ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

EVOLUTIONARY ENGINEERING AND MOLECULAR CHARACTERIZATION OF STRESS-RESISTANT *Saccharomyces cerevisiae* MUTANTS

Ph.D. THESIS

Ceren ALKIM

Department of Advanced Technologies

Molecular Biology-Genetics and Biotechnology Programme

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

STRESE DİRENÇLİ *Saccharomyces cerevisiae* MUTANTLARININ EVRİMSEL MÜHENDİSLİĞİ VE MOLEKÜLER KARAKTERİZASYONU

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TEMMUZ 2012

Ceren Alkım, **a Ph.D.** student of ITU **Graduate School of Science, Engineering and Technology** student ID **521062204**, successfully defended the **thesis** entitled **"Evolutionary Engineering and Molecular Characterization of Stress-Resistant *Saccharomyces cerevisiae* Mutants**", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

Thesis Advisor :	**Prof. Dr. Zeynep Petek ÇAKAR** Istanbul Technical University	.....
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Date of Submission : 06 July 2012
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To my family,

FOREWORD

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July 2012

Ceren ALKIM
(Molecular Biologist)

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ABBREVIATIONS

AVG : Average
bp : Base Pair
BPS : Bathophenantroline Disulphonate
cDNA : Complementary Deoxyribo Nucleic Acid
CDW : Cell Dry Weight
$CoCl_2$: Cobalt Chloride
C_T : Cycle Threshold
DNA : Deoxyribo Nucleic Acid
ds : Double Strand
EMS : Ethyl Methane Sulfonate
et al. : And Others
etc. : *et cetera*
Fe/S : Iron-sulfur
h : Hour
H_2O_2 : Hydrogen Peroxide
HPLC : High Pressure Liquide Chromatography
HSPs : Heat-Shock Proteins
ISC : Iron Sulfur Cluster
KAc : Potassium Acetate
MAT : Mating Type
µg : Microgram
µL : Microliter
µm : Micrometer
mM : Micromolar
mg : Milligram
mL : Milliliter
min : Minute
MPN : Most Probable Number
NAD : Nicotinamide Adenine Dinucleotide
ng : Nanogram
NRAMP : Natural Resistance-Associated Macrophage Protein
OD : Optical Density
ORF : Open Reading Frame
PCR : Polymerase Chain Reaction
RNA : Ribonucleic Acid
RPM : Revolution per minute
RT-PCR : Real-Time Polymerase Chain Reaction
SD : Synthetic Defined
wt : Wild Type
YMM : Yeast Minimal Medium
YPD : Yeast Extract- Peptone – Dextrose

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EVOLUTIONARY ENGINEERING AND MOLECULAR CHARACTERIZATION OF STRESS-RESISTANT *Saccharomyces cerevisiae* MUTANTS

SUMMARY

Saccharomyces cerevisiae is one the best model organisms widely used for genetics, molecular biology and metabolic studies. Besides its use in scientific area, it is one of the oldest microorganisms used for ages for industrial applications like brewing and baking. *S.cerevisiae* is a unicellular eukaryotic organism, which can be found in haploid and diploid state, and hence can reproduce asexually by budding or can induce meiosis to generate new progeny of haploids from diploids (so called sporulation event). It shares high degree of conservation in basic function with higher eukaryotes like human. Owing to these functional similarities, *S. cerevisiae* can be used in research related to cancer, aging and other human diseases.

In nature and/or industrial applications, microorganisms are exposed to a variety of stress types like osmotic, temperature, dehydration, starvation, metal ions *etc*. Scientists are interested in the basis of how microorganisms cope with all these types of stresses. Additionally, they are searching for the answers how stress tolerance could be increased. Producers are keen on the increase in yield and for this reason; they are searching for stress-tolerant microorganisms.

In this thesis study, firstly, cobalt-resistant *S.cerevisiae* mutants were obtained by evolutionary engineering. Phenotypic, physiological and genetic characterization was then carried out to identify the molecular basis of cobalt resistance in *S.cerevisiae*.

Evolutionary engineering was applied to wild type *S. cerevisiae* cells. They were treated with a chemical mutagen EMS (Ethyl Methane Sulfonate) in order to increase genetic diversity in the initial population to which evolutionary engineering has been applied. This mutagenized culture was cultivated at varying cobalt concentrations in the culture medium along with the wild type to determine the initial stress level to be applied. Cobalt stress was then applied to this mutagenized culture. The cobalt concentration was increased gradually for each successive population, from the first population that treated with 0.5 mM $CoCl_2$ up to the 25^{th} population that received 8 mM $CoCl_2$. The final population was used for selecting eight individual mutants. Eight individual mutants, wild type and the final population were tested for cobalt resistance. It was found that the evolved strain and the final population could grow at high cobalt concentrations at which the wild type could not show any sign of survival. One of the most cobalt-resistant individual mutants was chosen and genetic stability assay was applied. It was shown that the cobalt-resistance phenotype was a genetically stable trait in the mutant tested.

This evolved strain was termed CI25E. CI25E was crossed to the opposite mating type wild type in order to investigate the genotypic properties of this mutant. Classical genetic tests showed that the spores' progeny from a cross between CI25E and the original wild type displayed a complex resistant phenotype suggesting that the phenotypic trait was dependent on several genes. It was also found that diploid obtained from the cross of CI25E and the original wild type showed semi-dominance for cobalt stress. Phenotypic characterization revealed that CI25E exhibited cross-resistance to Fe^{+2}, Mn^{+2}, Zn^{+2} and Ni^{+2} ions and it was highly sensitive to Cu^{+2}, H_2O_2, caffeine and ethanol. Nevertheless, it was found that *COT1* was 2-3 fold upregulated in the CI25E and that deletion of this gene reduced but did not abolish resistance to cobalt.

In order to clarify the cobalt resistance mechanisms of the evolved strain whole genome transcriptomic analyses were conducted to wild type, CI25E and CI25E that was deleted for *COT1*. Sampling for microarray analysis was performed when the cultures were in their exponential phase of growth. The expression profiles of mutants were compared to that of the wild type. The results showed that a large proportion of the genes induced in this evolved strain was involved in iron homeostasis. This leads to upregulation of more than 25 genes that can be classified in activation of systems of iron uptake at the cell surface, mobilization of intracellular stores of iron, and metabolic adaptation to iron limitation.

In the light of these results, *AFT1* was deleted which encodes a transcriptional activator of the iron–regulated genes (iron regulon) in CI25E and it was found that the mutant had a diminished cobalt resistance, while it had lost the resistance to iron, manganese, zinc and nickel. Altogether, these results indicated that *AFT1* is an important gene in cobalt resistance, mediating this effect likely through increasing metabolic activity for iron uptake. Moreover, the localization of Aft1p both in wild type and CI25E was determined by fluorescence imaging. To this end, the two strains were transformed with a centromeric vector that expressed an Aft1 protein and this chimeric protein was visualized by immunofluorescence. It was shown that more than 80% of Aft1 protein was found to be present in the nucleus of the CI25E cells in the absence of cobalt, and addition of 2 mM cobalt only slightly increased this percentage of nuclear localization. This finding may explain the apparent constitutive upregulation of iron regulon in the cobalt-evolved strain.

In addition to this molecular event, upregulation of several genes encoding transmembrane transporters of amino acids, in particular those implicated in sulfur metabolism (cysteine, S-adenosylmethionine and sulfate) were also found. In addition, the transcript levels of several genes belonging to cell wall organization category were found to increase both in CI25E and CI25E *cot1*Δ.

Biological analysis of downregulated genes revealed three main classes of genes whose expression was significantly downregulated, namely, genes related to seripauperin multigene family members, NAD biosynthesis, and pheromone response genes. A strong downregulation of the seripauperin (*PAU*) genes was observed. Those genes are known to be repressed by oxygen or by high levels of heme, while they are induced under hypoxic state.

Transcriptomic profiles of CI25E and CI25E *cot1*Δ were compared to each other. It was found that only a few genes were found to have significant difference in expression.

Cobalt and iron accumulation ability was investigated for wild type, CI25E, and their *cot1*Δ and *aft1*Δ mutants. Those strains were incubated in the presence of cobalt, iron and both cobalt and iron. CI25E seemed to hold less iron than the wild type. This may actually explain the iron-starved character of the mutant. CI25E also held less cobalt compared to the wild type. High cobalt-resistance phenotype of CI25E might be originated from its efficiency of cobalt efflux mechanisms and/or the ability to reduce the uptake of cobalt.

In this thesis study, a cobalt hyper-resistant *S.cerevisiae* mutant was obtained and characterized in detail. The complexity of cobalt resistance genotype could not be completely clarified at the molecular levels but raised several hypotheses including a close connexion between cobalt and iron metabolism and identification of genes/ pathways that were not so far associated with cobalt resistance. Therefore, it should be relevant to continue molecular research to enlighten the mechanism of cobalt resistance and have a better understanding of metal homeostasis in both *S.cerevisiae* and other eucaryotes.

STRESE DİRENÇLİ *Saccharomyces cerevisiae* MUTANTLARININ EVRİMSEL MÜHENDİSLİĞİ VE MOLEKÜLER KARAKTERİZASYONU

ÖZET

Saccharomyces cerevisiae, moleküler biyoloji, genetik ve metabolik çalışmalarda sıklıkla kullanılan en iyi model organizmalardan biridir. Bilimsel alanlarda kullanımı dışında, *S.cerevisiae* endüstriyel uygulamalarda da –ekmek ve bira yapımı gibi- çok uzun süredir kullanılan en eski mikroorganizmalardandır. *S.cerevisiae* tek hücreli bir ökaryot organizma olup, haploid ve diploid formlarda bulunabilmekte; bu sayede de hem eşeysiz; tomurcuklanarak hem de eşeyli; mayoz bölünme sonucu diploid hücrelerden yeni haploid nesiller oluşturarak üreyebilmektedir (sporülasyon aşaması). İleri ökaryot organizmalarla göstermiş olduğu temel fonksiyonlar açısından benzerlik, insanlar için de geçerlidir. Bu gibi fonksiyonel benzerlikler sayesinde, kanser, yaşlanma ve birçok hastalık mekanizmaları *S.cerevisiae* hücreleri kullanılarak araştırılmaktadır.

Doğada veya endüstriyel uygulamalarda maya hücreleri, ozmotik, sıcaklık, susuzluk, açlık, metal stresi gibi pek çok farklı türde strese maruz kalırlar. Bilim adamları ise maya hücreleri ve diğer başka mikroorganizmaların bu kadar farklı stres çeşidiyle nasıl mücadele ettiklerini anlamaya çalışmaktadırlar. Bilim adamlarının ilgilendikleri bir diğer konu da stres direncinin nasıl artırılabileceğidir. Üreticiler ise verim artışı istedikleri için strese dirençli mikroorganizma arayışındadırlar.

Bu tez çalışmasında ilk olarak, evrimsel mühendislik yöntemi ile kobalta dirençli *S. cerevisiae* mutantları elde edildi. Ardından, kobalta dirençli *S.cerevisiae* mutantlarında, kobalt direncinin moleküler mekanizmasını anlamak amacıyla fenotip, genotip ve fizyolojik analizler gerçekleştirildi.

Evrimsel mühendislik yaban tip *S.cerevisiae* hücrelerine uygulandı. Bu amaçla ilk olarak başlangıç popülasyonunda genetik çeşitliliği arttırmak için kimyasal bir mutajen olan etil metan sülfonat (EMS) yaban tip maya hücrelerine uygulandı. Daha sonra, uygulanacak ilk stres değerini belirlemek için yaban tip ve EMS ile muamele edilmiş maya hücreleri farklı kobalt konsantrasyonu içeren besi yerinde üretildi. Ardından, EMS uygulanmış maya hücrelerine başlangıç kobalt stresi olarak 0.5 mM $CoCl_2$ kabul edildi. Bu değerde büyütülmüş maya kültürü birinci popülasyon olarak adlandırıldı ve evrimsel mühendislik kobalt stresini artan bir şekilde uygulayarak 25. popülasyon elde edilene kadar devam ettirildi. En son elde edilen popülasyon 8 mM kobalt stresine maruz bırakılmış olup yaban tipin dayanabileceği kobalt stresinin çok üzerinde olduğu belirtilmiştir. Daha sonra, son popülasyondan sekiz mutant birey seçildi. Sekiz mutant birey, yaban tip ve son popülasyonun kobalt direnci ölçüldü ve mutant bireylerle son popülasyonun yaban tipin en ufak bir yaşam belirtisi bile gösteremediği kadar yüksek kobalt konsantrasyon değerlerinde çok rahat üredikleri gözlemlendi. En dayanıklı mutant bireylerden biri seçildi ve genetik kararlılık testi uygulandı. Kobalt direncinin ilgili mutant bireyde genetik olarak kararlı olduğu gösterildi.

Evrimsel mühendislik ile elde edilen suş CI25E olarak adlandırıldı. Bu suş, farklı bir eşleşme tipine sahip yaban tip maya ile çaprazlandı ve diploid hücre elde edildi. Elde edilen bu diploid hücreden tetradlar mikromanipülator yardımı ile ayrıştırıldı. Diploid hücre ve tetradların sporları ile yapılan klasik genetik deneyler sonucunda kobalt stresinin mutant bireyde birden çok gene bağlı olduğu ortaya çıkarıldı. Aynı zamanda kobalt direncini kazandıran genetik faktör ya da faktörlerde yarı-baskınlık bulunduğu anlaşıldı. CI25E ve yaban tiple yapılan fenotipik analizler sonucunda ise CI25E'nin demir, nikel, manganez ve çinkoya da çapraz direnç sergilediği gözlemlendi. Ayrıca bu mutantın hidrojen peroksit, bakır, tuz ve etanole de hassasiyeti olduğu bulundu. Mutant bireyde kobalt direnciyle doğrudan ilgisi olduğu bilinen *COT1* geninin anlatım düzeyine bakıldı. Ortamda kobalt olmaması durumunda bu genin anlatımının arttığı gözlemlenmiştir. Ayrıca CI25E'de *COT1* geninin silinmesi yüksek düzeyde uygulanan kobalt stresi için CI25E'de hassasiyet yarattı.

Mutant bireyde kobalt direnç mekanizmasının aydınlatılması amacıyla transkriptomik analiz CI25E, CI25E *cot1*Δ ve yaban tip için gerçekleştirildi. Bu amaçla, yaban tip, CI25E, CI25E *cot1*Δ minimal besi yerinde ve stres olmayan ortamda logaritmik evrelerine kadar üretildi. Transkriptomik analiz için örnekleme bu aşamada yapıldı. CI25E ve CI25E *cot1*Δ mutantlarının artan veya azalan anlatım durumları yaban tipe göre karşılaştırıldı.

Yapılan bu çalışma ardından, CI25E ve CI25E *cot1*Δ mutantlarında yaban tipe göre anlatımın en çok artış gösterdiği gen grubunun demir homeostasisi ile ilgili olduğu görüldü. Anlatımı artan bu 25 gen, hücre yüzeyinden demir alımı, hücre içi demir kaynaklarının mobilizasyonu ve demir yokluğuna adaptasyon gibi sistemlerle ilgili genlerdir.

Bu sonuçlar ışığında, bir transkripsiyon aktivatörü kodlatan, *AFT1* geni mutantta silindi ve kobalt direncinin oldukça azaldığı ve aynı zamanda da demir, manganez, nikel ve çinkoya olan çapraz direncin de kaybedildiği görüldü. Tüm bu sonuçlar *AFT1*'in kodladığı Aft1 proteininin kobalt direnç mekanizmasında önemli olduğunu göstermektedir. Aft1'in bu etkisini de demir alımıyla ilgili metabolik faaliyetleri arttırarak sağladığı düşünülmektedir. Aft1 proteininin CI25E'de kobalt direnç etkisini araştırmak amacıyla bu mutantta ve yaban tipte Aft1 proteininin lokalizasyonu kobalt varlığında ve yokluğunda incelendi. Bu amaçla, yaban tip ve CI25E, Aft1 proteinini kodlatan sentromerik bir vektörle transforme edildi ve immünofloresans mikroskopi yöntemiyle şimerik proteinin lokalizasyonu belirlendi. İlginç olarak CI25E mutantında ortamda kobalt olmaksızın Aft1 proteinlerinin ~%80 kadarının hücre çekirdeğinde lokalize olduğu ve bu durumun 2 mM kobalt eklenmesiyle çok değişmediği gösterildi. Bu sonuç, CI25E'de *AFT1* regülonunun sürekli aktif olmasını da açıklar niteliktedir.

CI25E ve CI25E *cot1*Δ suşlarında demir homeostazisi dışında anlatımı artan farklı gen grupları arasında özellikle kükürt metabolizmasında (sistein, S-adenozilmetiyonin ve sülfat) rol alan amino asitlerin transmembran taşıyıcı proteinleri ve hücre duvarı organizasyonunundan sorumlu proteinleri kodlayan genler yer almaktadır.

Anlatımı mutant suşlarda azalan gen grupları üç ana grup dikkat çekmektedir: Bunlar *seripauperin* multigen ailesindeki genler, NAD biyosentezinden sorumlu genler ve feromon cevap mekanizmasıyla ilgili genlerdir. Seripauperin (*PAU*) genlerinde anlatım düzeylerinin özellikle mutantlarda önemli ölçüde azaldığı gözlemlendi. Bu

genlerin anlatımının oksijen veya yüksek düzeyde heme nedeniyle azaldığı daha önceki çalışmalarda gösterilmiştir. Anlatımın arttığı durumlar ise hipoksik şartlar olarak nitelenmiştir.

CI25E ve CI25E *cot1*Δ mutantları da kendi aralarında gen anlatım düzeyleri açısından karşılaştırıldı ancak anlatım düzeyi değişen gen sayısı ve aynı zamanda anlatım düzeyleri açısından anlamlı sonuçlar elde edilemedi.

Yaban tip, CI25E ve CI25E *cot1*Δ mutantları için kobalt, demir ve kobalt/demiri tutma durumları bu suşların *cot1*Δ ve *aft1*Δ mutantları ile birlikte değerlendirildi. Test edilen her koşul için CI25E'nin demiri yaban tipe göre hücre üzerinde veya hücre içerisinde çok daha az tuttuğu gösterildi. Bu sonuç neden CI25E'nin sürekli demir alma eğiliminde olduğunu açıklar niteliktedir. Ayrıca, hücre içi kobaltın yaban tipe kıyasla daha az olduğu da gösterildi. Bu nedenle mutanttaki yüksek kobalt direnci, kobalt iyonlarını hücre içerisinden etkin bir şekilde atmaya veya hücre içerisine az kobalt almaya bağlanabilir.

Sonuç olarak, bu çalışmada kobalta dirençli *S.cerevisiae* mutantları elde edilerek bu mutantların kobalt direncinin moleküler mekanizması aydınlatılmaya çalışıldı. Kobalt direnç mekanizmasının dayandığı anahtar noktalar bulundu, ancak, kobalt direncinin genetik karmaşıklığı nedeniyle dirençle ilgili başlıca gen isimlerini belirlemek henüz mümkün olmadı. Bununla birlikte, daha önceki çalışmalarda kobalt direnci ile ilişkilendirilmemiş olan gen grupları bu çalışmada belirlenmiş oldu. Mayada ve diğer ökaryot canlılarda, metal homeostasisini daha iyi anlamak için moleküler karakterizasyon çalışmalarına devam etmek ve kobalt direnç mekanizmasını aydınlatmak, kobalt ile ilgili çeşitli uygulamalar açısından önemli katkılar sağlayabilecektir.

1. INTRODUCTION

1.1 Metals: Transition Metals and Their Functions

Metals are important to biological functions of living systems. They serve catalytic and structural functions, fulfill a variety of physiological processes, including many important and/or central life processes. The most abundant metals inside the cells are sodium (Na^{+}), potassium (K^{+}), calcium (Ca^{+2}) and magnesium (Mg^{+2}) with their role from nervous systems to muscle movements. Moreover, proteins that contain metal ions constitute one fourth of proteins in Protein Data Bank 'PDB' (Argüello *et al.*, 2012; Bleackley and MacGillivray, 2011 and Shi *et al.*, 2005). However, transition metals contribute to the structure of the proteins more than the alkali and alkali earth metal ions mentioned above. Their unique redox and coordination chemistry might be the reason for their protein abundance (Bleackley *et al.*, 2011).

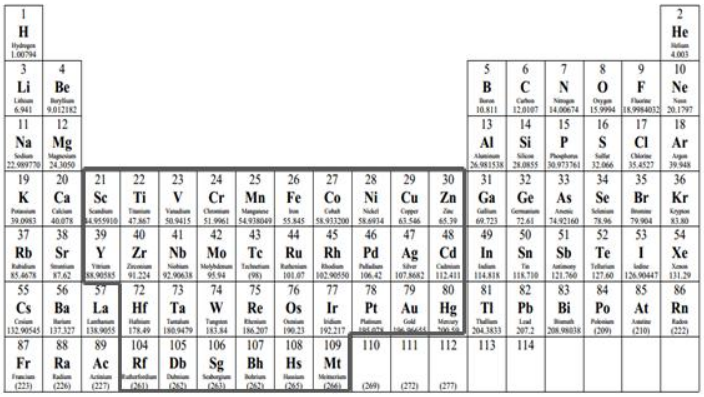


Figure 1.1 : Transition metals on the Periodic Table shown inside frame (Petrucci *et al.*, 2002).

Iron (Fe), copper (Cu), manganese (Mn), zinc (Zn), cobalt (Co), molybdenum (Mo), tungsten (W), chromium (Cr), vanadium (V), and nickel (Ni) are some of the transition metals which are needed for protein activity (Figure 1.1). All organisms are thought to require iron and zinc; but copper, cobalt, manganese, nickel, and molybdenum are only used by some prokaryotes and eukaryotes (Galaris and Pantopoulos, 2008 and Frassinetti *et al.*, 2006).

1.2 Cobalt in the Environment

Cobalt is a metal with an atomic number of 27 and its molecular weight is 58.9. Cobalt belongs to transition metals and is located between iron and nickel on the periodic table. It can be found in a wide range of oxidation states; 0, +2, +3 and +4. Abundance of cobalt in earth's crust has an average concentration of 25 mg/kg. Cobalt is generally found in nature in association with nickel and arsenic. However, it is refined through the melting process of nickel, copper, and lead ores containing arsenic (Kazantzis, 1981 and De Boeck *et al.*, 2003).

1.3 Cobalt in the Organisms

1.3.1 In vitamin B_{12}-containing proteins

The identification of coenzyme B_{12} in the early 1960s revealed the metal-carbon bond importance in enzymatic reactions for the first time. B_{12} is composed of a corrin skeleton, which compromised a cobalt ion following the deprotonation procedure (Lenhert and Hodgkin, 1961).

