together, indicating that the majority of correlation indeed is ancestral.

5.3.1 A thermodynamic consideration: Is correlation even possible?

Cellular growth is dependent on energy metabolism, the most important process of which is degradation of ATP to ADP. This process is coupled to thermodynamically unfavorable reactions during different parts of the life cycle, such as DNA replication, protein synthesis and cytokinesis. In yeast, ATP is produced by alternative pathways with opposing properties in yield (ATP produced per units of resource) and rate (ATP produced per units of time): respiration only vs respiration together with fermentation. In its

natural aerobic environment, *S. cerevisiae* has evolved to rely primarily on fermentation, which is usually interpreted as being competitively advantageous with a higher rate of production of ATP (or alternatively, as a way to kill competitors with ethanol).

At external glucose levels below 0.8 mM, *S. cerevisiae* makes maximal use of the substrates by inhibition of ethanol production and instead completely respires pyruvate to CO_2, using O_2 as electron acceptor [77]. This is a consequence of a thermodynamic constraint, where the free energy difference between carbon substrate and product is used to (1) phosphorylate ADP to ATP, and (2) drive the pathway. Shifting the usage of energy between these processes leads to a trade-off between yield and rate: the more ATP that is produced from a limited resource, the slower is the pathway [72].

Extrapolating this to growth means that the rate of biomass accumulation (which is linked to population size increase) is inversely correlated to ATP yield. And since ATP is limiting of growth yield, growth rate is inversely correlated to maximum population density. In other words, there is an evolutionary constraint due to a trade-off between between different fitness components.

All of the above applies when energy is limiting. What happens when nitrogen is limiting? One might believe that there should exist a similar trade-off, so that there is a selective advantage of increased growth rate with consequent decrease in yield (or vice versa). But nitrogen restriction is different from energy restriction in this respect. Here, carbon is available in excess so no diauxic shift from respirofermentative growth to solely respiration occurs. The correlation that we observe between rate and yield is therefore fully consistent with thermodynamics, but is distinct from what has been observed during carbon restriction [72, 89].

Moreover, the ATP produced does not limit growth yield, since energy is available in excess. Hence, we can have a fast flow through the pathway, fast biomass accumulation, and accept the consequence of a lower yield of ATP since it does not translate into a lower yield of biomass.

We observed that population density continued to increase after external nitrogen had been consumed (paper IV, figure 1F), indicating an internal storage of nitrogen. This privatization creates a selective advantage when external

nitrogen has been consumed and enables selection for maximum utilization of nitrogen resources. We therefore propose that improved internalization and storage capacity of nitrogen is key to evolutionary optimization of population efficiency, allowing for population expansion beyond the point where external resources are depleted.

5.4 Resolving the genetic components underlying the phenotypic variation

5.4.1 The theory of linkage analysis

We set up two criteria that must be fulfilled for ascribing adaptive mechanisms to the evolution of a trait: (1) Correlation of performance in fitness components and (2) independence between the genetic determinants of that correlation. We have seen that the first criterion is fulfilled with respect to the 28 nitrogen sources. What about the second criterion?

As mentioned earlier, the majority of correlation is expected to derive from the shared common ancestor. This ancestral correlation is difficult to resolve at a genetic level, but the population-specific correlation between components can be mapped by analyzing the linkage between genetic markers and fitness variations in crosses between parents with different phenotypes (*linkage analysis*).

A potential problem with this method is that we have strongest evidence for coevolution of fitness components on the variation that derives from the ancestral population, whereas we are only able to perform linkage analysis, and hence to show non-pleiotropy, for the more recent population-specific differences. We must therefore assume that the amount of pleiotropy is the same for ancestral and non-ancestral genetic variants. It is hard to imagine why this would not be the case, but it nevertheless presents a possible weakness with the method and should be kept in mind.

The basic idea behind linkage analysis is that trait variation is coinherited together with genetic markers that have known locations. By analyzing a lot of offspring that have or do not have the trait of interest, we can identify the genetic markers that are statistically linked with the occurrence of the trait.