Metabolism of microorganisms needs the function of B_{12}-cofactors. In prokaryotes, B_{12} is associated with three classes of enzymes; adenosylcobalamin-dependent isomerase, methylcobalamin-dependent methyltransferase, and B_{12}-dependent reductive dehalogenase. Among B_{12}-utilizing organisms, prokaryotes are able to synthesize B_{12} *de novo* both in aerobic (*Pseudomonas denitrificans etc.*) and anaerobic (*Salmonella typhimurium*, *Bacillus megaterium* and *Propionibacterium shermanii etc.*) manner. Although microorganisms are the only natural sources for B_{12} derivatives, other organisms such as archea and human need B_{12} coenzymes for their metabolic activities (Krautler, 2009). These organisms which could not synthesize B_{12}, must take it up from their environment. Methionine synthase (MetH), methylmalonyl-CoA mutase (MCM) and ribonucleotide reductase (RNR II) are the B_{12}-dependent enzymes found in eukaryotes. Among these enzymes, MCM is essential to maintain human life (Banerjee and Ragsdale, 2003; Scott and Roessner, 2002 and Krautler, 2009).

1.3.2 Cobalt in noncorrin proteins

Besides B_{12}-dependent enzymes, there are other proteins which contain cobalt solely. One of these proteins is methionine aminopeptidase (MAP) which removes the N-terminal methionine residue from the nascent polypeptides. It is found in a wide range of organism from bacteria (*Escherichia coli*, *Pyrococcus furiosus*, *Methanococcus jannaschi* etc.) and yeast (*Saccharomyces cerevisiae*) to swine and humans (Chiu *et al*., 1999). Another enzyme containing noncorrin cobalt is nitrile hydratase (NHase) which functions in biotransformation of nitriles into corresponding amides (Asano *et al.*, 1980). There are two classes of NHase, cobalt-containing NHase and iron-containing NHase. NHase is widespread in bacteria and it is still defined as a bacterial enzyme. It has been isolated from diverse genera of Proteobacteria, Actinobacteria, Cyanobacteria and Firmucutes but there is no molecular evidence for the NHase activity from yeasts, molds and plants (Prasad and Bhalla, 2010). Kobayashi and Shimizu (1999), reported other seven-noncorrin proteins (Table 1.1).

Table 1.1 : Known cobalt-containing proteins to date (Kobayashi and Shimizu,1999).

Enzyme or Protein	Source	Cofactor Content	Postulated role for Cobalt
Prolidase	Archea	1-2 Co per subunit	Hydrolysis
Glucose isomerase	Actinomycetes	1 Co for 4 subunits	Isomerization
Methylmalonyl-CoA carboxytransferase	Bacteria	1 Co, 1 Zn per subunit	Carboxytransferation
Aldehyde decarboxylase	Algea	1 Co-porphyrin per αβ-subunit	Decarboxylation for aldehyde
Lysine-2,3-aminomutase	Bacteria	0.5-1 Co per subunit	Mutation
Bromoperoxide	Bacteria	~0.35 Co per 2 subunits	Bromination
Cobalt-porphyrin containing protein	Bacteria	1 Co-porphyrin per protein	Electron carrier

1.4 Industrial Cobalt Applications and Human Health

There is a wide variety of industrial cobalt applications. Magnets, high temperature alloys, cobalt steels, tungsten carbide cutting tools and implants are the most used materials obtained from cobalt and they are utilized in the production of gas turbines, jet engines, orthopedic implants *etc.* During cobalt refining and application process, cobalt exposure occurs and acute cobalt exposure could cause severe health problem (Kazantzis, 1981).

In the mid-seventies and later, an epidemic of lung disease appeared among diamond polishers, which is called nowadays as "cobalt lung" because it might be produced upon inhalation of cobalt with metallic carbides or diamond dust (Lison *et al.*, 1996). De Boeck *et al.* (2003) stated, "inhalation and skin contact are the main occupational exposure routes of cobalt". The respiratory tract, the skin, the hematopoietic tissues, the myocardium or thyroid gland could be affected upon cobalt exposure. Metals, including cobalt, could trigger oxidative stress, cause impaired DNA repair, and inhibit enzyme functions involving in cell cycle progression, proliferation, apoptosis or differentiation. Moreover, cobalt is accepted as possibly carcinogenic to humans. Cobalt shows its genotoxic effects by producing reactive oxygen species and by damaging DNA repair systems (Beyersmann and Hartwig, 2008).

$CoCl_2$, cobalt metal and tungsten carbide-cobalt particle (WC-Co) mixture are three most studied cobalt compounds. These compounds might induce carcinogenesis by a possible mechanism depicted in Figure 1.2 (Lison *et al.*, 1995).

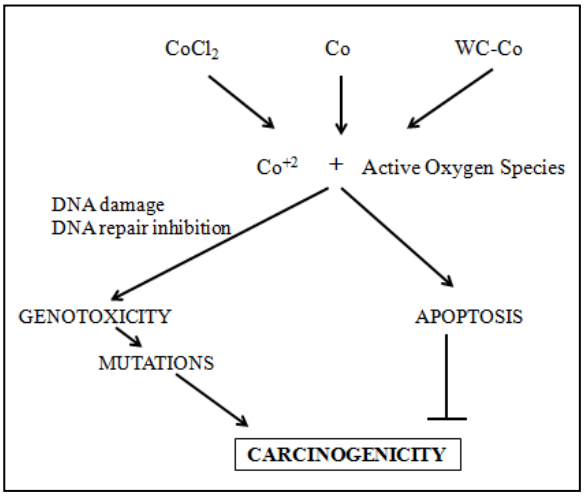


Figure 1.2 : Possible modes of action of $CoCl_2$, Co metal and WC–Co particle mixture, hard metal dust (Lison *et al.*, 1995).

1.5 The Model Organism *Saccharomyces cerevisiae*

Saccharomyces cerevisiae is a unicellular eukaryotic organism that is been used for an ever-increasing number of molecular-cell biology and biotechnology studies. Ö. Winge first performed yeast genetics studies in 1935 at the Carlsberg Laboratory in Copenhagen. Another pioneering and leader laboratory in the history of yeast genetics was that of Carl C. Lindegren first in Washington University and later at the University of Illinois (Roman, 1986). There are countless *S. cerevisiae* strains all around the world and it must be noted that all these strains are obtained from the interbred stocks of Winge, Lindegren and other valuable scientists whose names are not mentioned (Sherman, 2002). For example, the progenitor strains for *S. cerevisiae* S288c, which is the first eukaryotic organism whose complete genome was sequenced, was delivered initially to other laboratories by C.C. Lindegren (Goffeau *et al.*, 1996; Mortimer and Johnstone, 1986). *S.cerevisiae* A364A, W303, FL100, CEN.PK, S1278b, SK1 and BY4716 strains were also commonly used in several yeast laboratories (Schacherer *et al.*, 2007).

There are about 12 million base pairs and 16 chromosomes in *S.cerevisiae* genome. The genome consists of about 6000 open reading frames (ORFs) which make approximately 70% of whole genome sequences. It is considered very dense compared to other higher eukaryotes, like in the case of human genome with about 5% genes content. This is because of the rareness of the non-coding DNA sequences on the genes. This property makes *S.cerevisiae* a valuable tool for identification of genes and their functions and finding homologous sequences on other eukaryotes, including human (Goffeau *et al.*, 1996; Dujon 1996). Its DNA is nearly 3.5-fold more than that of *Escherichia coli* however; this relatively high DNA content compared to bacteria does not impede *S. cerevisiae* cells to be preferred for genetic research. Because it grows fast, yeast cells could be separated from each other (dispersed), replica-plating technique can be applied, mutants can be obtained easily, its genetic system is understood in detail, different kinds of transformation technique can be performed, and distinct types of auxotrophy can be created in *S.cerevisiae* cells. The yeast genome contains mitochondrial DNA, 2-μm circle plasmids and dsRNA viruses apart from the chromosomal DNA (Sherman, 2002).

S.cerevisiae cells could survive and grow in both haploid (n) and diploid (2n) states. Some of the differences between haploid and diploid cells of *S.cerevisiae* are shown in Table 1.2 (Sherman, 2002).

Table 1.2 : Size and composition of yeast cells (Sherman, 2002).

Characteristics	Haploid cell	Diploid cell
Volume (μm^3)	70	120
Size (μm)	2-5	5-7
Composition 10^{-12} g		
Wet weight	60	80
Dry weight	15	20
DNA	0.017	0.034
RNA	1.2	1.9
Protein	6	8

There are two types of haploid cells, which are determined by the existence of either *MATa* or *MATα* allele in the corresponding loci of a yeast cell. *MAT* refers to mating type for those abreviations. A diploid strain can be obtained by mixing the same amount of different mating types of *S.cerevisiae* cells. When either haploid or diploid cells grow in vegetative state, mother cell produces the daughter cell in the form of a bud. For that reason, this process is called as budding. The subsequent process is nuclear division and this follows the formation of the cell wall. The schematic representation of *S.cerevisiae* cell cycle phases is shown in Figure 1.3 (Walker, 1998).

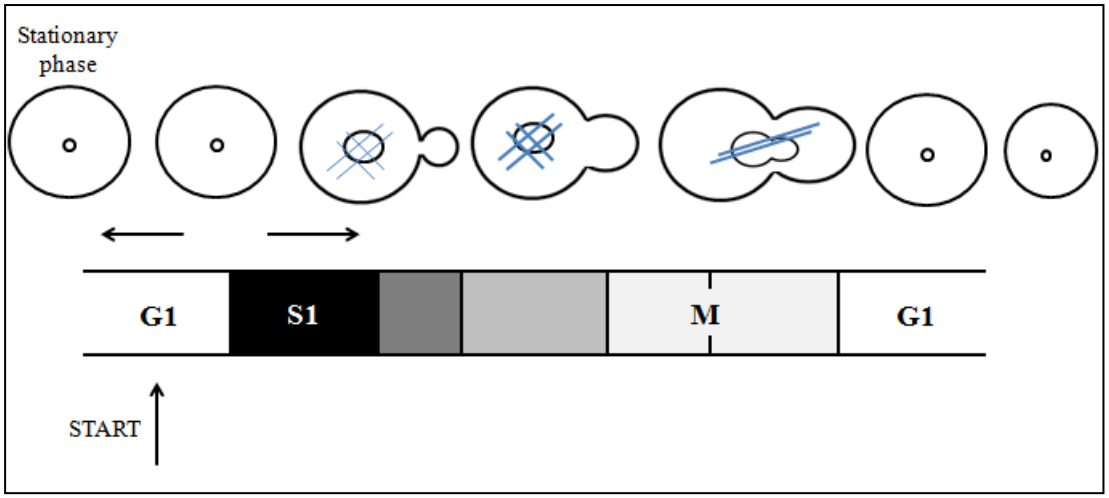


Figure 1.3 : Schematic representation of *S.cerevisiae* cell cycle phases (Walker, 1998).

A mother cell can produce about 20 to 30 buds and the bud scare number on the cell wall can be used to estimate the age of the mother yeast cell.

When a *S.cerevisiae* laboratory strain is grown in complete medium like YPD (2% 'w/v' glucose, 2% 'w/v' peptone, 1% 'w/v' yeast extract) and in synthetic medium (2% 'w/v' glucose, 0.67% 'w/v' yeast nitrogen base) at 30°C, its doubling time at exponential growth phase is 90 and 140 min, respectively. The defined medium could be used to grow *S.cerevisiae* cells and investigate their growth physiology.

1.6 General Mechanism to Maintain Cobalt Homeostasis in Yeast *S.cerevisaie*

S.cerevisiae can be used as a model organism in studying the metabolism of a variety of metals like iron, copper, zinc, manganese, cobalt and nickel. Although molecular cobalt uptake in *S. cerevisiae* could not be explained in detail there are some suggestions (Bleackley and Ross, 2011). Fuhrmann and Rothstein (1968) proposed that metal ions can be attached to the anionic groups of yeast cell wall and transported into the cell by a non-selective manner. In the case of cobalt ions, firstly, they can bind to the cell wall and then pass through it without spending energy. They also suggested that cobalt, nickel, and zinc ions use specific magnesium and manganese channels by using metabolic energy. Lesuisse *et al.* (1987) stated that cobalt is transferred through broad-specificity divalent metal transporters and iron uptake can be inhibited competitively by cobalt. In another study, Joho *et al.* (1991) obtained both cobalt and nickel-resistant mutants which have the ability to take less magnesium. Moreover, the addition of magnesium possibly prevents the toxic effects of cobalt and nickel on those mutants because of the existence of the magnesium transporter which also transfers cobalt and nickel into the cells. It was also demonstrated that *S. cerevisiae* low affinity iron uptake system allows cobalt passage into the cell (Dix *et al.*, 1994). The suggested cobalt uptake mechanisms are illustrated in Figure 1.4.

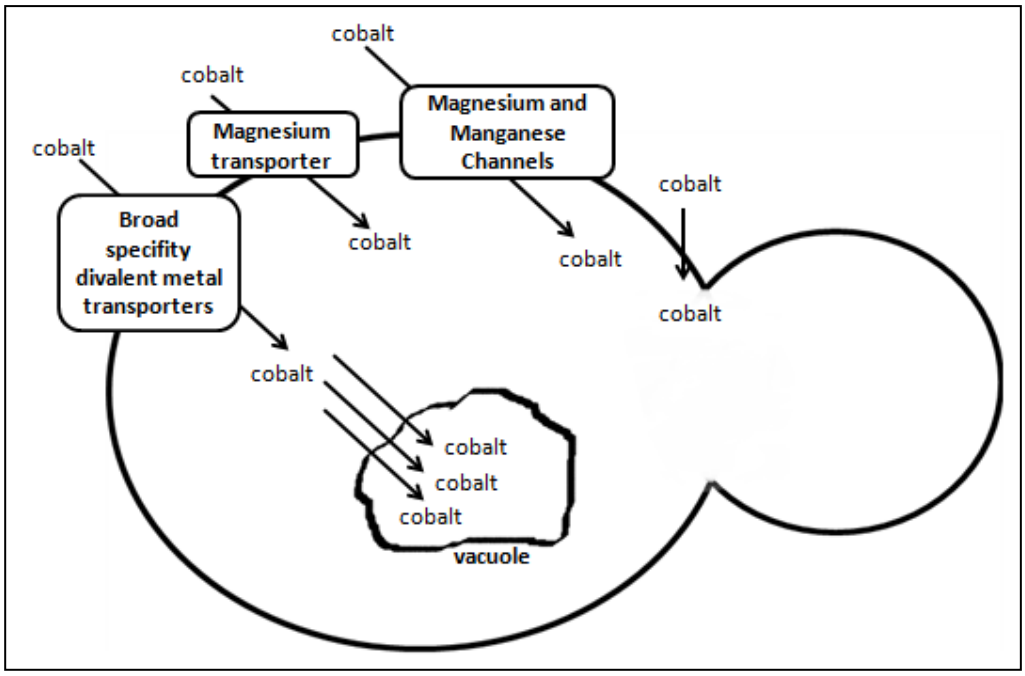


Figure 1.4 : Suggested cobalt uptake mechanisms in *S.cerevisiae* and cobalt fate inside the cell.

Saccharomyces cerevisiae is also a valuable tool to unravel the molecular details of metal homeostasis and tolerance mechanisms. Yeast cells like many other organisms always deal with metals for catalytic activity of enzymes and/or maintaining proper protein structure. The excess amount of metals is considered as toxic by the metabolism and they are omitted from the system by the available tolerance mechanisms. Yeast cells respond to metal toxicity by transcriptomic, proteomic and metabolic adaptations (Wysocki and Tamas, 2010). The metal tolerance mechanisms were shown to be governed by different ways. One of them is reducing uptake of metal ions, which is realized by increased metal-binding capacity on the cell wall and by regulating ion pumps. Another defence mechanism against adverse effects of metals is accumulating metals inside a particular organelle like vacuole for *S.cerevisiae* (Jennings, 1993 and Jin *et al.*, 2008).

In the case of cobalt, it was shown that both reduction of uptake and sequestration systems are used to fight against the toxicity of cobalt ions. Cobalt-resistant mutants of *S.cerevisiae* were obtained by repeated sub-culturing of the samples in the presence of cobalt. Reduced cobalt uptake was observed in these cobalt-resistant mutant cells (White and Gadd, 1986).

The vacuole in yeast cells is a dynamic organelle which also provides metal accumulation like; Mn^{+2}, Fe^{+2}, Zn^{+2}, Co^{+2}, Ca^{+2}, Sr^{+2}, Ni^{+2}, K^{+}, Li^{+}, and Cs^{+}. Ramsay *et al.* (1997) showed that vacuoles are indispensable for preventing toxic effects of cobalt, manganese, nickel and zinc.

1.7 Metabolic Engineering and its Limitations

Advances in recombinant DNA technology, beginning in the 1970s, have helped scientists to study in detail both prokaryotic and eukaryotic genomes and further innovations has revolutionized biology. Nowadays, pharmaceutical proteins could be obtained with high yield or a mutant strain could be improved for extreme conditions with high productivity (Porro *et al.*, 2005). At 1991, the rational methodology for mentioned examples was defined by Bailey as "Metabolic Engineering" and described by Stephanopoulos (1999) as "the directed improvement of product formation or cellular properties through the modification of specific biochemical reactions or introduction of new ones with the use of recombinant DNA technology". Theoretical analysis obtained from biochemical information along with genetic engineering applications enable to alter the promoter strength of a specific gene, to carry out gene deletions and even new biochemical pathways could be introduced into the cell (Ostergaar *et al.*, 2000). Metabolic engineering of yeast strains e.g. *S.cerevisiae* has been an attractive approach for industrial applications over classical strain improvement methods because the accumulation of unfavourable mutations could be eliminated and expression of heterologous genes could be performed (Nevoigt, 2008).

However, metabolic engineering could not be realized for all cellular activities due to the complex interactions of metabolic networks and unexpected metabolic regulations and/or responses. Additionally, directing the metabolic pathways requires the understanding of the entire metabolic network which is nowadays still not feasible. All those limitations had caused to emerge alternative approaches (Bailey *et al.*, 1996 and Çakar, 2009).

1.8 An Inverse Metabolic Engineering Strategy to Obtain Improved Microbial Phenotypes: Evolutionary Engineering

Metabolic engineering strategy is used in strain improvement programs with the aim of increasing rate, titer and yield in the corresponding phenotypes. Despite the well-known metabolic systems, it is not very straightforward to engineer genetic changes in even the simplest bacterium; *E.coli*, because metabolic networks are highly interconnected and all unique pathways could affect the whole system. The complete knowledge of the biosynthetic pathways and/or metabolism that is desired to be engineered is not available for many host strains. The phenotype that would be ameliorated through metabolic engineering might be another limitation for such applications because sometimes the desired trait is poorly described genetically or it involves more than 50 genes. Inverse metabolic engineering has been applied as an alternative strategy to metabolic engineering. It consists of three main stages: identification of the desired phenotype/trait, determination of the genetic basis for the corresponding phenotype and lastly, application of experiments to introduce the gene/genes of interest in another tractable organism (Bailey *et al.*, 1996).

The desired phenotypes have been successfully improved or created via evolutionary engineering which is an approach in inverse metabolic engineering (Patnaik, 2008). Evolutionary engineering has been providing strains with specific character and/or complex phenotypes in complementation with high-throughput characterization and genome-wide microarray analysis. It is sometimes used to increase the fermentation-based product yield in order to meet biofuel demands (Koppram *et al.*, 2012) or to obtain a complex/specific phenotype for studying its molecular mechanisms (Çakar *et al.*, 2005 and 2009).

There are a variety of evolutionary engineering applications that have been reported in the literature. In one of these studies, *Acetobacter aceti* was cultivated in batch or continuous growth conditions in the presence of gradually increasing acetate concentrations. The culture which gained higher acetate resistance was used to identify the molecular mechanisms of acetate resistance by two-dimensional protein electrophoresis ‘2DE’ (Steiner and Sauer, 2001). Steiner and Sauer (2003) showed in their subsequent study that *A.aceti* gained an acetate resistance level comparable to that of the industrial strains by growing them through hundreds of generations in the presence of acetate. In another study that used a boron-tolerant bacterium *Bacillus*

boroniphilus DSM 17376, evolutionary engineering approach was applied successfully. Individual boron-resistant mutants which had 16-fold higher resistances to boron than the wild type strain were obtained (Şen *et al.* 2011). In another application of evolutionary engineering, it was aimed to obtain *S. cerevisiae* which could grow anaerobically on xylose. For this reason, firstly, a recombinant *S. cerevisiae* strain which could over-express the genes needed for a functional xylose metabolic pathway was used. Then, a mutant population was obtained that had the ability to ferment xylose at the end of the long-term chemostat selection. (Sonderegger and Sauer, 2003). A recent study conducted by Sanchez and co-workers (2010) involved evolutionary engineering of a metabolically engineered *S.cerevisiae* strain to improve its ability to co-ferment pentoses such as xylose and arabinose. The strain with the desired phenotypes also helped to identify the changes which make the new mutant strains better in terms of utilisation of those pentoses. Pentose fermentation studies also involve *Pichia stipitis*. This yeast can produce ethanol from lignocellulosic feedstock upon acid hydrolysis. However, this process creates a hydrolysate which contains some inhibitory materials to this strain. For that reason, by using evolutionary engineering, hydrolysate-resistant *P. stipitis* were obtained and their ethanol production was compared to that of the wild type strain (Huang *et al.* 2009).

1.9 The Aim of the Study

In light of the information provided in the introduction section, the aim of the study presented in this thesis was first to obtain cobalt hyper-resistant *S.cerevisiae* mutants by an inverse metabolic engineering strategy called evolutionary engineering, and second, to use these mutants for molecular characterization of cobalt resistance in *S.cerevisiae* with an ultimate aim of using that molecular information in understanding the mechanism of cobalt-related diseases in humans, and potential cobalt bioremediation and/or biomimetic applications.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Strains and their conservation conditions

Strains of *Saccharomyces cerevisiae* used in this study were: CEN.PK 113.7D *Mata URA3 HIS3 LEU2 TRP1 MAL2-8^{c} SUC2* and CEN.PK 113.1A *Matα.* BY4741 (*MAT**a*** *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0), BY4742 (*MAT**α*** *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0) (Brachmann *et al.*, 1998) and its gene deletion derivative *aft1*Δ, *aft2*Δ, *cot1*Δ and *zrc1*Δ from the Yeast Knock-Out strains collection (YKO strain collection, Open biosystems Thermo SCIENTIFIC). *Escherichia coli* DH5α cells were used for molecular genetics applications.

Stock cultures were prepared from 1 mL of exponentially growing cells harvested in 1.5 mL microfuge tube at 10000 *g* for 5 min. The supernatant was discarded and the pellet was resuspended in 30 % (v/v) glycerol. The stock of the cells was stored at -80ºC.

2.1.2 Media

The Yeast Minimal Medium 'YMM' was used in this study (Burke *et al.*, 2000) also referred as synthetic defined (SD) medium (Table 2.1).

Table 2.1: YMM contents.

Content	Percentage in YMM (w/v)
Glucose	2.00 %
Yeast Nitrogen Base without amino acids and ammonium sulphate	0.67 %
Agar (for solid media)	2.00 %

When glycerol and ethanol were used as carbon sources instead of glucose, they were added as 1 and 2 % (v/v) of final concentrations, respectively.