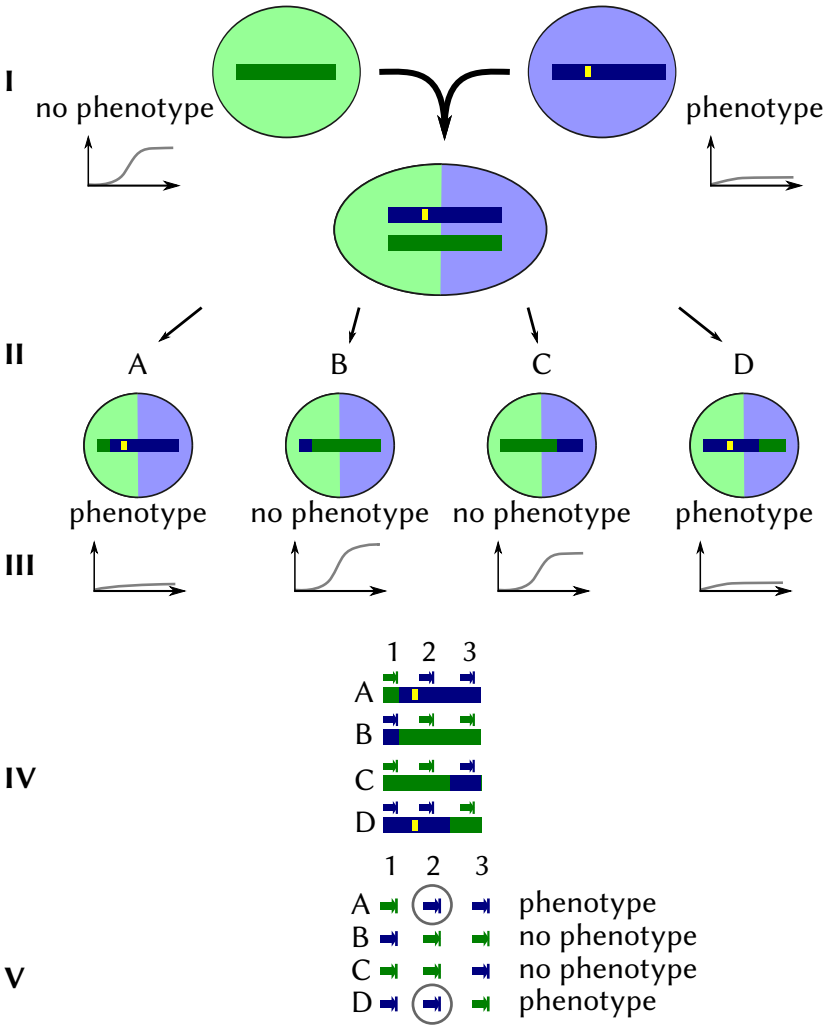

Figure 7: Workflow of linkage analysis. (**I**) Diploid hybrids were created by making pairwise crosses between two genetically well-characterized parentals. This was done for all pairwise combinations of the four strains, making six crosses in total. In the figure, one of the parentals have a mutation (yellow) that gives rise to a growth defect. (**II**) Hybrids were induced to go through homologous recombination and meiosis, generating chimeric spores, half of which will carry the causative mutation. (**III**) The F_1 segregants were phenotyped in the 28 different environments and fitness components (lag, growth rate, yield) were extracted. (**IV**) Genotyping at polymorphic sites was performed with parental-specific PCR primers, allowing for determination of haplotypes at approximately 160 sites for each segregant. (**V**) By combining phenotype and genotype data, linkage between trait variation and markers can be mapped. In the example, offsprings A and D have marker 2 in common, indicating a linkage disequilibrium between marker 2 and the causative mutation.

The result is a genetic region (quantitative trait locus, QTL) that contains a mutation that influences the trait. The complete process behind linkage analysis in yeast is outlined in figure 7.

To perform linkage analysis, two parental strains with differing phenotypes are sequenced and crossed, generating hybrid diploids (I). These hybrids are then sporulated to create four haploid offspring (II). During sporulation of the hybrids, chromosomes are broken up through homologous recombination. The offspring will therefore have chimeric chromosomes. Since the parents are phenotypically different, the offsprings will have different performances (III). Given that most traits are determined by multiple genes, there will be a more or less continuous distribution of phenotypes among the offsprings.

The offsprings are then genotyped at sites where the parental strains are known to be different (IV). This way, we can determine from which parent each region has been inherited. Finally, we can match the phenotypic variation with the genotypic variation to determine which markers are inherited together with the phenotype. This will give us a number of QTLs that are physically or genetically linked to the trait that is investigated (V).

5.4.2 Linkage analysis on natural strains

Pairwise crosses were performed between the four strains (European, West African, North American and Sake), in total six crosses. 24 hybrids were generated for each combination and each hybrid was sporulated to 96 offsprings, giving 576 offsprings in total.

I used the `R/qtl` package to perform linkage analysis [9]. Recombination frequencies between markers were translated to a genetic map using the Kosambi mapping function and non-parametric interval mapping was performed with 2 cM interpolation [57]. *S. cerevisiae* spores are inherited four-and-four in tetrads and this interdependency complicates a direct calculation of significance levels. I therefore performed 20,000 random simulations of recombination frequencies within each tetrad and used this to filter the observed QTLs on 5% FDR. This left 230 stable QTLs, of which a maximum of 5% should be false positives.