In order to select transformants with *URA3* deletion, 5FOA (5'-fluoro orotic acid, Euromedex) was supplemented to YMM solid media to a final concentration of 0.05 % (w/v).

Yeast extract-peptone-dextrose 'YPD' medium (Sherman *et al.*, 1978) was used for routine growth of the yeast cells (Table 2.2).

Table 2.2: YPD (YEPD, YP Dextrose) contents.

Content	Percentage in YPD (w/v)
Glucose	2.00 %
Peptone	1.00 %
Yeast extract	1.00 %
Agar (for solid media)	2.00 %

200 µg/mL Geneticine (Wach *et al.*, 1994) and 100 µg/mL nourseothricine (Hentges *et al.*, 2005) containing YPD plates were prepared for selection reagents upon yeast transformation experiments. G-418 (Roche) and ClonNAT (Werner Bioagents) stocks were prepared by dissolving the corresponding powder in distilled water. The solution was filter sterilized and then stored at -20ºC.

Minimal sporulation medium, abbreviated as KAc (Burke *et al.*, 2000), was utilized in order to manipulate with diploids and the tetrads (Table 2.3).

Table 2.3: Sporulation medium 'KAc'.

Content	Percentage in KAc Medium (w/v)
Potassium Acetate	1.00 %
Agar (for solid media)	2.00 %

E.coli cells were cultivated on LB liquid or solid medium (Bertani, 1951) (Table 2.4).

Table 2.4: LB Medium content.

Content	Percentage in LB Medium (w/v)
Tryptone	1.00 %
Yeast Extract	0.50 %
NaCl	0.50 %
Agar (for solid media)	2.00 %

Ampicillin was added from a stock solution of 150 mg/mL to a final concentration of 150 µg/mL. Ampicillin (Roche) stock solution was prepared from its powder by dissolving it in water (filter sterilized and stored at -20ºC). X-gal (5-bromo-4-chloro-

indolyl-β-D-galactopyranoside) and IPTG (Isopropyl β-D-1-thiogalactopyranoside) were added to LB ampicillin plates for blue/white screening by spreading 50 µL each from 100 mM IPTG and 4% 'w/v' X-gal solutions. IPTG and X-gal were solubilised in water and dimethyl formamide respectively. They were then filter-sterilized before being placed at -20ºC.

2.1.3 Chemicals, solutions/buffers, kits/enzymes and equipment

The chemicals and solutions/buffers used in this study are indicated in Tables 2.5 and 2.6, respectively.

Table 2.5: Chemicals used in thesis study.

Chemicals	**Company**	**Country**
5-fluoro-orotic acid	Euromedex	France
Acetic acid glacial	MERCK	Germany
Agarose	Applichem	Germany
Aluminium Chloride hexahydrate, $AlCl_3 6H_2O$	MERCK	Germany
Ammonium iron (II) sulfate hexahydrate, $(NH_4)_2Fe(SO_4)_2 6H_2O$	MERCK	Germany
Ammonium sulphate	VWR BDH PROLABO	UK
Ampicillin	Roche	Switzerland
Amyloglycosidase (3500 U)	Roche	Switzerland
Bacto Peptone	BD DifcoTM	USA
Calcium Chloride hexahydrate, $CaCl_2 6H_2O$	Sigma ALDRICH	USA
Cobalt chloride, $CoCl_2$	Fluka	USA
Copper (II)Sulfate pentahydrate, $CuSO_4 5H_2O$	Sigma ALDRICH	USA
Cyanine 3-	Amersham Bioscience	Sweden
DAPI, 4',6-diamidino-2-phenylindole	Sigma ALDRICH	USA
DNA markers	Fermentas	Lithuania
Ethanol	J.T.Baker	Netherlands
Ethylenediaminetetraacetic acid disodium salt dihydrate, EDTA	MERCK	Germany
G-418 (Geneticine) solution	Roche	Switzerland
Glucose	VWR BDH PROLABO	UK
Glucose Standard Solution	Sigma	USA
Glycerol	Sigma ALDRICH	USA

Table 2.5 (contd.): Chemicals used in thesis study.

Chemicals	**Company**	**Country**
Glucose oxidase/peroxidase reagent	Sigma ALDRICH	USA
Hydrogen peroxide	MERCK	Germany
IPTG, dioxane-free	Fermentas	Lithuania
Lithium acetate	Sigma ALDRICH	USA
Lithium Chloride anhydrous, $LiCl_2$	Sigma ALDRICH	USA
Magnesium Chloride hexahydrate, $MgCl_26H_2O$	MERCK	Germany
Manganese (II) Sulfate monohydrate, $MnSO_4H_2O$	Sigma	USA
NaCl	Carlo Erba	Italy
NaOH	Carlo Erba	Italy
Nickel(II)Chloride hexahydrate, $NiCl_26H_2O$	MERCK	Germany
Nourseothricine (ClonNAT)	Werner Bioagents	Germany
o-Dianisidine dihydrochloride	Sigma	USA
PEG-4000	Sigma	USA
Potassium acetate	MERCK	Germany
Potassium acetate	Sigma ALDRICH	USA
RNase A	Roche	Switzerland
Salmon Sperm DNA	Sigma	USA
SDS	Sigma ALDRICH	USA
Sorbitol	MERCK	Germany
Trehalase (from porcine kidney)	Sigma	USA
TrisHCl	MERCK	Germany
Tryptone	MERCK	Germany
X-gal	Fermentas	Lithuania
Yeast Extract	MERCK	Germany
Yeast nitrogen base without amino acid and ammonium sulphate salt	BD DifcoTM	USA
Zinc Sulfate heptahydrate, $ZnSO_47H_2O$	Sigma ALDRICH	USA
β-Glucuronidase/Arylsulfatase	Roche	Switzerland

Table 2.6: Name and content of the solutions and buffers.

Buffer/Solutions	
Potassium phosphate buffer	50 mM, pH 7
Sodium thiosulfate	10% ‘w/v’
Glycerol	30% ‘v/v’
Solution I	50 mM TrisHCl pH8, 10 mM EDTA, 100mg/L RNase A
Solution II	200 mM NaOH, 1 % SDS
Solution III	Potassium Acetate, Acetic acid glacial

Laboratory equipment used in this study is indicated in Table 2.7.

Table 2.7: Laboratory equipment used in this study.

Equipment	**Company**	**Country**
Autoclave	Tuttnauer Systec Autoclave 2540ml - 2870ELVC	Switzerland
Autoclave	Tomy SX 700E	China
Balance	Precisa BJ 610 C - 620 SCS	Switzerland
Benchtop Centrifuge	Beckman Coulter Allegra 25R	USA
Bioanalyser 2100	Agilent Technologies	USA
Deep Freeze	Sanyo (-80°C) Ultra Low MDT-U40865	Japan
Refrigerator	Arçelik 3011 NY	Turkey
Digital Color Camera	Leica, DFC 300FX	Germany
Elecrophoresis Tank	Midicell PrimoTM EC 320 - 330	USA
Flame Atomic Absorption Spectrometer	Varian AA 280FS	USA
Fluorescent microscope	Leica, DM4000B	Germany
Glass slides for microarray analysis	Agilent	USA
Hybridization Chamber	Discovery from Ventana Medical System	USA
Incubator	Nüve EN400 - EN500	Turkey
Laminar Flow Hood	Biolab Faster BH-EN 2003	Italy
Laser Scanner	Axon Instruments, GenePix 4000B	USA
Light Microscope	Olympus CH30	USA
Magnetic Stirrer	Labworld	Germany
MicroDismembrator	Braun	Germany
Microfuge	Eppendorf Centrifuge 5424	Germany
Micromanipulator	Singer® MSM 300	UK
Micropipettes	Eppendorf	Germany
Microplate Reader	Biorad Model 3550 UV	USA
NanoDrop 1000 Spectrophotometer	Thermo SCIENTIFIC	USA

Table 2.7 (contd.): Laboratory equipment used in this study

Equipments	**Company**	**Country**
Objective	Leica, 0.6 Oil HCX Plan-Apochromat	Germany
Orbital Shaker Incubators	Certomat S II Sartorius	Germany
pH meter	Mettler Toledo MP220	Switzerland
Power supply for Electrophoresis	EC Apparatus Corporation EC 250-90	
Real Time PCR equipment	Roche, Light Cycler 480 II	Switzerland
Rotor	Beckman Coulter JA-30.50i	USA
Spectrophotometer	Biocrom® S-11	UK
Spectrophotometer	Shimadzu UV-1700	Japan
Thermal cycler	Bio-Rad My cyclerTM	USA
Thermal cycler	HyBaid PCR express	UK
	TC-412 Techne	USA
Thermomixers	Eppendorf, Thermomixer Comfort	Germany
	Eppendorf, Thermomixer Compact	
Ultracentrifuge	Beckman Coulter, Avanti J-30I	USA
Ultrapure Water System	USF-Elga UHQ	USA
Vortex mixer	Heidolph	Germany
Water Bath	Memmert wb-22	Switzerland

Software and websites used in this study are demonstrated in Table 2.8.

Table 2.8: Software and websites used in this study.

Software and websites	
Microsoft Office 2007	
FW software, Leica	
GenePix software version 3.01	
Limma package of Bioconductor	www.bioconductor.org
Plateform Transcriptome-Biochips of Toulouse	http://biopuce.insa-toulouse.fr
Primer3Plus	http://frodo.wi.mit.edu/primer3/
R computing environment	www.R-project.org
Saccharomyces genome database	http://www.yeastgenome.org/
YEASTRACT	http://www.yeastract.com
FunSpec - Web-Based Cluster Interpreter of Yeast	http://funspec.med.utoronto.ca/
A five-tube MPN table	http://www.jlindquist.net/generalmicro/102dil3a.html

Table 2.9: Kits and enzymes used in this study.

Kits and Enzymes	Company	Country
Fusion DNA polymerase	FINNZYMES, No. F-540S	Finland
High Pure RNA Isolation Kit	Roche	Switzerland
IQ™ SYBR® Green Supermix	BIO-RAD	USA
iScript™ cDNA Synthesis Kit	BIO-RAD	USA
Label Star Reverse Transcriptase	QIAGEN	Germany
Light Cycler 480 SYBR Green I Master	Roche	Switzerland
MinElute spin columns	QIAGEN	Germany
pGEM-T-easy vector	Promega, No. A1360	USA
QIAquike Gel Extraction Kit	QIAGEN, No. 28706	Germany
Restriction enzyme, BamHI	Biolabs	USA
Restriction enzyme, NcoI	Biolabs	USA
Restriction enzyme, PstI	Biolabs	USA
SV Total RNA Isolation System	Promega	USA
T4 DNA ligase	Biolabs, No. M0202S	USA
Taq DNA polymerase	Biolabs, No. M0267S	USA
Transcriptor High Fidelity cDNA Synthesis Kit	Roche	Switzerland
Yeast DNA Purification Kit (MasterPureTM)	EPICENTRE, MPY2-70921	USA

Kits and enzymes ysed in this study is indicated in Table 2.9.

2.2 Methods

2.2.1 Cultivation of the cells

2.2.1.1 Regular growth of the yeast and bacteria cultures

Precultures of yeast cells were prepared in 100 or 250-mL shake flasks and incubated overnight at 30°C and 150 rpm. The main cultures were prepared by inoculation from those precultures. The initial optical density of the cultures were generally set to 0.25 which corresponds to 10^6 cells/mL. Cultures were cultivated in liquid medium at 30ºC in shake flasks (100/250/500 mL) at a shaking speed of 150 rpm. The medium volume was kept at one-fifth of the total flask size. The above-mentioned cultivation steps were applied throughout this study unless otherwise stated.

Escherichia coli cells were cultivated in LB medium at 37ºC and 150 rpm. They were placed at 37ºC whenever they were inoculated to solid media.

2.2.1.2 Sporulation of the yeast diploid cells

MATa and *MATα* parent cells were inoculated to solid YPD medium and they were incubated overnight at 30ºC. The next day they were mixed by taking nearly the same amount of cells by using micropipette tips. They were placed at 30ºC for about 4-6 h. The formation of zygotes was checked under the microscope. If the mixture contained zygotes, the culture was incubated further overnight at 30ºC (Burke *et al.*, 2000). At the end of 24 h time period, the mixture containing the diploid cells was transferred to sporulation medium as a patch for 2 days of incubation at 30ºC. The formation of tetrads was then observed under the light microscope.

2.2.2 Growth analysis

2.2.2.1 Spectrophotometric measurements

Growth of the cultures was monitored by measuring optical density at 600 nm 'OD_{600}' using Biocrom® S-11 (USA) or Shimadzu UV-1700 (Japan) spectrophotometers. Growth fitness was evaluated by dividing OD_{600} of yeast cultures exposed to various stress conditions to those of the untreated ones.

2.2.2.2 Maximum specific growth rate determination

Growth curves of the cultures were drawn by plotting OD_{600} values versus time. The natural logarithm of the OD_{600} values was obtained to calculate the maximum specific growth rate (μ_{max}), which corresponds to the maximum slope from this semi-logarithmic representation.

2.2.2.3 Cell dry weight determination

1.5 mL-microfuge tubes were dried in 80ºC oven for 48 h. They were then transferred to desiccators (Finemech Bola-Star Vitrium, USA) for 15 min. Their weight was determined by using microbalances (Precisa 620C SCS, Switzerland). Cultures (1.5 mL) were added to pre-weighed microfuge tubes. After 5 min centrifugation at maximum speed, the supernatant was discarded. The tubes were dried for 48 h at 80°C and weighed. Cell dry weight 'CDW' was expressed as mg per mL.

2.2.3 Determination of stress resistances

Stress resistances of the different yeast cells were determined by Most Probable Number (MPN) assay, spot test and replica plating.

2.2.3.1 Most Probable Number (MPN) assay

Resistance to different stress types was estimated by a high-throughput, most probable number (MPN) assay (Russek and Colwell, 1983). Viable cell numbers were determined by serial dilutions in 96-well plates containing 180 µL YMM. Dilutions were made in the range of 10^{-1} to 10^{-8} for five parallel samples. The most probable number of survivors was estimated by using published MPN tables (Lindquist, 2008) based on the ability of yeast cells to grow at higher dilutions.

Cells were grown in YMM containing 2 - 8 mM $CoCl_2$ or 0.8 mM H_2O_2 for 72 h. The viable cell numbers were determined from the MPN table. The resistance to these various stress conditions was expressed as 'survival rate', which was calculated by dividing the number of stress-treated viable-cells to that of non-treated cells (Çakar *et al.*, 2009).

2.2.3.2 Spot test

Spot test was performed as described previously (Memarian *et al.*, 2007), with slight modification. It was used to determine the sensitivity or resistances of yeast strains to various compounds. Cells were cultivated in 50 mL-culture tubes containing 10-mL liquid YMM until logarithmic growth phase at 30ºC and 150 rpm. Equal numbers of yeast cells were harvested by centrifugation at 10000 *g* for 5 min and the supernatant was poured off. The pellets were resuspended in 100 µL water and diluted serially as 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} in 96-well plates. Two µL from these diluted suspensions were dropped onto solid control plates and plates containing stress factors in their media. They were incubated at 30ºC.

2.2.3.3 Replica plating

Replica plating was used for yeast cells to compare different phenotypes, as described previously (Lederberg and Lederberg, 1952). Different yeast cultures were grown in solid rich medium (YPD). That Petri dish was called the "Master plate" and plates which contained different chemical materials were prepared in either rich

'YPD' or 'Yeast Minimal Medium'. Master plate was inoculated with those other Petri plates along with a control plate in order to reproduce the original special pattern of colonies. The plates were incubated at 30ºC for 1-2 days.

2.2.4 Evolutionary engineering procedure to obtain cobalt-resistant *Saccharomyces cerevisiae* mutants

In this section, evolutionary engineering to obtain cobalt-resistant *S.cerevisiae* mutants will be described in detail.

2.2.4.1 EMS mutagenesis of the wild type *S.cerevisiae*

EMS mutagenesis was performed with slight modifications as described previously, (Lawrence, 1991). Briefly, a 20 mL culture of *Saccharomyces cerevisiae* CEN.PK 113.7D in 100 mL flask of YPD medium was cultivated overnight at 30°C and 150 rpm. About $3.5x10^8$ cells/mL were taken, washed with 50 mM potassium phosphate buffer (pH 7), diluted to a concentration of $5x10^7$ cells/mL in 50 mM potassium phosphate buffer (pH 7) and delivered to five tubes. 150 µL of EMS was added to culture tubes except the one that was used as control. Tubes were vortexed and placed to a 30°C incubator at 150 rpm. After 15, 30, 60 and 90 min, a tube was taken and freshly prepared 5 mL sodium thiosulfate (10%, w/v) was added in order to inactivate EMS. The solution was mixed, centrifuged for 10 min at 3000 *g*. The pellets were then washed twice with YMM without glucose and resuspended in 20 mL YPD in a 100 mL flask. After 24 h of incubation at 30ºC, 150 rpm the optical density (OD_{600}) of the cultures was measured.

2.2.4.2 Determination of the initial cobalt stress level for the EMS mutagenized culture

Cultures that have been treated with EMS for 60 and 90 min were used for the determination of the $CoCl_2$ stress levels. After precultivation in 20 mL YMM in 100 mL flasks, they were reinoculated into 3 mL of YMM containing 0.5, 1, 1.5, 2, 2.5, 5, 7.5, 10, 15 and 20 mM $CoCl_2$ at an initial OD_{600} of 0.3 (2-4 x 10^6 cells/mL). OD_{600} was measured regularly throughout the 72 h incubation.

2.2.4.3 Selection strategy to obtain cobalt-resistant mutant generation

The preculture of EMS-mutagenized strain was inoculated in YMM and YMM containing 0.5 mM $CoCl_2$ by adjusting initial OD_{600} as 0.3 **(**2-4 x 10^6 cells/mL**)**. The culture volume was 20 mL and 100 mL flask was used. Optical density measurement was done after 24 h of incubation at 30°C and 150 rpm. The OD_{600} value of the culture incubated with cobalt were divided to that of control culture and the growth fitness was determined. The culture that was grown for 24 h in the presence of 0.5 mM $CoCl_2$ was accepted as the first cobalt-resistant generation. A new inoculation was made from the first generation to fresh YMM and YMM that contained 1 mM $CoCl_2$. Those cultures were also grown for 24 h and their OD_{600} values were measured. The first generation, which was inoculated into cobalt-containing medium and incubated for 24 h, was called the second cobalt-resistant generation. These cultivations were repeated throughout 25 successive generations, by gradually increasing the $CoCl_2$ concentration at each successive cultivation. For each generation, stock cultures were prepared in YMM with 30% 'v/v' glycerol, and kept at -80ºC.

Throughout the 25 generations, the $CoCl_2$ concentration was increased from 0.5 mM up to 8 mM $CoCl_2$ level at the 25^{th} or final mutant generation.

2.2.4.4 Random selection of individual mutants from the final population

The final mutant population (25^{th} generation) was inoculated to solid YMM plates. Eight single colonies were chosen randomly as individual mutants (Table 2.10). Eight single colonies, 25^{th} final population and the wild type were tested on solid YMM plates for their cobalt resistances.

Table 2.10: Names of the individual mutants obtained by evolutionary engineering.

	Name of the individual mutants
1^{st} mutant	**CI25A**
2^{nd} mutant	**CI25B**
3^{rd} mutant	**CI25C**
4^{th} mutant	**CI25D**
5^{th} mutant	**CI25E**
6^{th} mutant	**CI25F**
7^{th} mutant	**CI25G**
8^{th} mutant	**CI25H**

2.2.5 Determination of resistance level to cobalt and other stress types

The precultures of the strains were inoculated to 10 mL liquid YMM in 50-mL culture tubes and they were incubated at 30°C, 150 rpm for 18-20 h. Cells were then collected by centrifugation at 10000 *g* for 5 min and supernatants were discarded. Cultures' dilutions were prepared (10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}) and two µL of each dilution were dropped onto the solid media that contained 2.5; 5; and 10 mM $CoCl_2$ in YMM. The same cultures were tested in parallel on YMM plates which contained 20 mM $MnCl_2$, 10 mM $ZnCl_2$, 50 mM $(NH_4)_2Fe(SO_4)_26H_2O$, 0.25/0.5 mM $NiCl_2$, 1 M $MgCl_2$, 0.1 mM $CuSO_4$, 10 mM caffeine, 0.5 M NaCl, 8% 'v/v'ethanol and 1 mM H_2O_2.

The individual mutants were also tested for their growth fitness in liquid media. Precultures of the individual mutants, 25^{th} population and wild type were grown overnight in 50-mL culture tubes and 10 mL culture volume, at 30°C and 150 rpm. They were then inoculated to 10 mL YMM and YMM that contained 5 mM $CoCl_2$. After 24 h of incubation, their OD_{600} were measured and the corresponding growth fitness values were calculated.

2.2.6 Genetic stability analysis of individual mutants

Selected mutant strain which was taken as frozen stock from -80ºC was inoculated to YPD solid medium and incubated at 30ºC. An overnight culture was prepared with 10 mL of YMM in a 50-mL tube inoculated with cultures from the YPD agar plate streaked 2 days before with the selected mutant. A 10-mL liquid YMM culture in a 50-mL culture tube was prepared by setting its OD_{600} as 0.3 (2-4 x 10^6 cells/mL). It was incubated at 30 ºC, 150 rpm for 24 h and a stock culture was prepared from this first cultivation. At the same time, this culture was again inoculated to fresh liquid YMM at an initial OD_{600} of 0.3 (2-4 x 10^6 cells/mL). At the end of 24 h, the previous steps were repeated for five times. All the five stock cultures and wild type precultures were then prepared in liquid YMM and tested on cobalt-containing and control agar plates.

2.2.7 Genetic characterization of individual mutants

One of the cobalt resistant mutants was crossed with haploid strain of *S.cerevisiae* having an opposite mating type on solid YPD (Delneri *et al.*, 1999). After about 6 h

of incubation at 30°C, the zygotes were selected from the mixture of wild type *S.cerevisiae* and the cobalt-resistant mutant culture by using a micromanipulator (Singer® MSM 300 micromanipulator). One of the separated diploids (2n) was grown on YPD medium, and then transferred to sporulation (KAc) medium. Upon 2 days of incubation in sporulation medium at 30ºC, about 25 tetrads were dissected by using a micromanipulator. The spores from the tetrads, their diploid and their parental strains were analyzed in cobalt-containing solid media.

2.2.8 Back-cross experiments

Back-cross experiments were carried out as described previously (Marullo *et. al*, 2009). Briefly, mutant strain and the wild type *MATα* cross was performed on YPD plates and the diploids were selected by using a micromanipulator (Singer® MSM 300). Diploid cells were incubated on YPD medium and they were then transferred to sporulation medium. Tetrad-containing sample was taken from the surface of the KAc plate upon 2 days of incubation at 30ºC and it was incubated in 5% β-Glucuronidase/Arylsulfatase (Roche) enzyme solution at 37ºC for 15 min. Tetrads were then dissected in order to separate 4 segregants of the corresponding tetrad in different regions of the YPD plate. Standard serial backcrossing of the mutant strain was performed against an isogenic wild type strain. The backcrossing procedures were generally carried out following phenotypic tests on solid plates containing cobalt for determining the resistant phenotypes. Both of the spore clones were crossed with a wild type having an opposite mating type and progeny clones were obtained. The corresponding diploids were tested along with the parental strains upon cobalt application on solid medium.

2.2.9 Obtaining cobalt-resistant mutants by chemical mutagenesis and direct selection on plates

Similar to previous studies, EMS mutagenized wild type culture was directly inoculated to solid YMM that contained 5 mM $CoCl_2$ and the plate was incubated at 30ºC until colonies appeared on the surface of the medium. These four mutant colonies were picked and cultivated for further analysis (Table 2.11).

Table 2.11: Names of the mutants obtained by direct selection.

	Name of the individual mutants
1st mutant	**ECo1**
2nd mutant	**ECo2**
3rd mutant	**ECo3**
4th mutant	**ECo4**

2.2.10 Growth analysis of the wild type and the mutants

Precultures of wild type and mutants were routinely grown in 100-mL Erlenmeyer flask with 20 mL working volume of YMM at 30ºC and 150 rpm. Precultures were grown at 30ºC on a rotary shaker until early stationary phase. They were used to inoculate fresh 100 mL YMM with and without 5 mM $CoCl_2$ in 500 mL flasks. Those flasks were incubated at 30ºC, 150 rpm. Cell growth was monitored spectrophotometrically by measuring OD_{600} (Biocrom® S-11, USA).

2.2.11 Detailed growth analysis and physiological characterization of wild type and mutants

Shake flask cultivation was performed in 500-mL flasks containing 100 mL of YMM with or without 5 mM $CoCl_2$. The inoculum was added to a final optical density (OD_{600}) of 0.3 (2-4 x 10^6 cells/mL). The yeast cells were incubated at 30ºC, 150 rpm for 48 h. Samples were withdrawn every 1.5th hour for spectrophotometric determination of cell growth.

2.2.12 Analytical procedures

2.2.12.1 Ethanol, glycerol, acetate production and glucose consumption during batch cultivation of wild type and CI25E

Supernatants of the samples used for subsequent High Performance Liquid Chromatography (HPLC) measurements were filtered through 0.2-mm-pore-size membrane filters (Sigma-ALDRICH). The concentrations of glucose, ethanol, glycerol and acetate were determined by using an HPLC system (Gilson Inc., Middleton, Wis.) equipped with an Aminex HPX-87H column (Bio-Rad) and a refractive index detector (RID-6A; Shimadzu, Kyoto, Japan) operating at 60°C. The mobile phase was 5 mM H_2SO_4 at a flow rate of 0.6 mL/min.

2.2.12.2 Quantitative assessment of glycogen and trehalose content

The procedure was performed as described previously (Parrou and François, 1997). Briefly, the cell pellet (collected from 25 OD_{600} units of culture) was suspended in 250 µL 0.25 M Na_2CO_3 in screw-top microfuge tubes (Corning, USA) and heated at 95ºC for 4 h. Then, 150 µL 1 M acetic acid was added to the mixture and subsequently the addition of 600 µL of 0.2 M sodium acetate buffer (pH 5.2) was realized into the cell suspension. Half of this mixture was incubated overnight at 57ºC in the presence of 100 mg of amyloglucosidase (Roche, Switzerland) in a hybridization chamber. The second half of the mixture was incubated overnight at 37ºC in the presence of trehalase (Sigma). The glucose released was determined using the glucose oxidase/peroxidase method (Cramp, 1967).

2.2.13 Microarray analysis

2.2.13.1 Design of the microarray experiment

Microarray analysis was performed for wild type, cobalt-resistant mutant 'CI25E', and cobalt-resistant CI25E *cot1*Δ mutant. Wild type and mutant strains were grown as three replicates in 100 mL of YMM in 500-mL flasks, and ~1.5 mL (4 times) were harvested when cells reached the logarithmic phase of growth (OD_{600}=3, 4.5 x 10^7 cells/mL). After centrifugation; the cells were resuspended in small microfuge tubes, centrifuged again for 1 min at full speed and the tubes were thrown into a liquid nitrogen bath.

2.2.13.2 cDNA synthesis and hybridisation to microarrays

Frozen culture (approximately 2×10^9 cells) were mechanically disrupted (MicroDismembrator Braun, Melsungen) and total RNA was isolated using SV Total RNA Isolation System (Promega) following the protocol of the manufacturer. The quantity and the quality of the extracted RNA were determined by nanodrop and microcapillary electrophoresis using a Bioanalyser 2100, respectively (Agilent Technologies, Wilmington, DE, USA). Incorporation of Cyanine 3 'Cy3' (Amersham Bioscience) was performed during reverse transcription of total RNA using Label Star Reverse Transcriptase (QIAGEN). Labelled cDNA from the microarray experiments was purified on MinElute spin columns (QIAGEN) and hybridized to Agilent yeast oligonucleotide microarrays containing 50-mer

oligonucleotide probes representing 6256 known ORFs (open reading frames) from the S288c strain of *S. cerevisiae*, according to the instructions from Agilent Technologies. Hybridization was carried out in an automatic hybridization chamber (Discovery from Ventana Medical System) for 10 h at 42°C. After hybridization, slides were washed in 2× SSC/0.2% (v/v) SDS, immersed briefly in iso-propanol and then dried under a stream of air. The hybridization signals were detected using GenePix 4000B laser Scanner (Axon Instruments), and transformed to numerical values using the integrated GenePix software version 3.01. The microarray hybridization and processing were carried out at the Plateform Transcriptome-Biochips of Toulouse (http://biopuce.insa-toulouse.fr).

2.2.13.3 Transcript data acquisition and analysis

Data analysis was done in R computing environment (www.R-project.org) using the limma package (Smyth, 2005) of Bioconductor (www.bioconductor.org).

The estimates used for the foreground and background intensities were the median of pixels intensity. Raw data were imported into R and spot quality weights were performed assigning a weight of 1 or 0 to each spot. Low-quality spots: non uniform spots, spots with low signal/background ratio or spots with low signal-to-noise ratio and empty or non validated spots, were down weighted.

Data were pre-processed by base 2 logarithmic transformation and within-array normalization was performed using the weighted global median (spots with zero weight were not included in the normalization). To achieve consistency of expression values between arrays, quantile normalization was performed across all the microarrays for each strain separately.

After normalization, the expression of a gene was calculated by the median of replicate spots within each microarray. Only the spots that had a weight of 1 on at least 4 replicates were considered. Genes without expression were marked as missing (NA). Next, if, for the experimental conditions (e.g., cobalt resistant mutant) there were at least two NA values for the replicates, then the gene was filtered out. After filtering, data dimensions were reduced to 5.261 (number of genes remaining given a signal) × 9 (3 strains x 3 replicates).

Gene expression data for strain wild type and mutant were pairwise compared by using the limma package (Smyth, G. K. 2004). Linear models and empirical Bayes

methods were used for assessing differential expression in microarray experiments. Genes with significant evidence for differential expression were identified with a modified *t*-test in conjunction with an empirical Bayes method to moderate the standard errors of the estimated log-fold changes. The *p*-values of interest were adjusted for multiple testing by the "BH" method (Benjamin and Hochberg, 1995).

2.2.14 Gene deletion and transformation experiments

2.2.14.1 PCR-based gene deletion

Disruption constructs for *AFT1*, *AFT2*, *COT1*, *ZRC1* and *URA3* genes were carried out using the method of Baudin *et al.* (1993) and Lorenz *et al.* (1995). The null alleles of those genes were created by one-step Polymerase Chain Reaction 'PCR' amplification which prepares gene deletion cassettes for the related genes. In this study, *AFT1*, *AFT2*, *COT1*and *ZRC1* open reading frame 'ORF' was replaced with a completely heterologous dominant resistance marker '*KanMX*'. *KanMX* module provided *S.cerevisiae* cells resistance to the drug Geneticin (G-418) expressing the aminoglycoside phosphotransferase activity (Jimenez and Davies, 1980).

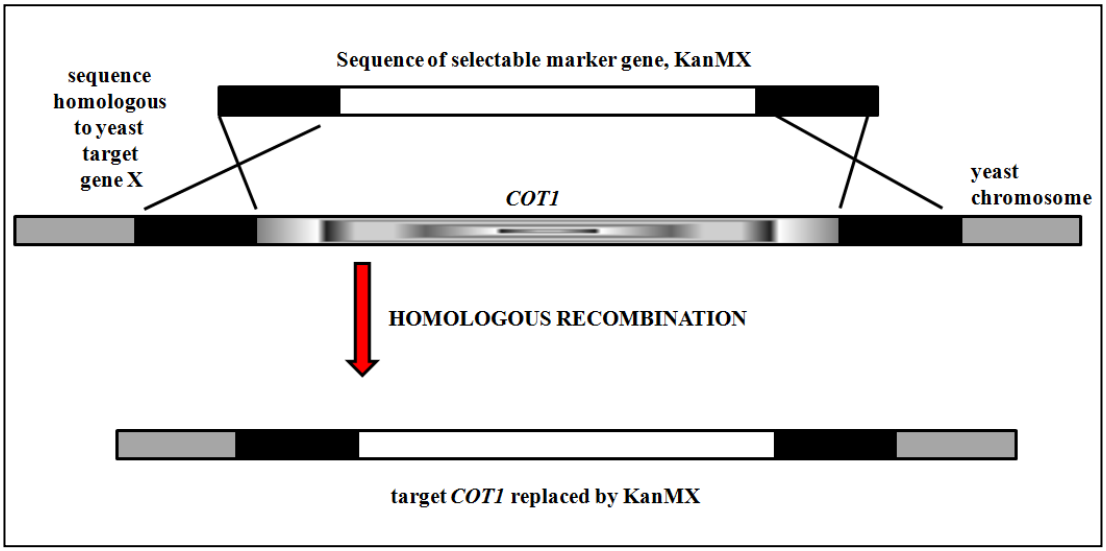


Figure 2.1 : A deletion cassette consists of sequences homologous (black) to each end of a target gene *COT1* (gradient path) and a selectable marker (white) sequence. The sequence homologous to the target yeast gene is approximately 30-50 nucleotide pairs in length (Alberts *et al.*, 2002).

2.2.14.2 Preparation of *aft1*Δ, *aft2*Δ, *cot1*Δ and *zrc1*Δ mutants

The primers flanking down and up-stream regions (~ -/+200) of the *KanMX* module were amplified by PCR using *aft1*Δ, *aft2*Δ, *cot1*Δ and *zrc1*Δ mutants derived from

the Yeast Knock-Out strains collection (YKO strain collection, BY background). The resulting product deletion cassette was then used for further yeast transformation experiment. Primers used for deletions and verifications of the target genes are shown in Table 2.12.

Table 2.12: Primers used for *aft1*Δ, *aft2*Δ, *cot1*Δ and *zrc1*Δ deletions and verification.

Primer Name	**Primer sequence 5'➔3'**
aft1::KAN_{MX4}_for	TCGGTGTCATTGACAAACCTC
aft1::KAN_{MX4}_rev	AGCACTACTATTTACACCAG
aft1_verification_for	CGCGAGATCCTAATAACCAAAC
aft2::KAN_{MX4}_for	CCTCAGTTTACTTTATACAAAG
aft2::KAN_{MX4}_rev	CGTGAACCATATTATAATCAACA
aft2_verification_for	TTAGAAATTTCAATAGCAAGTACT
cot1::KAN_{MX4}_for	TATAGCAATTGCCTGCGGAC
cot1::KAN_{MX4}_rev	TGCAAGACTATTGCTATTATATTA
cot1_verification_rev	TTCCAGTAAACTCTTCAATT
KAN_{MX4}_for	TGTTGATGCGCTGGCAGTG
KAN_{MX4}_rev	CCATACAATCGATAGATTGT
zrc1::KAN_{MX4}_for	TCTAATATGATTTCTTGGCATAG
zrc1::KAN_{MX4}_rev	CACACCGAAGTGAGCTTAATT
zrc1_verification_rev	ACATTTGAAAGATCCATGAAAGT

2.2.14.3 *cot1*Δ::*NatMX4* and *aft1*Δ::*KanMX4* double mutants and *ura3*Δ::*NatMX4* strains' preparation

Null mutants of *cot1*Δ::*NatMX4* and *ura3*Δ::*NatMX4* were constructed by replacing the gene of interest by selective cassettes *NatMX4* by homologous recombination *in vivo*. This sequence provides the resistance to nourseothricine (commercial name as clonNAT) which is an antibiotic for selection of the recombinant yeast cells (Goldstein *et al.*, 1999). *NatMX4* selective cassettes were amplified from a plasmid pGEM-T-ygp1_::*NatMX4*.

The primers used for the amplification of the gene deletion cassette contain two distinct regions (Table 2.13). One region is responsible for the homologous recombination at the target locus. The other region of the primers provides the amplification of the selectable *NatMX4* marker. Generally, 50 bases in the 5' end of the each forward and reverse primer is homologous to the 50 bases down- and upstream regions of corresponding genes' coding region, respectively.

*cot1*Δ::*NatMX4* & *aft1*Δ::*KanMX4* double mutant strains were obtained with *cot1*Δ::*NatMX4* deletant by deleting *AFT1* ORFs.

Table 2.13: Primer sequences for PCR-based gene deletion. The sites for the NAT_{MX} marker are shown in upper case and homologous recombination regions in lower case.

Primer Name	**Primer sequences 5' ➔ 3'**
cot1::NatMX_for	catagctatagaaagaaagttaacacaaagtacggaaagattgagtaaat CAGTATAGCGACCAGCATTC
cot1::NatMX_rev	taccgtataacgatttttaaaagtatttaattcttcacgctttttcgttataaagtcctt GACATGGAGGCCCAGAATAC
ura3::NatMX_for	tattaacccaactgcacagaacaaaaacctgcaggaaacgaagataaatc CAGTATAGCGACCAGCATTC
ura3::NatMX_rev	gctctaatttgtgagtttagtatacatgcatttacttata GACATGGAGGCCCAGAATAC

2.2.14.4 pRS416-*AFT1*-HA12x transformation

In order to localize Aft1p both in wild type and mutant cells, they were first transformed with an *ura3*::*NAT*mx cassette because plasmid carrying *AFT1*-HA12x has *URA3* marker. The transformation was verified by growing the transformant on the 5-Fluoroorotic Acid (0.05 % 'w/v'). The centromere vector (pRS416-*AFT1*) which contains Aft1p tagged with HA12x was then transformed to wild type and mutant *ura3* strains. This plasmid was a gift from Dr. Jerry Kaplan.

2.2.14.5 Yeast transformation by lithium acetate

Yeast transformation was realized according to Woods and Gietz (2001). Briefly, a preculture of the yeast cells was inoculated into 10 mL liquid YPD medium and incubated overnight at 30ºC, 150 rpm. The next day, this culture was diluted in 10 mL fresh YPD to an OD_{600} of 0.5 (~ $5x10^6$ cells/mL) and incubated for 3-5 additional hours until the culture OD_{600} reached about 2 (3×10^7 cells/mL). At this point, one mL of this culture was transferred into a microcentrifuge tube and the cells were harvested at 10000 rpm for 5 min. The supernatant was poured off and 100 µL of 100 mM lithium acetate solution was added to the cell pellet. After 30 min incubation at room temperature, cells were collected at maximum speed for 15

seconds. Lithium acetate was removed by a micropipette, followed by addition of the transformation mixture to the cell pellet (Table 2.14). After vigorous resuspension, the cell suspension was incubated at 30ºC for 30 min followed by 30 min of incubation in a water bath at 42°C (heat-shock phase). The cells were then centrifuged at 8000 rpm for 15 seconds and the cells pellet was resuspended in 150 µL sterile distilled water. This mixture was spread to selective media and incubated at 30ºC for 2-4 days to score for transformant cells.

Table 2.14: Yeast transformation mixture.

	Component	**Amount**
1	PEG (50% 'v/v')	90 µL
2	1 M LiAc	15 µL
3	ss-DNA (2 mg/mL)	4 µL
4	Plasmid DNA (0.1 – 1 µg) or Gene Deletion Cassette (0.5 – 5 µg)	X µL
5	Sterile dH_2O	18 µL

2.2.14.6 Transformation verification

Verification of correct gene deletion or insertion in the genome was done by PCR using colonies that grew on selective media. To this end, genomic DNA of the colonies was extracted and purified using MasterPure™ Yeast DNA Purification Kit (EPICENTRE, USA). The concentration of the genomic DNA was determined by NanoDrop 1000 spectrophotometer (Thermo SCIENTIFIC, USA). About 100 ng of genomic DNA was used for classical PCR in the presence of 0.25 mM dNTP and 0.25 µM primer sets. *Taq* DNA Polymerase (400 units, 5,000 units/mL; Biolabs, USA) was used for verification PCR analysis.

In order to check the correct genomic integration of the selection marker on the strains, forward and reverse primers were used wich can bind inside the geneticine or nourseothricine selection sequences on the upstream or downstream regions. Moreover, primers which can hybridize outside of the ORFs of the targeted genes were designed. These primers were used for the verification PCR analyses along with the others having the ability to bind inside the selection marker sequence. The deletant strains and the wild type cells were identified upon gel electrophoresis of PCR products, based on their size differences.

2.2.15 Sequence analysis of *AFT1, COT1* and *ZRC1* on the strains

2.2.15.1 Amplification of the target sequences

DNA sequences of *AFT1, COT1* and *ZRC1* in wild type and evolved mutants were analysed as follows: genomic DNA were extracted by using MasterPure™ Yeast DNA Purification Kit protocol (EPICENTRE, USA). The extracted DNA was used for the amplification of -1000 upstream and +200 downstream regions of *AFT1*, *COT1* and *ZRC1* genes on the corresponding strains (Table 2.15). The PCR product was purified from the gel and for some samples PCR purification was directly applied to the samples. QIAquick Gel Extraction Kit (QIAGEN, No. 28706) was used for both gel extraction and PCR purification experiments.

Table 2.15: Primers for sequence analysis.

Primer Name	**Primer sequence 5'➔3'**
AFT1_seq_for	AATAACACTAGTGAGGGAAGTA
AFT1_seq_rev	CCTGATCTTAGCACCGATATAT
COT1_seq_for	TATAGCAATTGCCTGCGGAC
COT1_seq_rev	TGCAAGACTATTGCTATTATATTA
ZRC1_seq_for	AAGGAAATGATATGAAAGTAGTTG
ZRC1_seq_rev	AGATGAGGAAGGATTGTGGC

2.2.15.2 Cloning of the target sequences

The purified PCR fragments were modified according to the A-tailing procedure by using *Taq* DNA Polymerase (Biolabs, USA) before performing ligation reaction with the pGEM-T Easy vector systems (Promega, USA). A-tailing and ligation reaction components are shown in Tables 2.16 and 2.17, respectively. The volumes were adjusted depending on the molar yield of the purified PCR product.

Table 2.16: A-tailing reaction components.

	Component	**Amount**	**Concentration/Activity**
1	PCR product	~1-7 µL	-
2	*Taq* DNA Polymerase Buffer with $MgCl_2$	1 µL	10X
3	dATP	X	Final concentration of 0.2 mM
4	*Taq* DNA Polymerase	X	5 U
5	Deionised water to a final reaction volume of 10 µL		

A-tailing of the PCR product was carried out at 70ºC for 15-30 min. These products could be further used for ligation reactions.

Ligation reaction mixture was prepared as shown in Table 2.17. The reaction components were mixed by pipetting and the mixture was incubated either for 1 h at room temperature or overnight at 4ºC.

Table 2.17: Ligation reaction components with pGEM-T Easy vector system.

	Component	**Amount**	**Concentration/Activity**
1	2X Rapid Ligation Buffer, T4 DNA ligase	5 µL	2X
2	pGEM-T Easy vector	1 µL	50 ng
3	PCR product	X µL	
4	T4 DNA ligase	1 µL	3 Weiss units/µL
5	Deionised water to a final reaction volume of 10 µL		

2.2.15.3 Transformation to bacteria

Transformation (Cohen *et al*., 1972) was performed by using thermo-competent *E.coli DH5α* cells. 1-5 µL ligation mixture was added to the microfuge tube that contained 100 µL of thermo-competent cells. The tube was incubated for 30 min on ice. The tube was then placed at 42ºC for 90 seconds. The tube was replaced again on ice for an additional 15 min. At the end of 15 min, cells were inoculated to ampicillin (150 µg/mL), IPTG and X-gal containing LB plates for blue/white screening.

The plates were incubated at 37ºC overnight and the next day white colonies were inoculated to LB ampicillin-containing liquid media separately by using toothpicks. The plasmid was extracted using alkaline extraction procedure (Birnboim and Doly, 1979). Three mL culture of overnight-grown bacteria was harvested with centrifugation at 13000 rpm (Table-top microcentrifuge, Eppendorf). The supernatant was discarded and the pellets were resuspended in 250 µL solution I (50 mM TrisHCl pH8, 10 mM EDTA, 100 mg/L RNase A). Then 250 µL solution II (200 mM NaOH and 1 % SDS ‘w/v’) was added to the resuspended cell solution. Finally, 350 µL solution III (Potassium acetate and Acetic acid glacial pH 5.5) was added to the mixture, and it was mixed by inverting the microfuge tubes. The tubes were placed in the centrifuge for a 10 min run at 13000 rpm. The clear supernatants were transferred to new microfuge tubes. Iso-propanol was added to the solutions containing the plasmids and the tubes were incubated at -20ºC for 15 min. They were

again centrifuged at 13000 rpm for 15 min. Supernatants were discarded and the pellets were washed with 70 % (v/v) ethanol. After evaporating ethanol from the pellets, 100 µL distilled water were added to the microfuge tubes. The plasmid solution was placed at -20ºC.

Restriction enzyme digestion was performed to test if the plasmid contained the insert or not. Plasmids which contained *COT1* and *ZRC1* were digested by using NcoI (Biolabs, USA) and BamHI+PstI (Biolabs, USA) respectively. The reaction for restriction digestion was realized as shown in Table 2.18.

Table 2.18: Restriction enzyme digestion reaction components.

	Component	**Amount**	**Concentration/Activity**
1	Plasmid	1-5 µL	-
2	Restriction enzyme	0.5 µL	
3	Restriction enzyme buffer	2.0 µL	-
4	BSA (depends on the enzyme)	2.0 µL	10X
5	Deionised water to a final reaction volume of 20 µL		

The reaction mixtures for restriction enzyme digestion were incubated at 37ºC for 3 h. They were then run on agarose gels. Gels were incubated in TAE containing ethidium bromide before taking photographs.

True plasmids were sent to sequencing which contained ORFs of *AFT1*, *COT1* and *ZRC1* genes along with the -1000/+200 bp regions. The sequence of wild type *AFT1*, *COT1* and *ZRC1* were aligned with the corresponding sequences of mutants.