Having identified the underlying QTLs, we need a criterion to assign pleiotropy by determining when two QTLs are identical. Interval mapping estimates the location of a QTL q between two markers **A** and **B** as a function of the recombination frequency between q and **A** and q and **B**, assuming that the phenotype co-segregates with q. In order to estimate whether two QTLs q_1 and q_2 were identical, I used the average distance between markers in each cross as the smallest allowed distance between two QTLs. Two QTLs that were identified at a distance smaller than this were considered to be identical and thus represent a pleiotropic QTL with regard to either fitness components or environments (or both).

5.4.3 QTLs are specific to fitness component, environment and genetic context

Using this definition of overlapping QTLs, three observations were made. First, as figure 2B in paper IV shows, most of the identified QTLs (87%) are ***unique to one fitness component*** (lag time, growth rate or yield). For example, the LOD plots for citrulline, leucine and isoleucine are shown in figures 2C and S3B and show that significant QTLs are found on different chromosomes for the different fitness components. This is reason to reject the hypothesis of pleiotropy or physical linkage as an important explanation of the phenotypic correlation between the fitness components. Hence, the second criterion of the test is fulfilled.

Most QTLs are unique to one fitness component.

Second, with the present cutoff levels, >50% of QTLs are ***unique to a single nitrogen source*** and <20% affect fitness in more than 3 environments (figure 2D). This indicates that adaptive selection has mainly acted independently in nitrogen restricted habitats with different selective pressures, leading to specialized metabolic pathways. Still, the level of pleiotropy identified is more than expected by chance according to the randomization tests, mainly due to the existence of a few highly pleiotropic QTLs. For example, a QTL on chromosome VI determines the efficiency of utilization of almost all nitrogen sources.

Third, as exemplified by the QTL on chromosome VI which penetrates only in crosses where the European strain is involved, the genetic ***background is highly relevant*** to the penetrance of QTLs. Each parental strain participates

in three crosses, making six crosses in total. However, 60% of QTLs are detected only in one particular cross (figure S4B). This indicates the existence of extensive population-specific epistatic interactions between genes and makes the case for adaptive differentiation stronger.

5.5 Assessment of the test

Different mechanisms underlie changes in genotype and phenotype frequencies in a population. The heritable adaptive process of natural selection increases the potential of an organism to survive and reproduce, whereas non-adaptive processes, like genetic drift or gene flow, occurs without consideration of improvements in fitness. Adaptive and non-adaptive processes interact in ways that are hard to predict and it is often not obvious what is most influential. Yet, distinguishing between the processes that have shaped organisms is an essential part in understanding why they behave the way they do.

Whereas adaptive and non-adaptive differentiation both induce changes in the gene pool over time, they should leave different marks on the genome. If the total fitness of an organism depends on distinct components that can be optimized, such as a shorter time before the reproductive phase begins, an increased rate of reproduction or improved utilization of resources, natural selection might be expected to improve these in concert to improve the total fitness in an environment. If different components are controlled by the same genes, stochastic non-adaptive processes might change them concertedly, but if they are controlled by different genetic loci, non-adaptive processes are unlikely to lead to concerted performance improvements in multiple fitness components.

Based on these assumptions, the following two criteria might allow us to distinguish between adaptive and non-adaptive processes during evolution by an abductive reasoning: If (1) *in a given environmental context, a phenotypic optimization of performance in one fitness component is correlated with a phenotypic optimization of performance in another fitness component*, and if (2) *the genetic loci that influence the performance in the first fitness component is distinct from the genetic loci that influence the performance in the second fitness component*, then an inference to the best explanation states that adaptive

differentiation is responsible for the performance improvements.

In other words, this test requires elucidating both the amount of phenotypic correlation between fitness components and the genetic basis for the correlation. This was done by measuring performance in three different fitness components of *S. cerevisiae* in environments that are likely to have exerted a selective pressure, and then perform linkage analysis on crosses between natural strains to map QTLs that underlie the genetic basis for the correlation.

Fitness measurements revealed a clear correlation between all three components, and linkage analysis showed that fitness components tend to be controlled by different QTLs. Hence, natural selection appear to have been an important contributor to genotypic and phenotypic variation in response to nitrogen restriction during *S. cerevisiae* evolution.