2.2.16 Determination of *AFT1* and *COT1* expression levels by quantitative RT-PCR upon pulse cobalt stress exposure

Wild type and the mutants were inoculated to YMM with an initial optical density of 0.05. They were incubated in 250 mL flasks at 50 mL culture volume at 30ºC and 150 rpm. When they reached the OD_{600} of about 0.5-0.6 (4-6 x 10^6 cells/mL), samples were taken for subsequent RNA extraction. Then 2 mM $CoCl_2$ was added to the cultures as a stress factor and they were cultivated at 30ºC and 150 rpm for 90 min. Sample cultures were then taken by setting the cell content the same as those of the control samples. These cultures were used for total RNA extraction as well and all RNA samples were stored at −80 °C until further processing.

RNA extraction was realized with SV Total RNA Isolation System (Promega, USA) and the concentration was determined with NanoDrop 1000 Spectrophotometer

(Thermo SCIENTIFIC, USA). iScript™ cDNA Synthesis Kit (*BIO-RAD*, USA) was used for the cDNA synthesis of the samples. Quantitative RT-PCR experiment was performed by using IQ™ SYBR® Green Supermix (*BIO-RAD*, USA) kit. All procedures were done according to the manufacturer's recommendations and the primers used for quantitative RT-PCR analyses are indicated in Table 2.19.

Table 2.19: Primers used for quantitative RT-PCR analyses.

Primer Name	**Primer sequence 5'➔3'**
ACT1_QPCR_for	ATTATATGTTTAGAGGTTGCTGCTTTGG
ACT1_QPCR_rev	AATTCGTTGTAGAAGGTATGATGCC
AFT1_QPCR_for	ATGCATCTAAAAGGCCATGC
AFT1_QPCR_rev	GGCAGTGGCAAGATTTCATT
COT1_QPCR_for	GCATGGTGTGTTTCTTCACG
COT1_QPCR_rev	GGAAGCCTTGCACGATAGAG

2.2.17 Cobalt and iron content determination by atomic absorption spectrophotometer

Cobalt and iron contents of the wild type and the mutants were determined by flame atomic absorption spectrophotometry (FAAS) upon pulse and continuous exposure to metal stress, as described in this section.

2.2.17.1 Cobalt and iron content determinations of the strains upon pulse metal stress application

Wild type and mutant with their deletant strains of *aft1*Δ, and *cot1*Δ were inoculated to 150 mL yeast minimal medium in 1 L flasks at an initial OD_{600} value of 0.35 (5 x 10^6 cells/mL). When they reached an OD_{600} of about 3 (4.5 x 10^7 cells/mL), they were transferred to 50 mL culture tubes in 10 mL culture volume. They were incubated for 90 min in the absence of the metal ions and also in the presence of 2.5 mM cobalt ($CoCl_2$), 2 mM iron (Ammonium iron (II) sulfate), and both 2.5 mM cobalt and 2 mM iron ions. Three tubes were prepared for each condition. After incubation, cells were centrifuged at 5000 rpm (Rotor, Beckman Coulter JA-30.50i, USA) for 10 min. The supernatants were discarded and the cell pellets were washed twice by using double distilled water to separate cells from any metal residues left on them. Cell pellets were dried at 110 ºC for 2 h. Cell dry weights were determined and the cells were hydrolyzed with 10 M HNO_3 solution for two h at 90 ºC. At the end of this process, samples were measured by using Varian AA 280FS (USA) model flame

atomic absorption spectrophotometer (FAAS). Measurements were carried out at 240.7 nm and 248.3 nm wavelengths for cobalt and iron, respectively.

2.2.17.2 Cobalt and iron content determination of the cells upon continuous metal stress application

Yeast precultures were inoculated into YMM and incubated overnight at 30°C, 150 rpm. After overnight incubation, precultures were inoculated into 10 mL YMM as a control, and 10 mL YMM containing 2.5 mM $CoCl_2$ and 2 mM ammonium iron (II) sulfate in 50 mL-culture tubes. The experimental procedure was performed as three replicates for each condition. The cultures were incubated for 24 h under these stress and non-stress (control group) conditions at 30°C, 150 rpm. After incubation, cells were centrifuged at 5000 rpm (Rotor, Beckaman Coulter JA-30.50i, USA) for 15 min. The supernatants were discarded and the cell pellets were washed twice by using double distilled water to separate cells from any metal residues left on them. Cell pellets were dried at 110ºC for 2 h. Cell dry weights were determined and the cells were hydrolysed with 10 M HNO_3 solution for two h at 90ºC. At the end of this process, metal contents were determined by using Varian AA 280FS (USA) model flame atomic absorption spectrophotometer (FAAS).

2.2.18 Confocal microscopy analysis

Fluorescent protein tagged (GFP fused to the N terminus of *AFT1* gene – vector used pRS426) cells were cultivated in YMM until the exponential growth phase in the absence and presence of cobalt stress (2mM), and then collected by centrifugation (3000 *g*, 5 min). Images were captured on a Leica DM4000B fluorescent microscope equipped with a Digital Colour Camera coolsnap camera (DFC 300FX) and a FW4000 software (Leica). A 63x / 1.4-0.6 Oil HCX Plan-Apochromat objective from Leica was used. The resolution in XY with this objective was 0.2 mm for DAPI and GFP. The camera has a resolution of 0.1 mm with the X63 objective (6.45µm × 6.45µm pixel size). Exposure times were 50 to 200 ms for DAPI (excitation k = 360/40 nm ; leica ; A4) and 100 to 300 ms for GFP (excitation k = 480/40 nm ; leica ; L5). Images were minimally adjusted for brightness and contrast using image J.

3. RESULTS

3.1 EMS Treatment of the Wild Type Strain

EMS mutagenesis was applied to wild type *Saccharomyces cerevisiae* for 15, 30, 60 and 90 min, as described in Materials and Methods, to increase genetic diversity of the starting population. After EMS treatment and incubation at 30°C, 150 rpm for 24 h, OD_{600} values were measured. Those data were used to calculate their growth fitness.

Table 3.1: OD_{600} values of EMS applied cultures and their corresponding growth fitness data upon 24 h incubation.

	OD_{600} values upon 24 h of incubation	**Growth fitness results**
Control (without EMS stress)	18.85	-
EMS application for 15 min	14.50	0.77
EMS application for 30 min	13.15	0.70
EMS application for 60 min	9.65	0.51
EMS application for 90 min	3.90	0.21

It was decided to use 90 min-EMS treated culture as the starting population for cobalt stress selection.

3.2 Direct Selection Mutants

EMS-treated mutant culture of *S.cerevisiae* was inoculated to 5 mM $CoCl_2$-containing solid YMM. The colonies which could grow upon incubation at 30ºC were picked and they were used to compare the efficiency of the mutagenesis/direct selection and evolutionary engineering strategies.

3.2.1 Direct selection mutant phenotypes

Individual mutants obtained from direct selection strategy (ECo1, ECo2, ECo3 and ECo4) were tested for their cobalt resistance phenotypes on 2.5 and 5 mM $CoCl_2$-containing medium.

The precultures of the direct selection mutants were grown as 10-mL liquid YMM cultures in 50-mL culture tubes at 150 rpm and 30ºC. When reaching mid-logarithmic phase of growth (OD_{600}=3, 4.5x10^7 cells/mL), population cells tested on solid YMM containing different concentratin of cobalt, along with the wild type. All the individual mutants obtained from direct selection strategy were more resistant to cobalt than the wild type (Figure 3.1). Interestingly, ECo1, ECo3 and ECo4 grew more poorly in the control medium than ECo2. Unlike ECo2, they seemed to have a growth defect which might be originated from the EMS application (Kwon *et al.*, 2012).

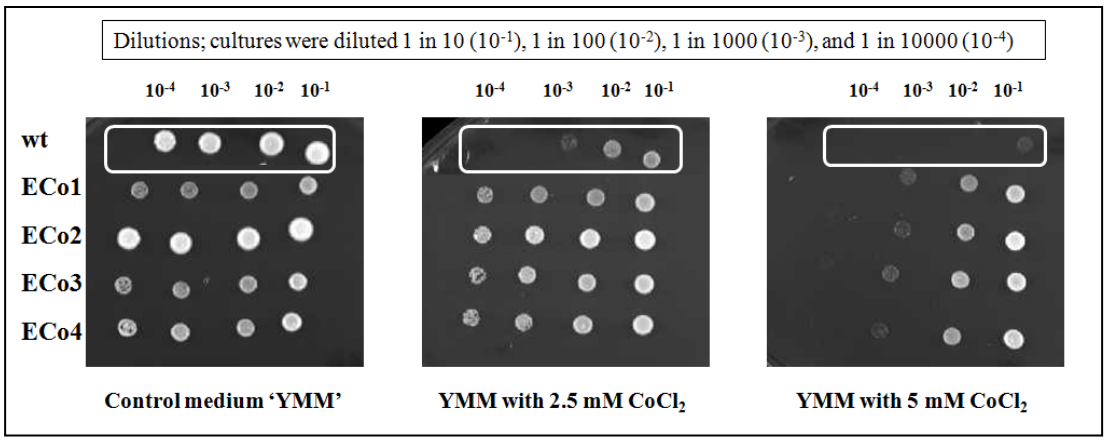


Figure 3.1 : Spot test results of mutants obtained by direct selection strategy with the wild type on media containing 2.5 and 5 mM $CoCl_2$ (wild type is indicated in a white frame). Cells were spotted (10^{-1} to 10^{-4}) in 1:10 dilution series and growth was monitored after 3 days of incubation at 30°C.

3.2.2 Genotype determination

Among the individual mutants obtained from direct selection strategy, ECo2 and ECo4 were selected for further genotype determination because ECo2 did not show any growth defect and ECo4 was one of the individual mutants with growth retardation. They were mated with opposite mating type wild type. Their corresponding diploids were dissected upon sporulating them on KAc media. The spore colonies of 'ECo2 X wt' grew without growth defects on the complete medium (YPD). However, among the 'ECo4 X wt' spore progeny, some spores grew to large sizes and some other grew to very small sizes (Figure 3.2).

Figure 3.2 : Tetrad dissection results of 'ECo4 X wt' diploid.

The diploids from the 'ECo2 X wt' and 'ECo4 X wt' crosses were tested with the parental strains for their cobalt stress resistances. The resistance to cobalt was found to be semi-dominant for both heterozygous diploides 'ECo2 X wt' and 'ECo4 X wt' (Figure 3.3).

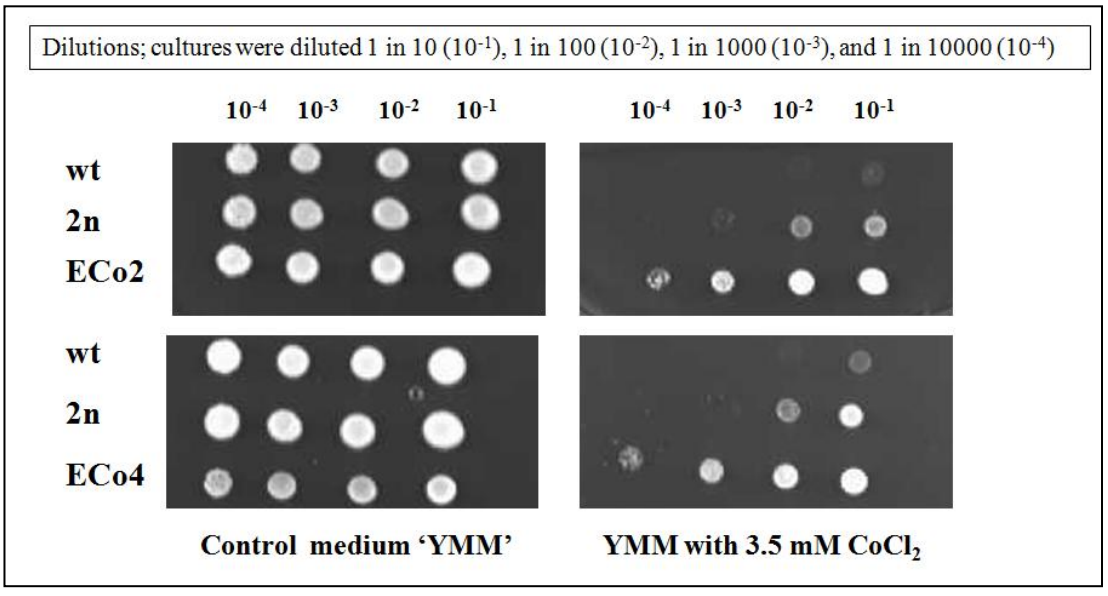


Figure 3.3 : Spot test results of wild type, diploid (2n strain obtained from genetic cross of ECo2 and ECo4 with opposite mating type wild type) and the haploid mutant ECo2 and ECo4 in the YMM and 3.5 mM $CoCl_2$-containing YMM.

Tetrads were dissected from the 'ECo2 X wt' and 'ECo4 X wt' diploids respectively and their growth on cobalt-containing media were investigated. 2^+:2^- segregation of the spores were observed neither for 'ECo2 X wt' nor for 'ECo4 X wt' spore progenies. It can be suggested that direct mutants have mutations in more than one

gene that might be involved in cobalt resistance. Nonetheless, more tetrads should be selected to conclude definitively on this segregation trait (Figure 3.4 and 3.5).

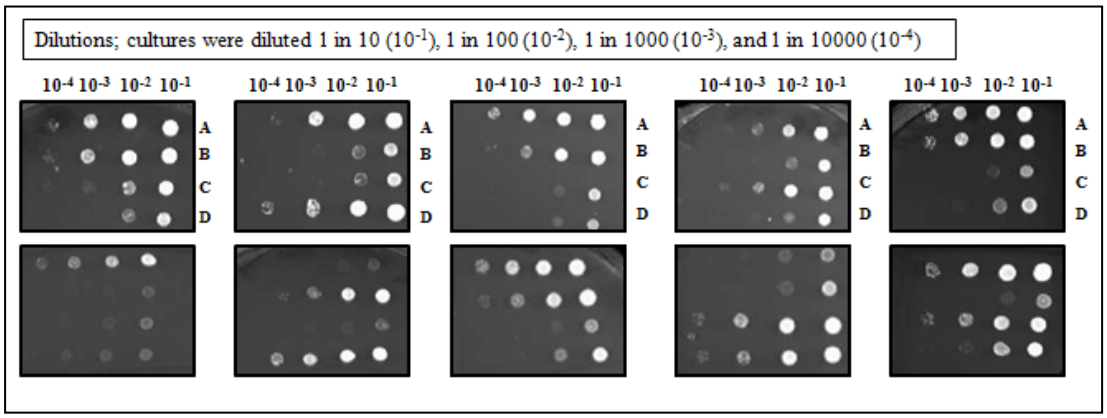


Figure 3.4 : Tetrad analysis results of 10 tetrads obtained from the diploid 'ECo2 X wt' on YMM with 3.5 mM $CoCl_2$ (A, B, C, D represent different spores).

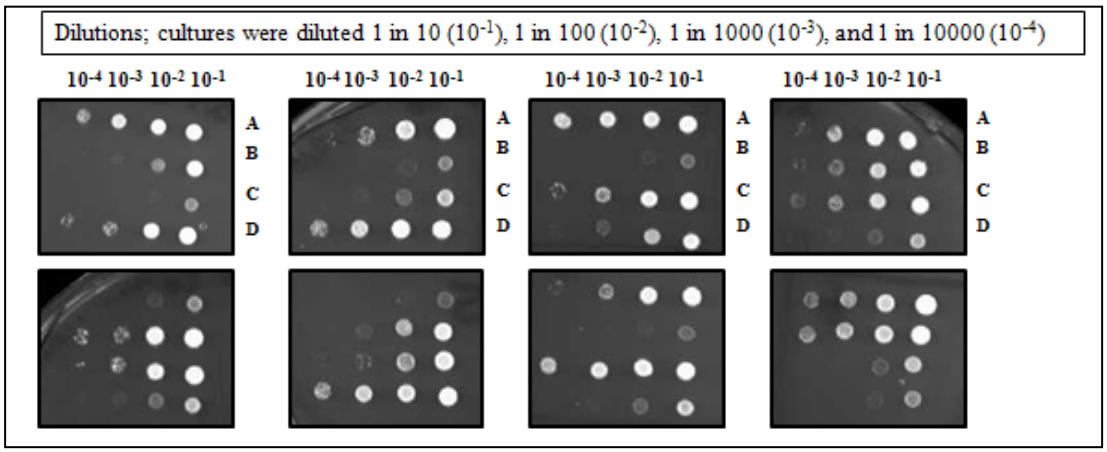


Figure 3.5 : Tetrad analysis results of 8 tetrads obtained from the diploid 'ECo4 X wt' on YMM with 5 mM $CoCl_2$ (A, B, C, D represent different spores).

3.3 Evolutionary Engineering Strategy to Obtain *S.cerevisiae* Resistant to Cobalt Ions

The strategy that was adopted for cobalt-resistant *S.cerevisiae* cells was as follows: An EMS-treated starting population was subjected to sequential cultivation in the presence of increasing concentrations of cobalt (0.5 mM to 8 mM). Individual clones were then selected for their capability to grow in the presence of 5 mM cobalt. The clone with the highest resistance was then phenotypically and genetically characterized.

3.3.1 Screening for cobalt stress resistance of wild type and EMS-treated cells

Wild type 'wt' culture that was incubated for 60 and 90 min in the presence of EMS were named as wt 60' and wt 90'. They were used with the wild type for the $CoCl_2$ stress resistance screening. They were grown as 20 mL-YMM cultures in 100 mL-flasks and those precultures were inoculated into YMM containing 0, 2.5, 5, 7.5, 10, 15 and 20 mM $CoCl_2$. The optical density of the cultures was measured after 24 h incubation and the growth fitness was calculated (Table 3.2).

Table 3.2: Stress resistances (as growth fitness) of the original and 60'/90' min EMS mutagenized wild type cells upon exposure to varying levels of cobalt stress, after 24 h of incubation.

	Growth fitness of the cultures (OD_{600} of stress applied sample / OD_{600} of control sample)					
	2.5 mM Cobalt	5 mM Cobalt	7.5 mM Cobalt	10 mM Cobalt	15 mM Cobalt	20 mM Cobalt
wt	0.52	0.37	0.27	0.17	0.11	0.10
wt 60'	0.37	0.21	0.16	0.11	0.08	0.06
wt 90'	0.28	0.24	0.17	0.12	0.09	0.09

The concentration of cobalt used appeared quite strong prevailing significant growth of the wild type and EMS-treated strains tested (Table 3.2). For that reason, the experiments were repeated by lowering the concentration of these metal ions to 0.5, 1, 1.5, 2 and 2.5 mM (Figure 3.6).

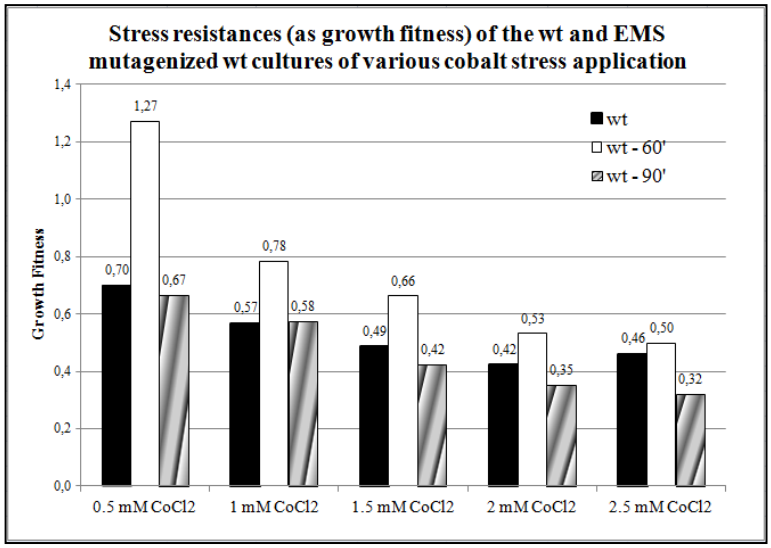


Figure 3.6 : Growth fitness of wt and EMS-mutagenized cultures (wt 60' and wt 90') after 24 h in the presence of 0.5, 1, 1.5, 2 and 2.5 mM $CoCl_2$.

The presence of 0.5 mM $CoCl_2$ in the growth medium did not decrease the growth significantly. For this reason, the evolutionary engineering strategy was applied to the EMS-treated cells using 0.5 mM $CoCl_2$ as the initial stress level for selection.

3.3.2 Cobalt-resistant populations obtained by evolutionary engineering

Cobalt resistant populations were obtained using a continuous selection strategy as described in Section 2.2.4.3 of Materials and Methods.

The OD_{600} values of each population and their corresponding control culture were measured three times (Table 3.3 and 3.4). Growth fitness of each population was calculated by dividing the arithmetic average of stress-treated population's OD_{600} result to that of its control population (Figure 3.7).

Table 3.3: OD_{600} results of populations under non-stress conditions ($OD_{1,2,3}$ correspond to three independent measurements of OD_{600}; OD_{avg} stands for arithmetic mean value of three independent OD_{600} measurements. St Dev indicates standard deviation of three measurements.).

Population number	OD_1	OD_2	OD_3	OD_{avg}	St Dev
1st	6.67	6.53	6.67	6.62	0.08
2nd	6.80	6.63	6.83	6.75	0.11
3rd	5.87	5.87	6.01	5.92	0.08
4th	5.94	6.01	5.97	5.97	0.04
5th	5.48	5.81	5.91	5.73	0.23
6th	5.61	5.78	5.78	5.72	0.10
7th	6.47	6.50	6.44	6.47	0.03
8th	6.77	6.67	6.60	6.68	0.09
9th	6.63	6.50	6.47	6.53	0.09
10th	7.10	7.16	6.93	7.06	0.12
11th	7.19	6.90	6.93	7.01	0.16
12th	6.86	6.90	6.86	6.87	0.02
13th	6.50	6.47	6.60	6.52	0.07
14th	6.77	6.67	6.63	6.69	0.07
15th	6.73	6.70	6.40	6.61	0.18
16th	6.63	6.50	6.50	6.54	0.08
17th	6.90	6.90	6.93	6.91	0.02
18th	6.93	6.86	7.10	6.96	0.12
19th	6.67	6.86	6.67	6.73	0.11
20th	6.63	6.67	6.57	6.62	0.05
21st	6.70	6.67	6.67	6.68	0.02
22nd	6.60	6.63	6.53	6.59	0.05
23rd	6.60	6.67	6.44	6.57	0.12
24th	6.86	7.06	6.93	6.95	0.10
25th	6.80	6.67	6.80	6.76	0.08

Table 3.4: OD_{600} results of stress-treated populations ($OD_{1,2,3}$ correspond to three independent measurements of OD_{600}; OD_{avg} stands for arithmetic mean value of three independent OD_{600} measurements. St Dev indicates standard deviation of three measurements.).

Population number	$CoCl_2$ (mM) concentration for the corresponding populations	OD_1	OD_2	OD_3	OD_{avg}	St Dev
1st	0.50	5.54	5.38	5.45	5.46	0.08
2nd	1.00	5.41	5.38	5.28	5.36	0.07
3rd	1.50	2.67	2.71	2.74	2.71	0.04
4th	2.00	1.65	1.58	1.78	1.67	0.10
5th	2.50	1.82	1.91	1.88	1.87	0.05
6th	3.00	1.82	1.82	1.85	1.83	0.02
7th	3.50	3.60	3.60	3.37	3.52	0.13
8th	3.75	3.50	3.50	3.50	3.50	0.00
9th	4.00	1.65	1.55	1.72	1.64	0.09
10th	4.25	2.28	2.24	2.21	2.24	0.04
11th	4.50	4.13	4.49	4.09	4.24	0.22
12th	4.75	4.42	4.52	4.72	4.55	0.15
13th	5.00	4.62	4.55	4.49	4.55	0.07
14th	5.25	4.62	4.72	4.62	4.65	0.06
15th	5.50	5.18	5.08	4.85	5.04	0.17
16th	5.75	3.47	3.17	3.14	3.26	0.18
17th	6.00	4.49	4.46	4.46	4.47	0.02
18th	6.25	3.70	3.80	3.73	3.74	0.05
19th	6.50	3.30	3.27	3.07	3.21	0.13
20th	6.75	3.53	3.47	3.63	3.54	0.08
21st	7.00	3.60	3.53	3.70	3.61	0.09
22nd	7.25	4.06	4.16	4.03	4.08	0.07
23rd	7.50	3.17	3.23	3.20	3.20	0.03
24th	7.75	3.56	3.50	3.40	3.49	0.08
25th	8.00	2.15	2.08	2.05	2.09	0.05

Growth fitness of the populations had been fluctuated from one passage to another. It decreased gradually during the selection procedure, which lasted for 25 successive populations (Figure 3.7).

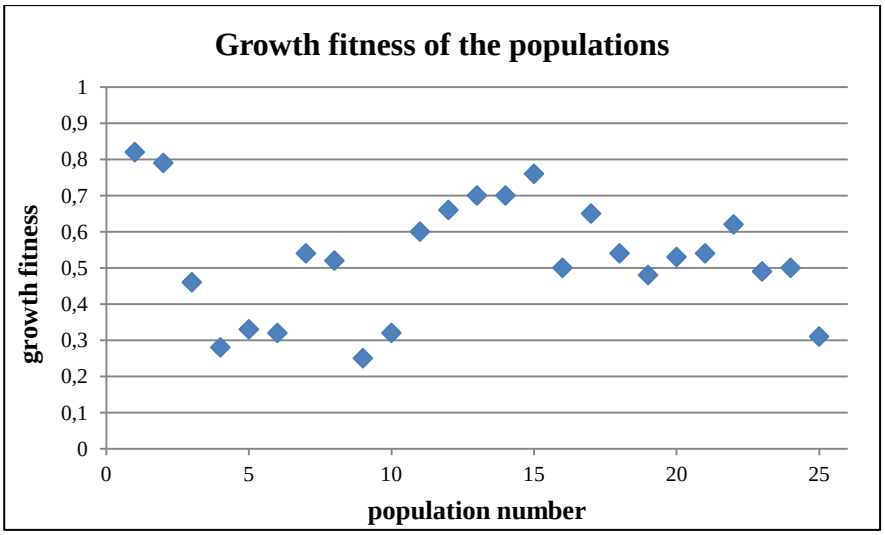


Figure 3.7 : Growth fitness of the successive populations.

3.3.3 Individual mutant selection

At 25th population, it was observed that the growth fitness decreased to 31%. It was decided to stop the selection for the cobalt-resistant strain at this stage. Since a mutant population was obtained in this study, it was thought that the intrinsic resistances of individual cells in the population could be highly different from that of the population (Çakar *et al.*, 2005). To this end, 25th population was diluted from 10 to 10^6-fold serially and 10^5 and 10^6-fold diluted samples were inoculated into YMM solid plates. Eight individual colonies were randomly selected from these solid plates (CI25A, CI25B, CI25C, CI25D, CI25E, CI25F, CI25G, and CI25H). They were tested in 2.5, 5 and 10 mM $CoCl_2$-containing YMM in order to search for those exhibiting the highest resistance to 5-10 mM cobalt.

All the individual mutants and the final population could grow up to 10 mM $CoCl_2$, while growth of the wild type was already strongly impaired at 2.5 mM cobalt (Figure 3.8).

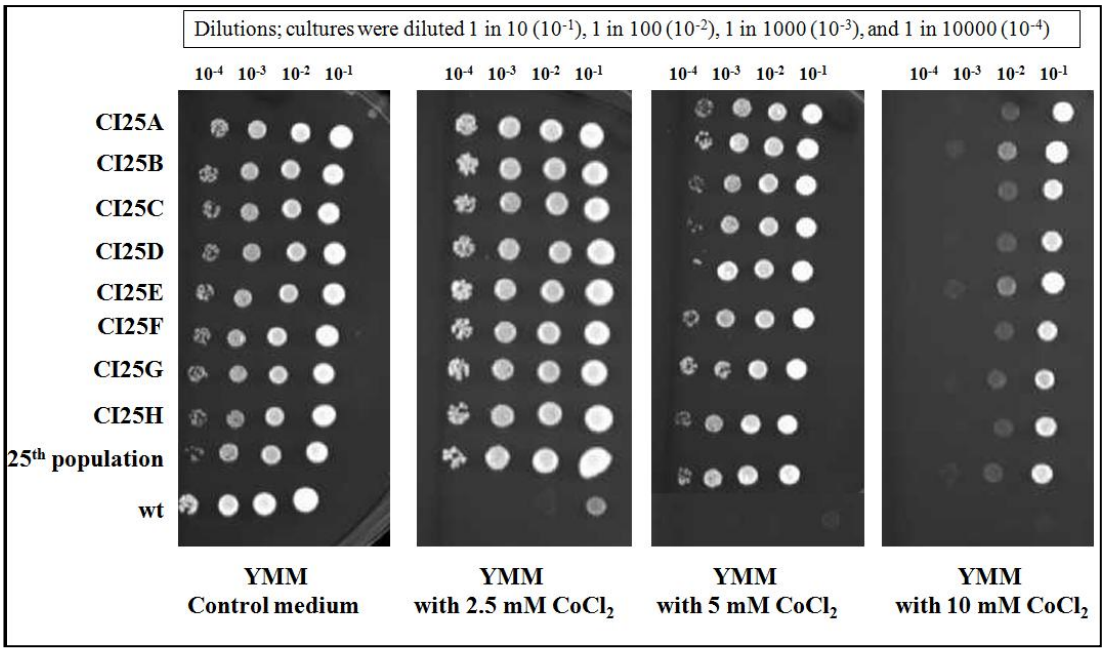


Figure 3.8 : Spot test results under control conditions and in the presence of varying cobalt levels (2.5, 5 and 10 mM $CoCl_2$) for individual mutants, wt and 25th population.

Parallel to this experiment, the individual colonies were inoculated in liquid YMM alone and in YMM containing 5 mM $CoCl_2$. The initial OD_{600} of the cultures were set to 0.3 and after 24 h of incubation OD_{600} values were measured (Figure 3.9).

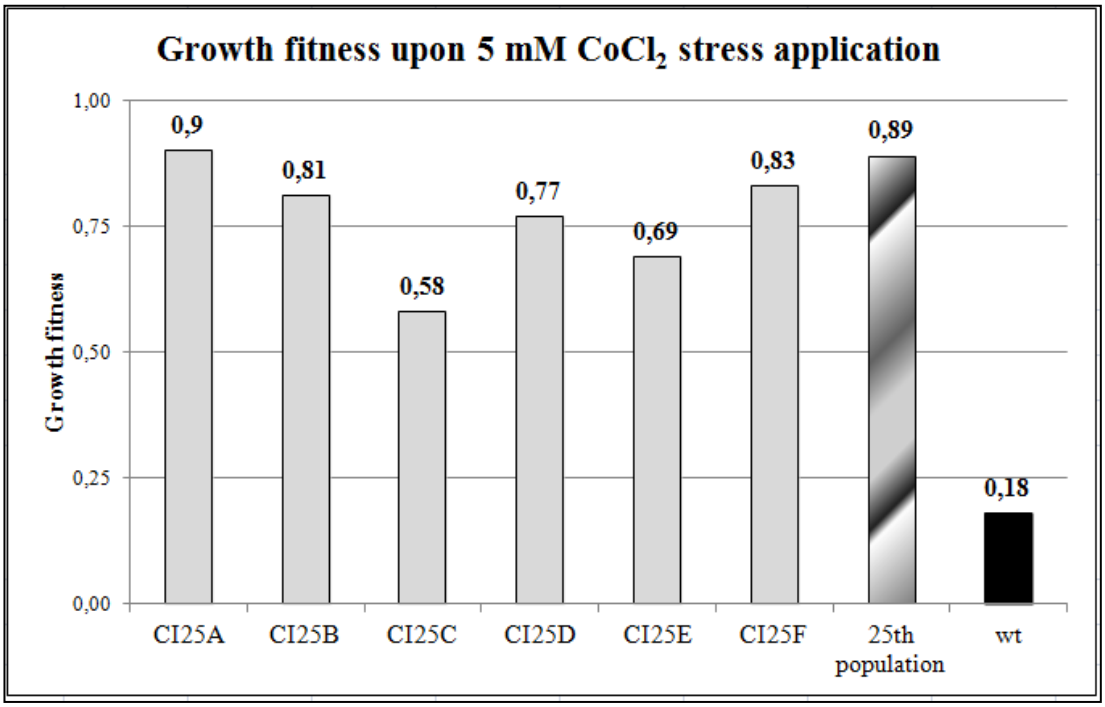


Figure 3.9 : Growth fitness data of wt, 25th population and individual mutants upon 24 h exposure to 5 mM $CoCl_2$.

Individual mutants obtained by evolutionary engineering were significantly more resistant to $CoCl_2$ both in liquid and solid media compared to the wild type (Figure 3.8 and 3.9). There were also differences in cobalt resistance levels between individual clones.

3.3.4 Phenotypic properties of the individual mutants

3.3.4.1 Estimation of stress resistance by spot test

Individual mutants were tested for their possible cross-resistances to different types of stress in solid media. For that reason, 8 individual mutants (CI25A, CI25B, CI25C, CI25D, CI25E, CI25F, CI25G, and CI25H), 25th population and the wild type were grown in YMM until their exponential growth phase of growth. They were serialy diluted and spotted onto the YMM agar plates supplemented with nickel (0.25 and 0.5 mM), copper (0.1 mM), zinc (10 mM), iron (50 mM), manganese (20 mM), magnesium (1 M), sodium chloride (0.5 M), ethanol (8% 'v/v'), hydrogen peroxide (1 mM), and caffeine (10 mM).

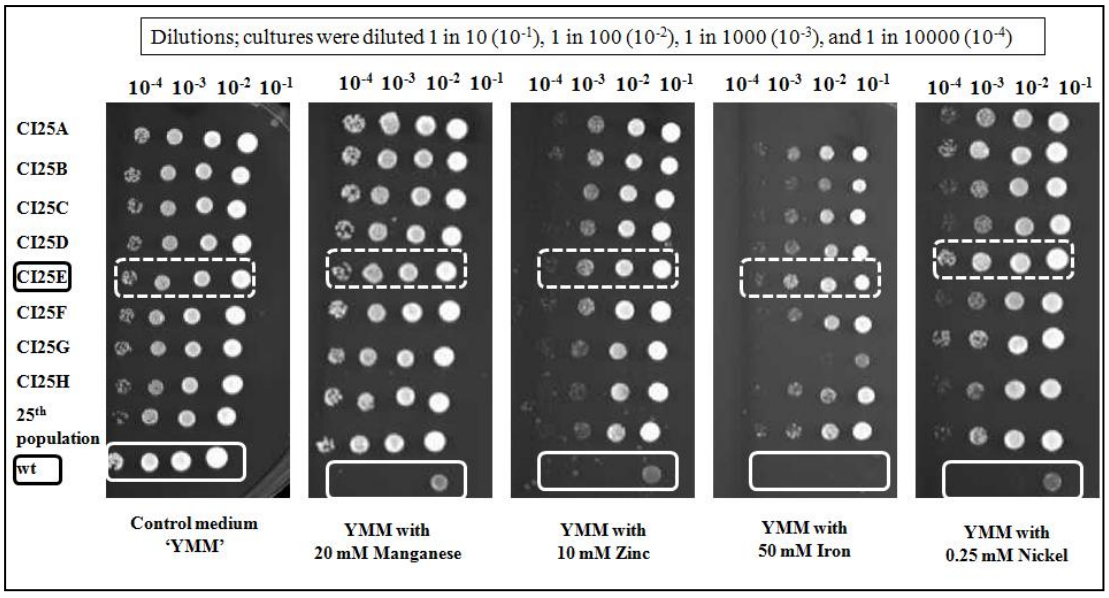


Figure 3.10 : Spot test results of wild type, individual mutants and the final population (25th population) on YMM plates containing different stress factors to determine cross resistances, if any (white compound box indicates wild type and white dashed box indicates the results of the most resistant mutant).

On both Figure 3.10 and 3.11, wild type and CI25E are shown inside white compound and dashed frames, respectively.

Cobalt resistant strains exhibited cross-resistance to zinc, manganese, iron and nickel (Figure 3.10).

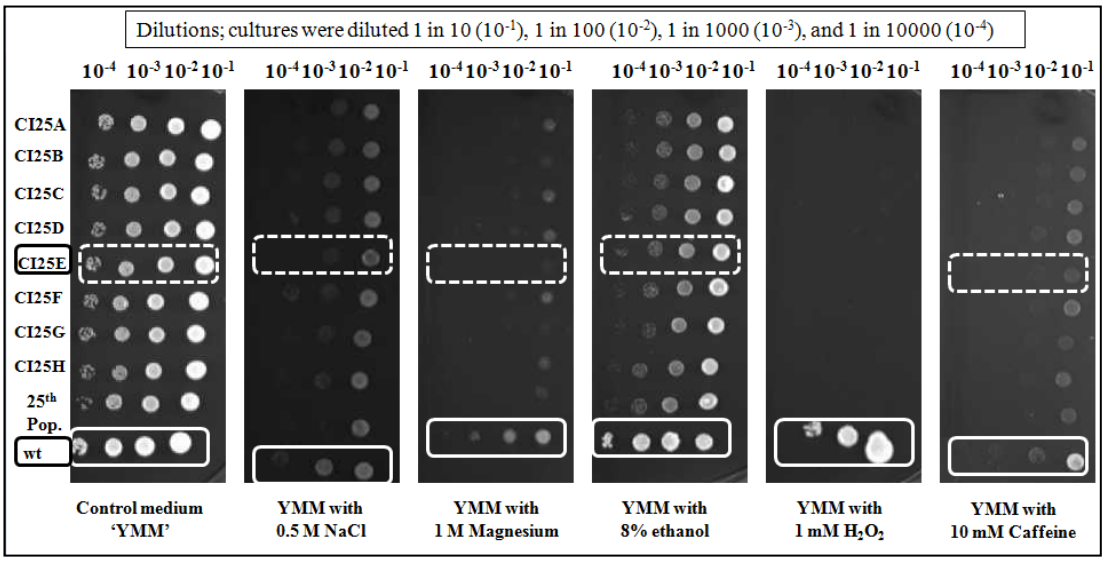


Figure 3.11 : Spot test results of wild type, individual mutants and the final population (25th population) on YMM containing NaCl, magnesium, ethanol, hydrogen peroxide and caffeine as stress factors.

In Figure 3.11, the stress types to which the individual mutants showed sensitivity are shown. The growth of individual mutants was highly sensitive to H_2O_2, sodium chloride, magnesium, ethanol, and caffeine.

3.3.4.2 Estimation of stress resistance by MPN Method

Stress resistance tests were applied under continuous stress conditions using MPN method. Selected individual mutants, final population and wild type were cultured in liquid YMM and YMM containing 5–8 mM $CoCl_2$ and 0.8 mM H_2O_2, respectively for 72 h. For each condition, a corresponding preculture was serially diluted for 8 times in five different columns of the 96-well plate. Resistance to cobalt and H_2O_2 was estimated upon determination of the viable cell numbers and survival rate. 'Survival rate' was calculated by dividing the number of stress-treated viable-cells to that of non-treated cells, as described previously (Section 2.2.3.1).

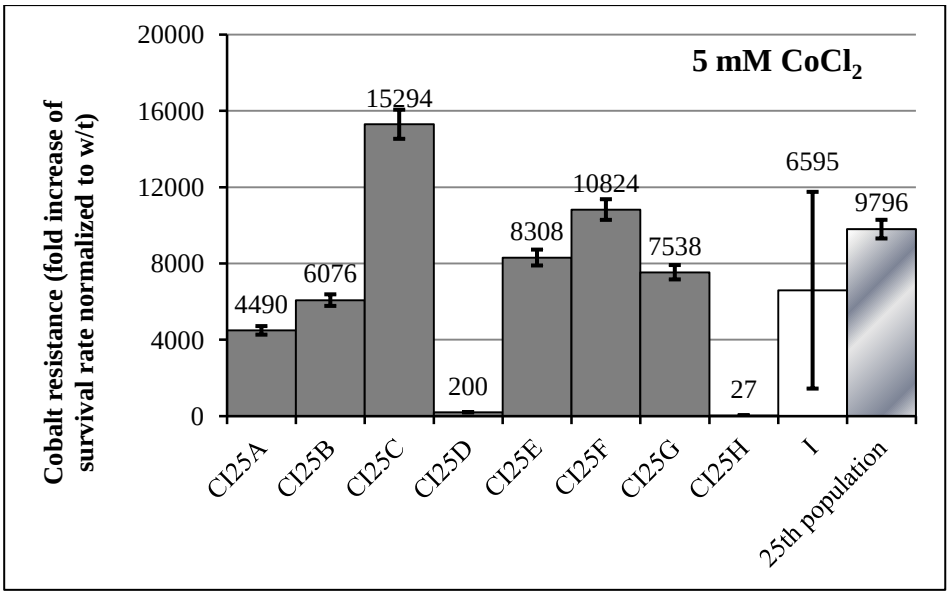


Figure 3.12 : Determination of survival rate of 25th population and individual mutants at 5 mM $CoCl_2$ continuous stress application, 72 h incubation results (I is the arithmetic average of the individuals CI25A to CI25H).

The cobalt resistance was expressed as the fold increase in survival rate normalized to that of wild type. The survival rate of the final population at 5 mM cobalt was about 9800-fold higher than that of the wild type (Figure 3.12). Moreover, the survival rate of individual mutants ranged from 27 to about 15'000-fold of the wild type. MPN method also demonstrated that the mutants obtained by evolutionary engineering were significantly more resistant to cobalt than the wild type.

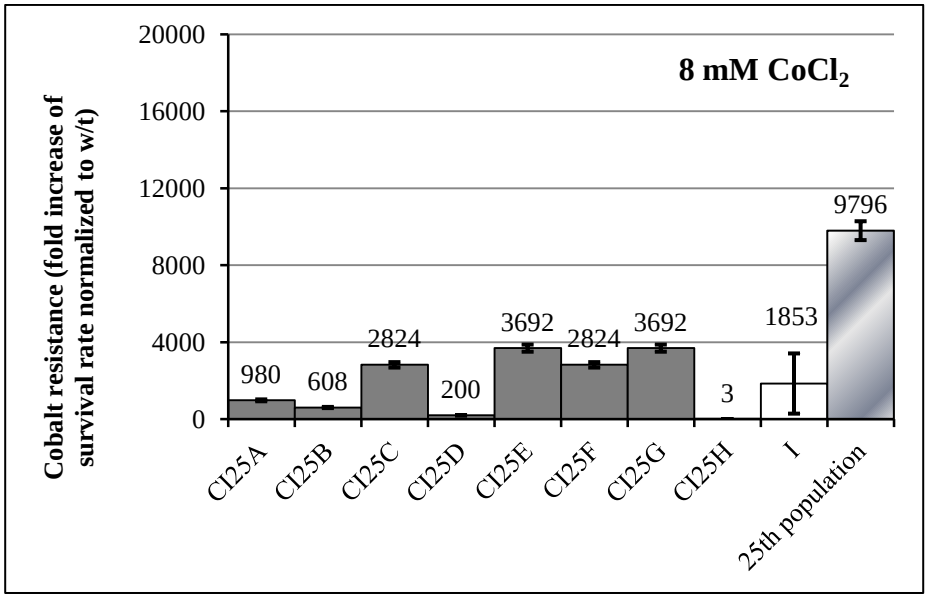


Figure 3.13 : Determination of survival rate of 25th population and individual mutants at 8 mM $CoCl_2$ continuous stress application, 72 h incubation results (I is the arithmetic average of the individual mutants CI25A to CI25H).

Isolated cells, CI25A to CI25H, harbored survival rates to 8 mM cobalt that were ranging from about 3–3692-fold higher than that of wild type. However, the 25th population's survival rate was about 10'000-fold of that of the wild type. 25th population showed the highest fold change in survival rate, among all cultures tested (Figure 3.13).

Cross-resistance to H_2O_2 stress was also evaluated by MPN method upon continuous exposure to this stress factor (Table 3.5).

Table 3.5: Hydrogen peroxide cross-resistance of cobalt-resistant individual mutants upon continuous H_2O_2 stress exposure, 72 h incubation results (I is the arithmetic average of the individual mutants CI25A to CI25H).

Culture name	**Number of cells/mL at Control Medium**	**Number of cells/mL at 0.8 mM H_2O_2**	**% Survival at 0.8 mM H_2O_2**	**Survival as fold of wt at 0.8 mM H_2O_2**
CI25A	7900000	23	0.0003	0.00001
CI25B	3300000	23	0.0007	0.00003
CI25C	4900000	23	0.0005	0.00002
CI25D	4900000	23	0.0005	0.00002
CI25E	7000000	23	0.0003	0.00001
CI25F	4900000	23	0.0005	0.00002
CI25G	3300000	23	0.0007	0.00003
CI25H	1700000	23	0.0014	0.00006
I	4737500	23	0.0006	0.00003
25th population	3300000	23	0.0007	0.00003
Wild type	3300000	790000	23.9394	**1**

The survival rates upon hydrogen peroxide (H_2O_2) stress were determined (Table 3.5). All the individual mutants and final population were highly sensitive to continuously applied hydrogen peroxide stress.

3.4 Genetic Characterization of One of the Individual Mutants 'CI25E'

3.4.1 Genetic stability test with CI25E

As described in Section 2.6.6, preculture of CI25E was inoculated into YMM at an initial OD_{600} of 0.3. After 24 h incubation, this culture was inoculated into fresh YMM again at an initial OD_{600} value of 0.3. This cultivation was repeated 5 times and at the end of each 24 h of cultivation, stock solutions of each culture were prepared. After obtaining five stock cultures in this way, their precultures were

inoculated into solid YMM and YMM containing cobalt (5 mM $CoCl_2$). CI25E showed approximately the same stress resistance pattern for each different subculture (Figure 3.14). It can thus be suggested that cobalt resistance of CI25E is a genetically stable trait.