In conclusion, it is likely that natural selection has had a strong influence on multiple parts of the life cycle of *S. cerevisiae* to make it more efficient in utilizing nitrogen. This test could probably be used to distinguish between adaptive vs. non-adaptive contributions during other environmental challenges, such as metal and metalloid stress. Although the test appears to work in yeast, caution is necessary if it is to be applied to other organisms, since it requires that life stages can be clearly distinguished, that they contribute fitness and that the genetic basis for phenotypic variation can be mapped by some means.

6 Conclusions, implications and questions for the future

Metal and metalloid contamination, whether it is natural or human-induced, can have detrimental effects on living organisms. This thesis has aimed to shed some light on why the yeast *Saccharomyces cerevisiae* behaves the way it does in response to metalloid stress.

The increasing usage of tellurium in electronic applications and subsequent accumulation of tellurium compounds in proximity of landfills and industrial facilities call for a better understanding of the biological effects of tellurium. However, the mechanisms behind tellurite toxicity has been an enigma since it was first discovered. Chapter 3 mapped the cellular processes that mediate toxicity in yeast. In contrast to bacteria, tellurite toxicity in yeast is associated with tellurium accumulation and is mediated by a functional sulfate assimilation pathway, perhaps due to bioassimilation of tellurium and production of reactive or labile tellurium intermediates (e.g. H_2Te). Yeast is particularly sensitive during mitochondrial respiration, indicating that tellurite targets mitochondrial function.

The genome-wide screen on tellurite stress revealed an enrichment of several functions that are involved in toxicity. The details have not been mapped out, so current discussions on toxicity mechanisms will with necessity remain speculative. Future studies should therefore investigate some of the revealed functions in closer detail. One question that deserves a more thorough investigation is whether tellurite is bioassimilated and if it is incorporated into proteins. The lack of a strong signal when components in redox regulation are deleted was surprising, given the potential formation of reactive tellurol compounds. It is tempting to speculate that tellurium might act as an electron acceptor during mitochondrial respiration but this remains to be shown.

Chapter 4 investigated the role of arsenite in misfolding and aggregation of newly synthesized proteins. We saw that arsenite may directly inhibit chaperone activity, making proteins with a high requirement of chaperones more aggregation-prone. These proteins are normally highly translated and abundant, and the cell might respond by diminishing the expression of these proteins to avoid overwhelming the PQC systems. We also saw

that substoichiometric amounts of arsenite may influence protein activity in the cell by forming aggregation seeds or disturbing the protein interaction network.

Protein aggregation in humans is linked to aging as well as several neurodegenerative disorders. As described in paper III, the aggregation-prone proteins that were identified in this study are to a large extent homologous to aggregation-prone proteins in several human diseases. This suggests that the basic mechanisms that govern protein aggregation in yeast might be relevant also during human disease processes. Given the extensive conservation of the PQC systems among eukaryotes, it is possible that diverse species employ common mechanisms to cope with arsenite-induced protein aggregates. The role of the different arms of the PQC network should therefore be investigated in more detail. Another question that remains is how representative arsenite is in inducing aggregation. Do other stresses affect the same proteins? An upcoming paper will investigate this issue.

The results presented here show that arsenite-induced formation of protein aggregates is linked to toxicity, but the cause of toxicity is not yet known. Aggregates might serve a protective role in inactivating reactive misfolded proteins, or they might negatively affect important cellular functions.

The fact that a cellular function exists does not mean that it is beneficial for the organism, but distinguishing between the processes that have shaped living organisms is no easy task. Chapter 5 investigated the possibility of giving an evolutionary explanation to why cells behave the way they do. We saw that independent optimization of fitness components is a signal that adaptive evolutionary processes have acted to enhance performance of a trait. The method should be possible to extend also to other stresses such as metals and metalloids. An upcoming paper will investigate the role of adaptive processes and pleiotropy during yeast adaptation to metals and metalloids. Caution should be taken however when applying this method to other organisms, since it assumes that life cycles can be clearly distinguished, that they occur in the same environment and that their genetic basis can be determined.

My PhD project has focused on delineating the general processes involved in toxicity of tellurite and arsenite and to better understand how processes that are affected by shifting environments change over time. Considering the

increasing challenges of multidrug-resistant microbes, a better understanding of cellular and evolutionary adaptations to metalloids is desirable. If any take-home message is to be given, perhaps it is this: Biological organisms are not left helpless. The plasticity of the PQC systems and of different pathways to handle metal and oxidative stress stem from the potential to acquire novel hereditary adaptations. Earth is the only known place in the universe that supports life, but even on earth, life has met many challenges. The potential to adapt has allowed life to endure significant challenges during its history and fine-tuned it for the world that we live in today. Life is a truly remarkable invention!

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