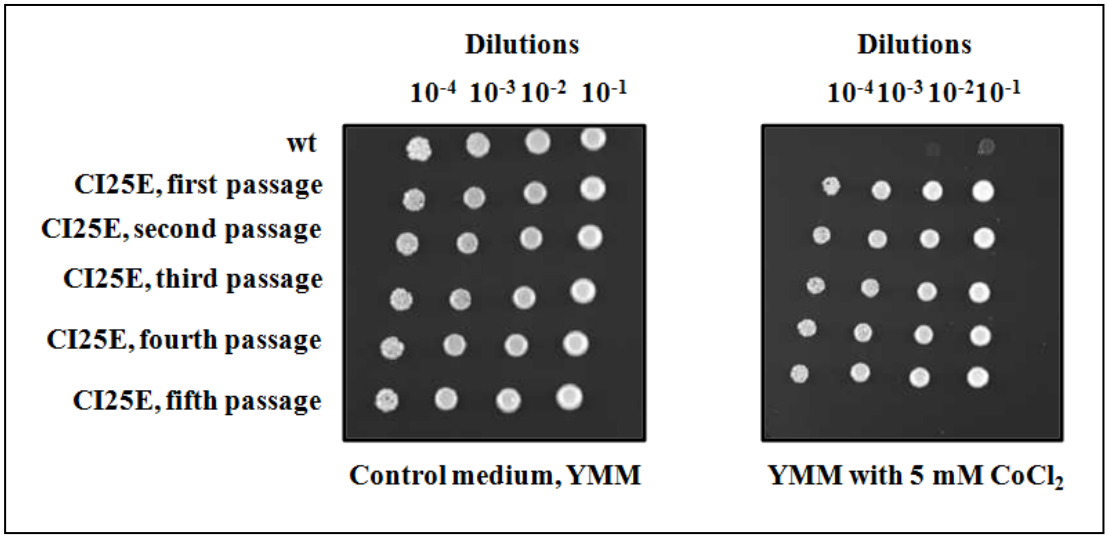


Figure 3.14 : Genetic stability test results of CI25E upon five successive passages in YMM without stress, resistance was determined in the presence of 5 mM $CoCl_2$.

3.4.2 Tetrad Analysis of 'CI25E x wt' diploid

The selected 'evolved' CI25E strain was backcrossed with the original wild type CEN.PK113-1A *MATα* to determine whether on the one hand, the cobalt phenotype was dominant or recessive, and on the other hand, if this resistant phenotype was associated with a single genetic event.

Heterozygote diploids "CEN.PK X CI25E" were sporulated by spreading cells onto KAc solid medium. After 2 days, tetrads were produced and dissected on YPD. The individual spores obtained from more than 25 independent tetrads, the haploid, CI25E, *S.cerevisiae MATα* and the "CEN.PK X CI25E" diploid were tested for resistance to cobalt on solid YMM. Diploid had semi-dominance for the cobalt resistance trait because it showed a better growth than the wild type in cobalt-containing medium (Figure 3.15).

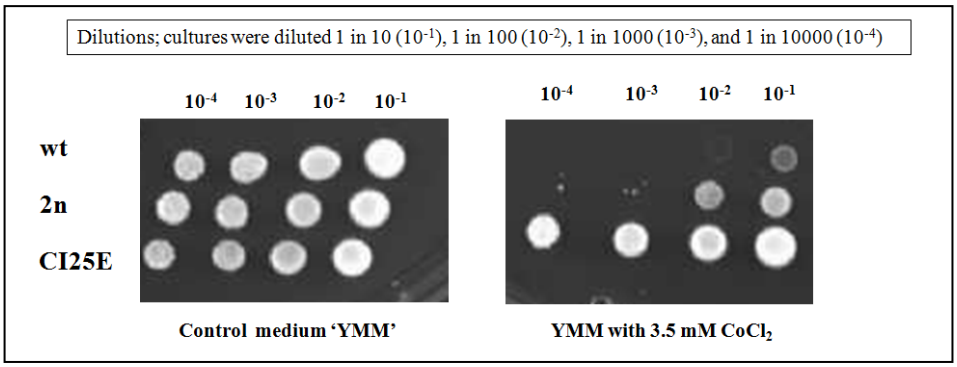


Figure 3.15 : Spot test results of wild type, diploid (2n strain obtained from genetic cross of CI25E with opposite mating type wild type) and the haploid mutant CI25E in YMM and YMM containing 3.5 mM $CoCl_2$.

In addition, it was observed that the spore's progeny from a cross between CI25E and the original wild type did not segregate 2^{+}:2^{-} for cobalt resistance. Actually, there was more than one type of segregation patterns among spore's progeny, which means that the phenotypic trait was dependent on several genes (Figure 3.16).

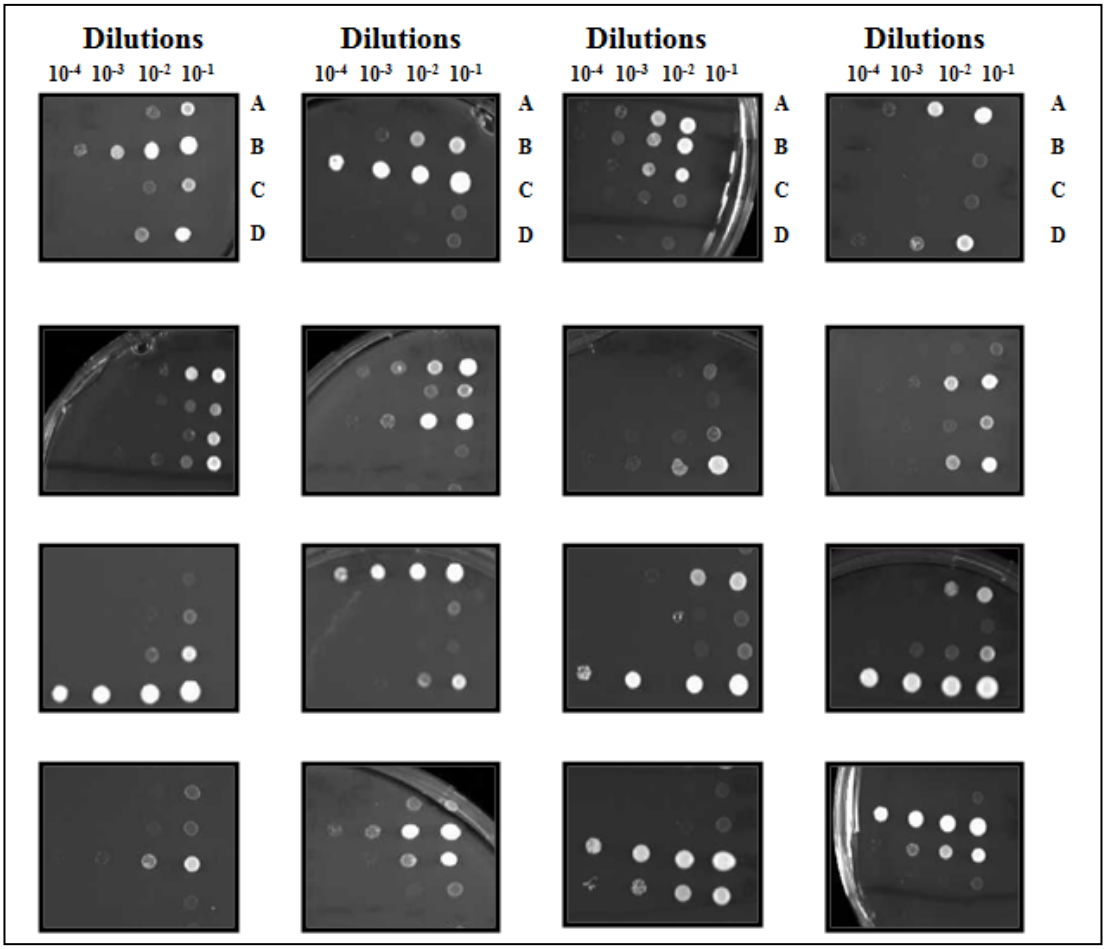


Figure 3.16 : Tetrad analysis results of 16 tetrads obtained from the diploid 'CI25E X wt' on YMM with 5 mM $CoCl_2$ (A, B, C, D represent the 4 spores from a single tetrad that was different for each plate).

As CI25E was found to be resistant to iron, manganese, and nickel; the dissected spores, the parental strains and corresponding diploid (2n) were also analysed for resistance to these metal ions. Nearly all the spores that were resistant to cobalt were also resistant to iron, nickel and manganese. As it was found as for cobalt resistance, the heterozygotes diploids exhibited semi-dominance to iron, manganese and nickel (Figure 3.17).

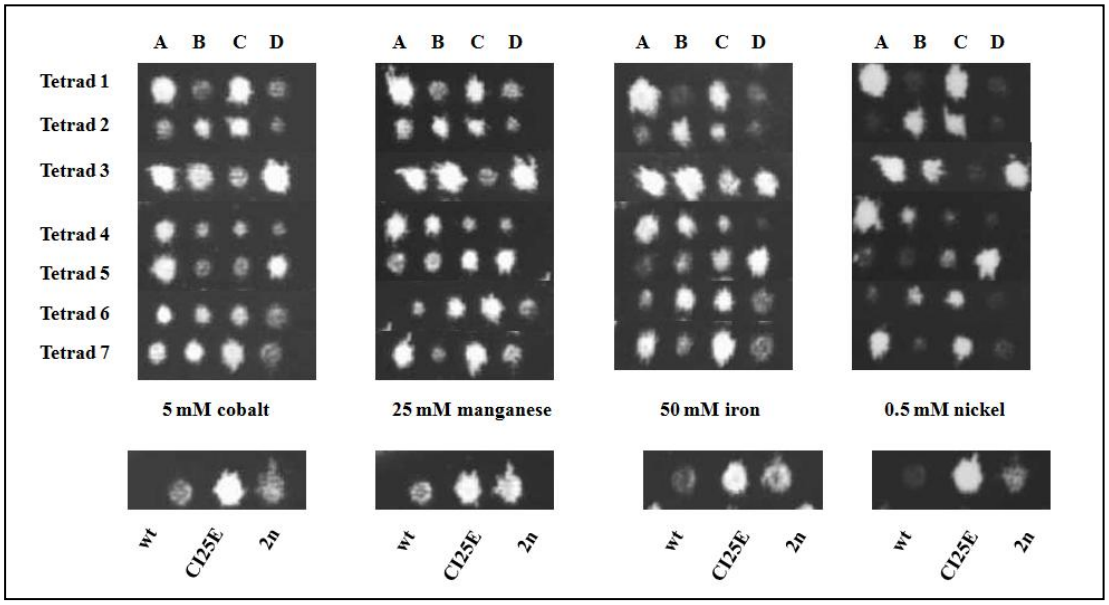


Figure 3.17 : Replica plating results of dissected tetrads derived from CI25E X wt diploid in the presence of cobalt, manganese, iron and nickel stress (A, B, C, D represent different spores).

Following sequential backcrossing is a mode to evaluate whether the trait may depend on a master gene, together with others playing a minor role. To evaluate this possibility, one spore from the ninth tetrad '9D' showing a similar cobalt resistance to the CI25E was backcrossed with wild type CEN.PK. The generated diploid was again found to exhibit a semi-dominant phenotype towards cobalt (Figure 3.18).

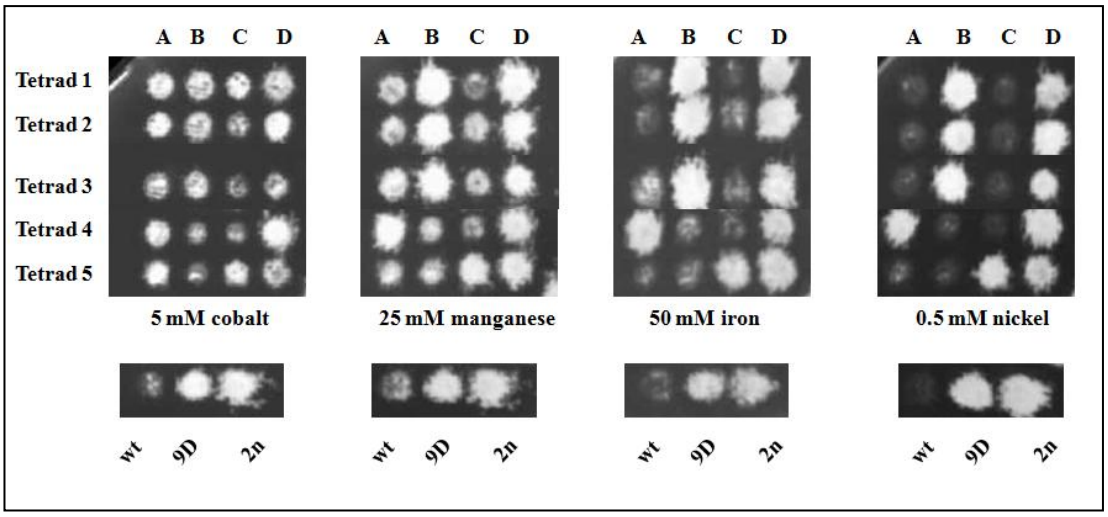


Figure 3.18 : Replica plating results of dissected tetrads derived from 9D X wt diploid in the presence of cobalt, manganese, iron and nickel stress (9D is a segregant from CI25E X wt diploid previously obtained).

Spores progeny obtained from '9D X wild type' displayed again a quite complex resistance phenotype suggesting that the phenotypic trait of cobalt was dependent on several genes. On the other hand, this second backcross led to a 2^{+}:2^{-} segregation for iron and nickel resistance (Figure 3.18).

3.4.3 Backcross analysis

Three successive backcrosses were realized and the cobalt resistance of the diploid progeny and their offsprings were determined. In this experiment, whole spores from the tetrads were crossed with the opposite mating type wild type different from the previous backcross realized for 9D.

CI25E was mated with *Saccharomyces cerevisiae* CEN.PK 113.1A *MATα* in solid YPD medium and the zygotes were taken by microdissection. Diploid cells were sporulated on solid KAc medium for 48h at 30ºC. The tetrads from the first progeny ($2n_1$) were dissected in order to separate four segragants of the corresponding tetrad in different areas of the YPD plate. All tetrad's spore clones were tested on solid plates containing cobalt for the resistance phenotypes. Among all the tetrads, third tetrad's spores '3A, 3B, 3C and 3D' were crossed with a wild type having an opposite mating type.

In Figure 3.19, the cobalt resistance phenotype, which was determined by spot test, is shown for the third tetrad's spores, the parental strains (wt and CI25E) and their corresponding diploid (2n).

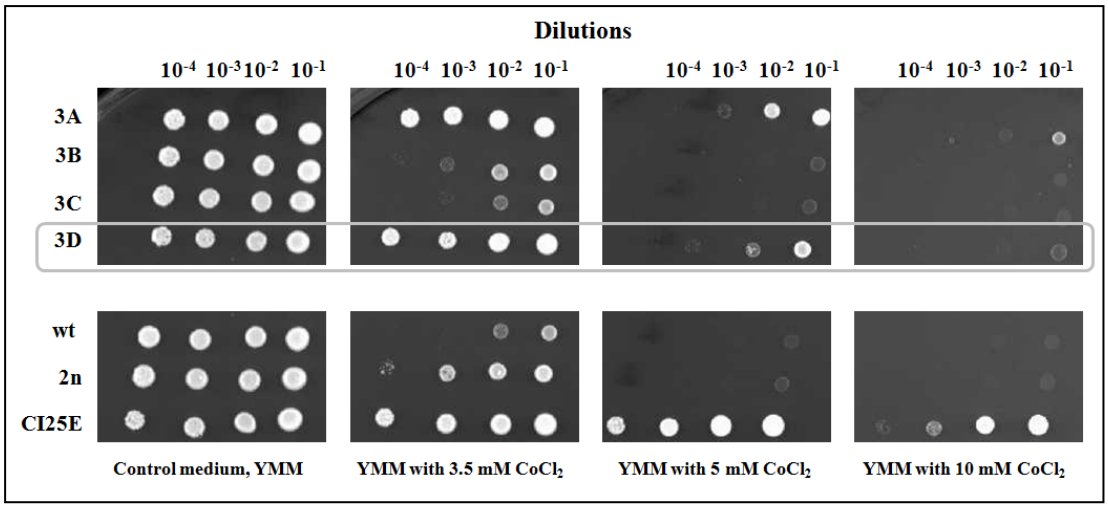


Figure 3.19 : Spot test results of dissected spores (3A, 3B, 3C and 3D) of third tetrad, derived from CI25E X wt diploid in the presence of 3.5, 5 and 10 mM $CoCl_2$.

'3A X wt', '3B X wt', '3C X wt', and '3D X wt' progeny clones were obtained subsequently. The corresponding diploids were tested along with the parental strains upon cobalt stress application. It was decided to continue the backcrossing procedures by using spores derived from progeny clones of '3D X wt'. In Figure 3.20, 6 tetrads derived from the cross of '3D X wild type' and their corresponding offsprings are shown.

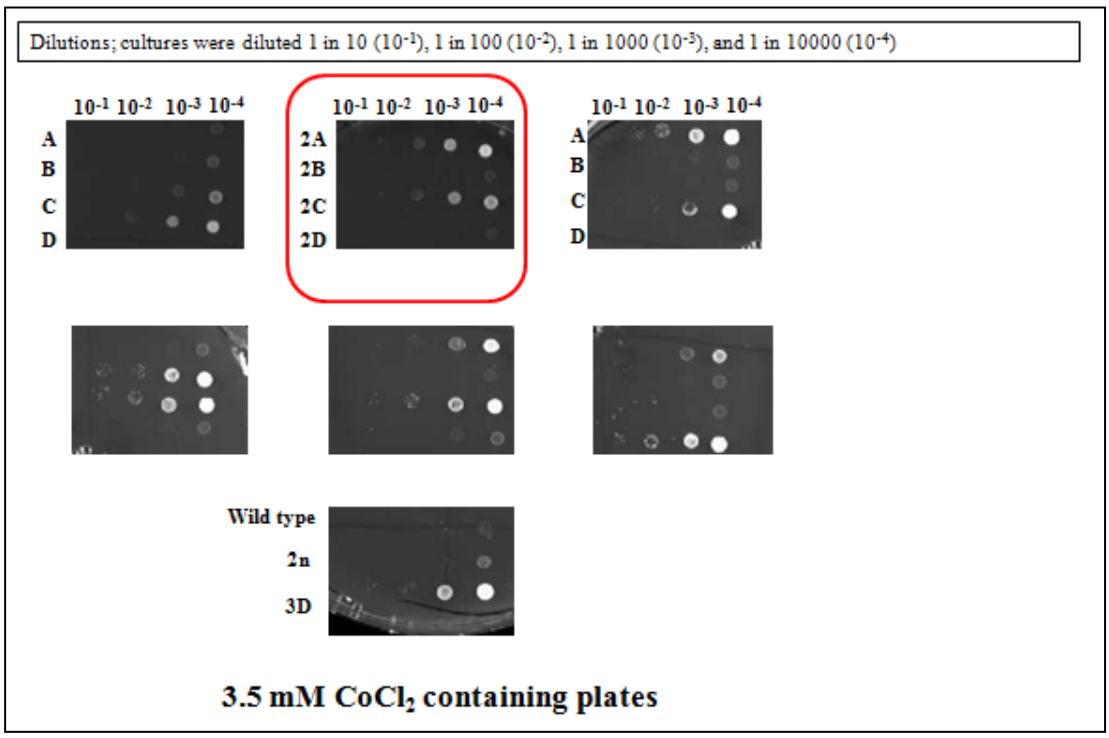


Figure 3.20 : Tetrads obtained from wild type X 3D diploid on cobalt-containing medium (Second tetrad is enclosed in frame).

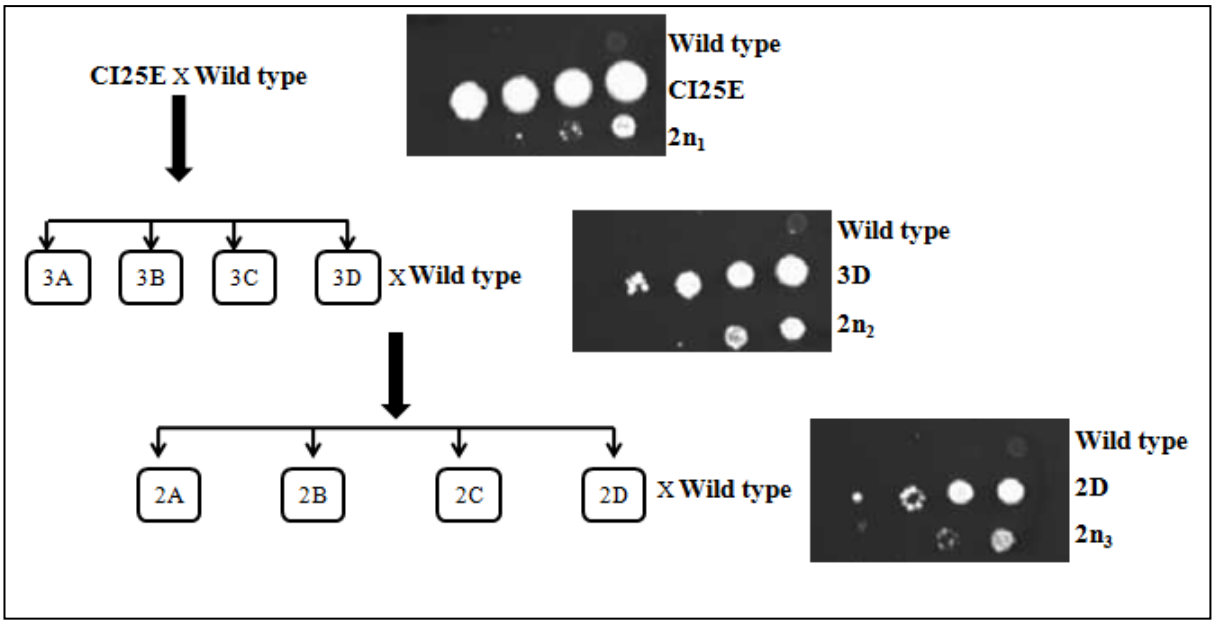


Figure 3.21 : The backcross diagram is shown on the left of the figure, and on the right, the parental strains and their corresponding progeny are shown on cobalt-containing plate (3.5 mM $CoCl_2$).

Haploid offsprings of this backcross were again crossed with the proper wild type strains. For this reason, 2nd tetrad's spores were mated with the opposite mating type wild type to obtain '2A X wt', '2B X wt', '2C X wt', and '2D X wt' progeny clones. The backcross examination was stopped at the end of these offsprings' phenotype test investigation.

To conclude, these three successive backcrosses indicated that the cobalt resistance resulted from a combination of several genes, and that each heterozygous diploid obtained exhibited a semi-dominant phenotype towards cobalt resistance.

The cross-resistance phenotypes of those progeny clones were also investigated through successive backcrosses. It was shown that CI25E had cross-resistance to manganese stress (Figure 3.10). It was observed that the semi-dominance to manganese was not abolished after three successive generations (Figure 3.22).

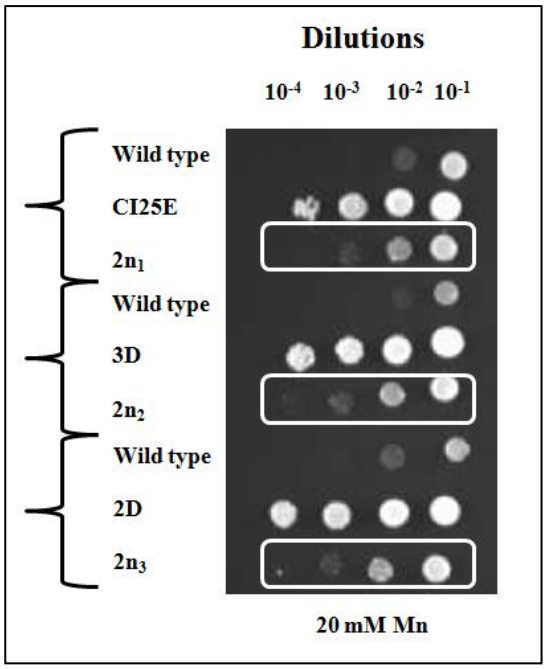


Figure 3.22 : Spot test of diploids on manganese medium and their parental strains after successive backcross.

However, backcrossing compensated sensitivity of the CI25E to H_2O_2 (1 mM), NaCl (0.5 M), copper (0.1 mM), ethanol (8% 'v/v') and caffeine stresses (Figure 3.23).

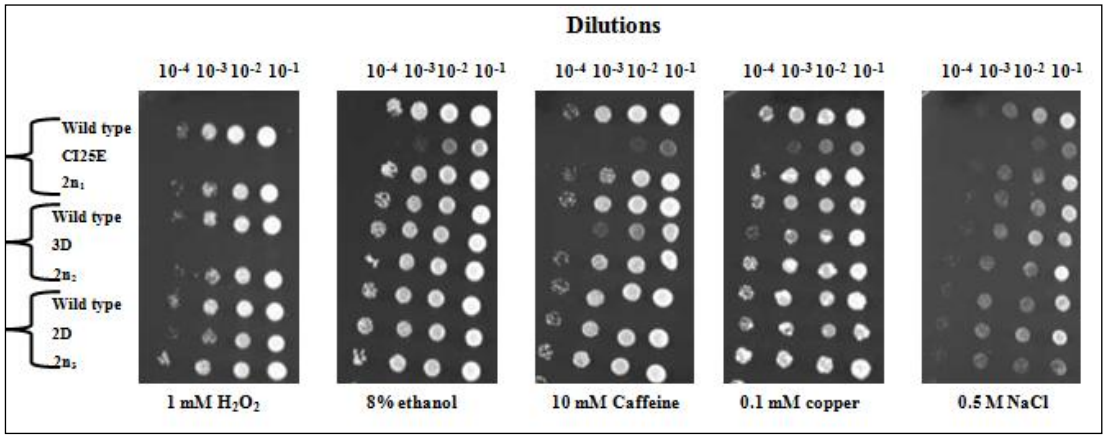


Figure 3.23 : Progeny clones obtained after backcross and the parental strains on H_2O_2, ethanol, caffeine, copper and NaCl containing YMM.

3.5 Comparison of Direct Selected and Evolved Mutants

Evolved strains and direct mutants were tested on cobalt-containing media. It was found that cobalt-resistant mutants obtained by evolutionary engineering were significantly more resistant to cobalt than the direct selection mutants (Figure 3.24). It can be suggested that evolutionary engineering is a powerful technology compared to direct selection techniques to obtain desired phenotypes with the high resistance.

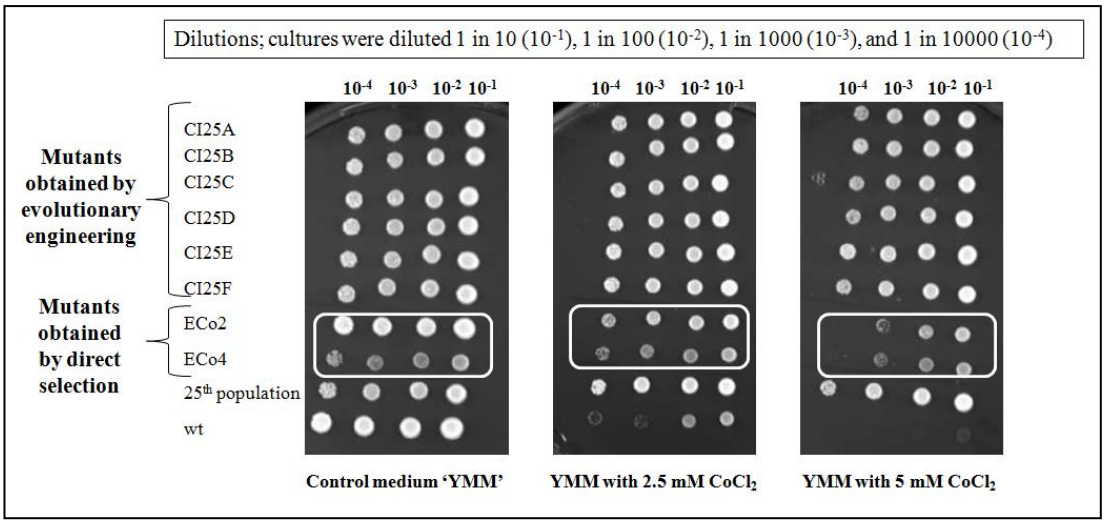


Figure 3.24 : Spot test results of mutants obtained by evolutionary engineering and direct selection strategy, the wild type and the final population on media containing 2.5 and 5 mM $CoCl_2$ (Direct mutants are indicated inside the white frame).

3.6 Gene Deletion Experiment

In *S.cerevisiae* cells *COT1* encodes a vacuolar transporter for cobalt and zinc that mediates uptake of these ions into the vacuole (Li and Kaplan,1998). *cot1*-null mutant shows growth defect on medium containing cobalt and displays increased sensitivity to cobalt (Conklin *et al.*, 1992). *COT1* is so far the sole gene known to be directly connected to the cobalt resistance. For that reason, wt *cot1*Δ, CI25E *cot1*Δ, 9D *cot1*Δ and ECo2 *cot1*Δ strains were obtained and tested for stress resistance.

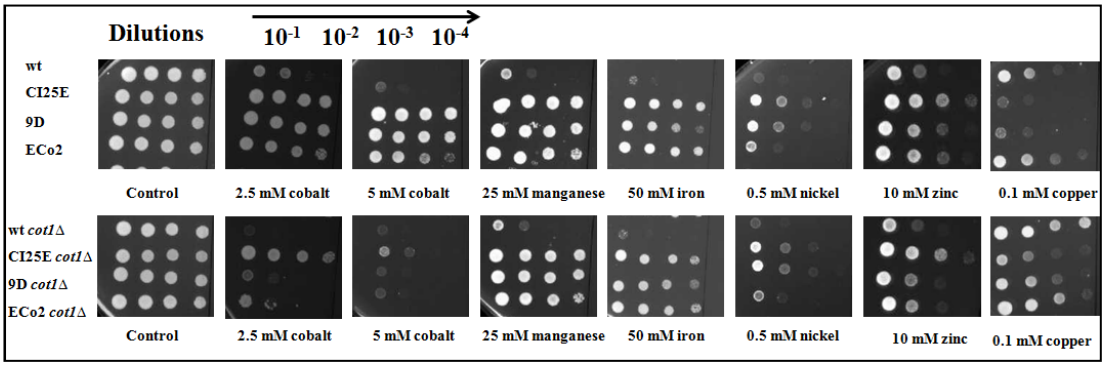


Figure 3.25 : Spot test of wt, CI25E, 9D, ECo2 and their corresponding *COT1*-deleted strains grown in YMM containing different concentrations of $CoCl_2$ (2.5 and 5 mM) and other metals (25 mM $MnSO_4H_2O$, 50 mM $(NH_4)_2Fe(SO_4)_26H_2O$, 0.5 mM $NiCl_26H_2O$, 10 mM $ZnSO_47H_2O$).

It was shown that wt *cot1*Δ could not grow even at mild cobalt stress (2.5 mM $CoCl_2$). CI25E *cot1*Δ showed little growth sensitivity in 2.5 mM $CoCl_2$ when

compared to CI25E grown in the same media. However, CI25E *cot1*Δ managed to survive at 5 mM $CoCl_2$ concentration. 9D *cot1*Δ lost cobalt resistance ability at the high $CoCl_2$ stress level (to 5 mM cobalt stress) and it could barely grow at 10^{-1} dilution at mild cobalt stress (2.5 mM). ECo2 *cot1*Δ lost cobalt resistance at 5 mM $CoCl_2$ stress. It was remarkable to see that *COT1* deletion did not abolish the resistance to manganese, iron or nickel in CI25E, 9D and ECo2. However, a slight decrease in zinc resistance was observed in all *COT1*-deleted strains (Figure 3.25).

3.7 Expression Level Determination of *COT1* for both Wild Type and CI25E

COT1 gene expression levels were determined in response to pulse cobalt stress application. RNA samples were taken before and after 2 mM $CoCl_2$ stress application which followed quantitative RT-PCR. Expression values of *COT1* were normalized to that of the *ACT1* (β-actin, housekeeping gene) transcripts for each corresponding samples. Expression analysis results showed that this gene has been upregulated more than 2-fold in CI25E cells relative to wild type cells in accordance with the finding of high cobalt-resistance phenotype of CI25E and the significant effect of *COT1* loss to its resistance phenotype (Figure 3.26).

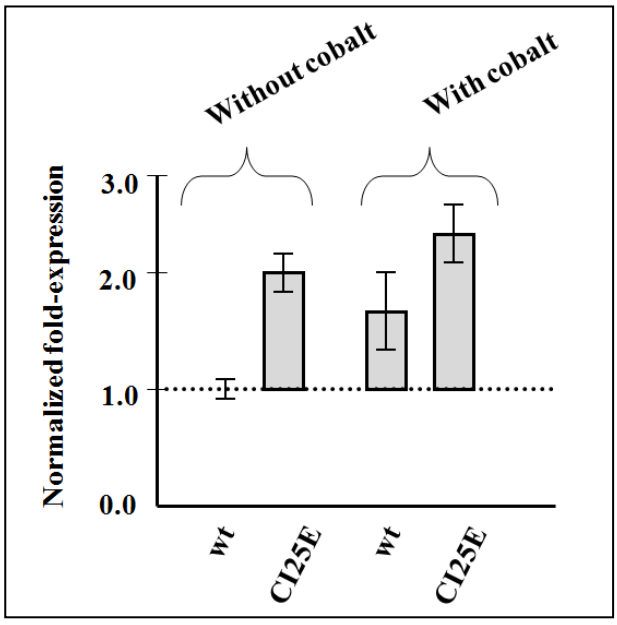


Figure 3.26 : Quantitative RT-PCR analysis of wt and CI25E before and after 2 mM $CoCl_2$ exposure.

The *COT1* gene of wild type was shown to be upregulated slightly upon 2 mM $CoCl_2$ stress exposure (Figure 3.26).

Both the gene deletion experiments and gene expression analysis for *COT1* of CI25E indicated the significance of this gene for the cobalt resistance of this mutant.

3.8 Growth Characteristics of Evolved–Cobalt Resistant Mutant

Wild type and cobalt-resistant mutants obtained by both evolutionary engineering and direct selection strategy were cultivated in liquid YMM and they were investigated in terms of their growth and physiological characteristics.

3.8.1 Shake flask cultivation of wt, CI25A, CI25E and ECo4

Among many individual mutants selected from the final population, growth profiles with and without cobalt treatment of two individual evolved cobalt resistant CI25A and C125E and one resistant clones (ECo4) isolated from direct EMS mutagenesis were characterized in detail. Precultures of wt, CI25A, CI25E and ECo4 were inoculated into 50 mL of YMM with and without 5 mM $CoCl_2$ in 250 mL flasks. Cultures were incubated at 30ºC at 150 rpm and OD_{600} values were monitored during about 3.5 days (Figure 3.27).

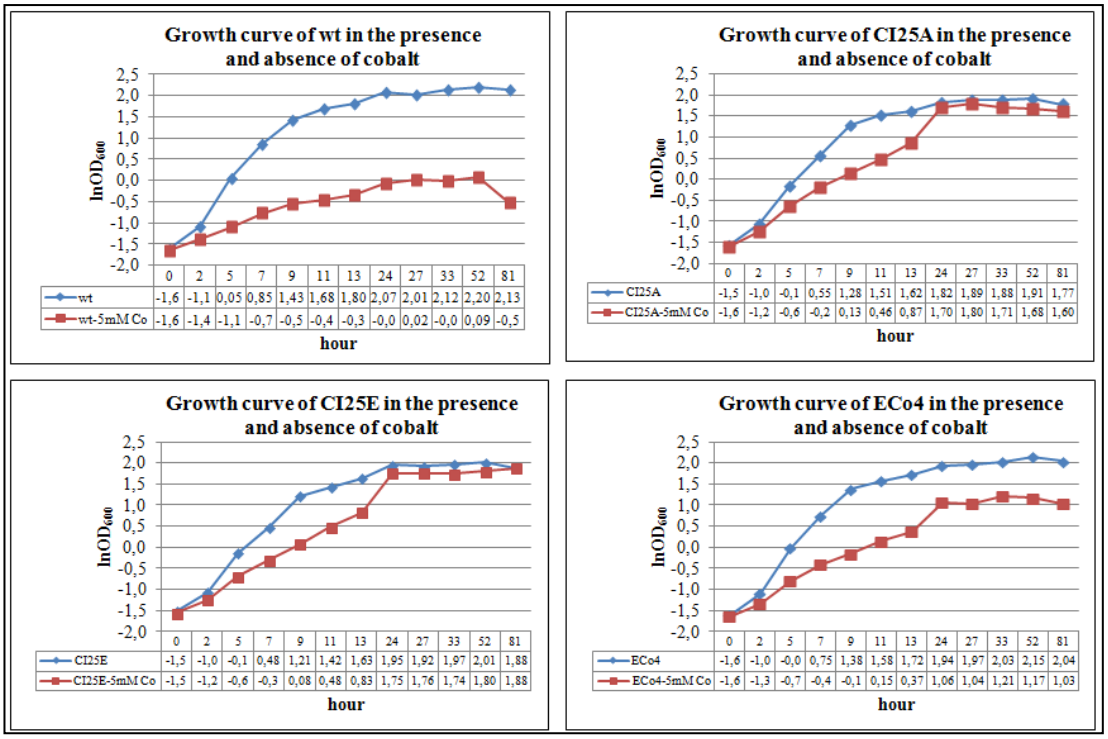


Figure 3.27 : Growth curve of wild type and the mutant both in the presence and absence of cobalt (Natural logarithm of the OD_{600} values are given).

Growth of both CI25A and CI25E were nearly half of their corresponding control cultures for the first 13 h in the presence of cobalt. However, after 24 h, they both reached an optical density similar to that in the absence of cobalt. On the other hand, the growth of the mutant ECo4 was also significantly affected, but it still grew at this high level of cobalt, while that of the wild type was strongly affected. Maximum specific growth rate of the cultures (μ_{max}, h^{-1}) were calculated and they are shown in Table 3.6 for each strain. Wild type could not grow in the presence of 5 mM $CoCl_2$. Moreover, the maximum specific growth rate of ECo4 in the presence of cobalt was lower than that of CI25A and CI25E.

Table 3.6: Maximum specific growth rates (μ, h^{-1}) of the cultures obtained from the batch cultivation in the presence and absence of cobalt stress.

Strain name	**μ, h^{-1} without cobalt**	**μ, h^{-1} with cobalt**	**μ(with cobalt) / μ(without cobalt)**
Wild type	0.37	0.12	0.32
CI25A	0.33	0.20	0.61
CI25E	0.32	0.19	0.58
ECo2	0.36	0.18	0.50

The ratio of the specific growth rate for cobalt treated and non-treated samples were calculated. This μ ratio for CI25A and CI25E were 2-fold higher than that of the wild type (Table 3.6).

3.8.2 Detailed comparison of growth physiology between wild type and CI25E

Cobalt-resistant mutant 'CI25E' and wild type were examined for their physiological traits by cultivating them in aerobic-batch condition in the presence and absence of cobalt. The metabolite production, glucose consumption and μ_{max} for cobalt-treated and non-treated samples were determined.

Batch fermentation was carried out in shake flasks in the presence and absence of 5 mM $CoCl_2$. Fresh precultures of wild type and CI25E were used to inoculate 100-mL fresh YMM in 500-mL flasks by adjusting the initial OD_{600} as 0.25. Main fermentation products, such as ethanol, acetate and glycerol and the consumption of glucose were monitored along with the growth and quantified by HPLC.

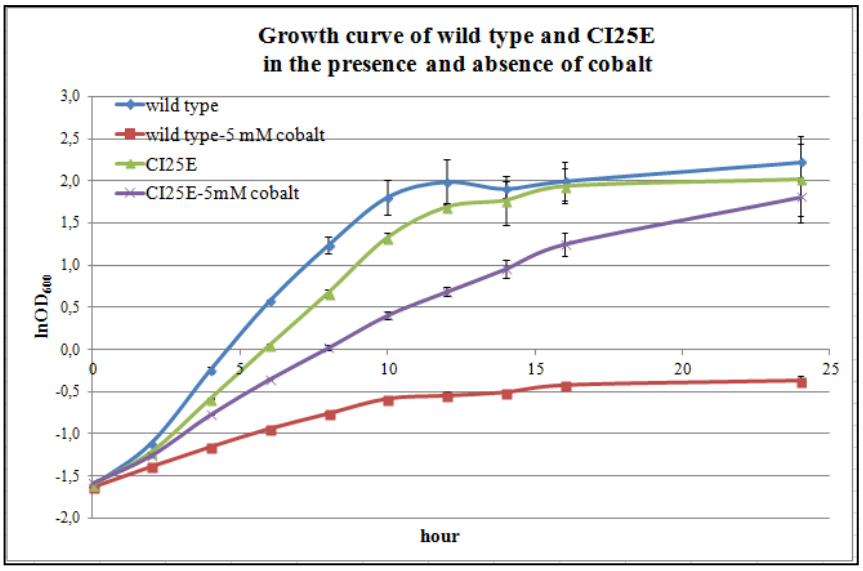


Figure 3.28 : Growth curves of wt and CI25E during batch cultivation in the presence and absence of 5 mM $CoCl_2$ (Standard deviations are indicated on the curves).

First, maximum specific growth rates were calculated by using the slope of the obtained graphs (Figure 3.28). Wild type and CI25E results in the absence of cobalt are shown separately from the cultures to which 5 mM cobalt stress was applied (Figure 3.29).

Growth rate of wt was slightly higher than that of CI25E in the absence of cobalt stress (Figure 3.29, left). Oppositely, the growth rate of CI25E in the presence of cobalt was nearly two-fold greater than that of wild type (Figure 3.29, right).

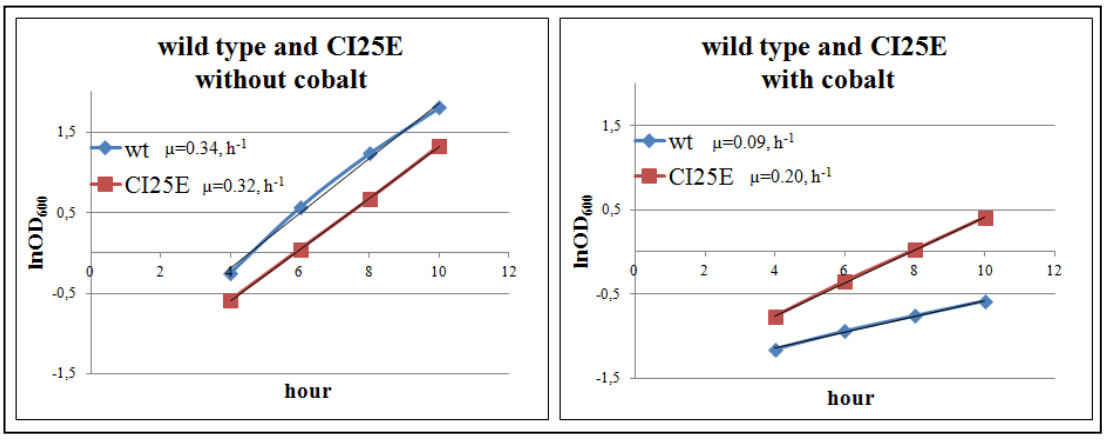


Figure 3.29 : Maximum specific growth rate (μ, h^{-1}) of wild type and CI25E grown in shake flasks in the absence (left side of the figure) and presence (right side of the figure) of cobalt stress.

Glucose consumption seemed to be in parallel with the maximum specific growth rates of the cultures. μ of the wild type was higher than that of CI25E and the wild

type started to consume glucose before CI25E, in the absence of cobalt. Wild type cells grown in YMM consumed the entire glucose before CI25E did. In cobalt-treated CI25E cells, glucose consumption started after about 12.5 h of cultivation (Figure 3.30).

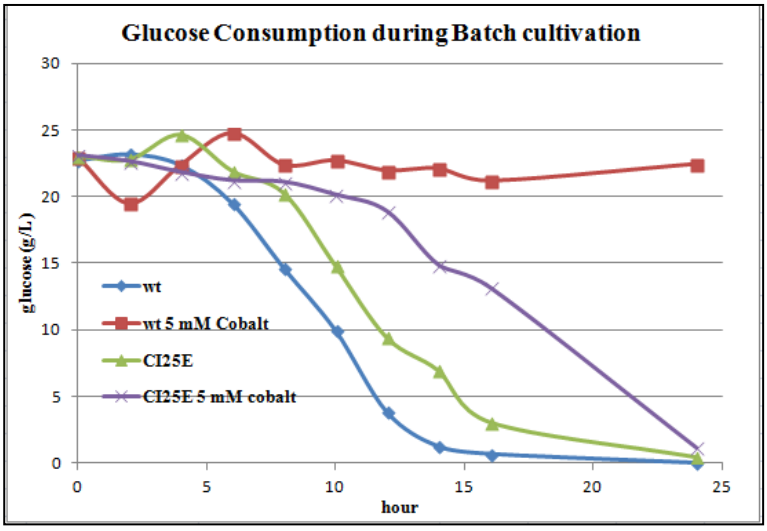


Figure 3.30 : Glucose consumption of wild type and CI25E in the absence and presence of 5 mM $CoCl_2$.

Wild type could not survive at 5 mM $CoCl_2$ stress, for that reason no metabolic products like ethanol, acetate or glycerol production were observed. However, it produced ethanol in the non-stress condition. CI25E produced ethanol both in the presence and in absence of cobalt. Additionally, it produced the highest ethanol concentration in the cobalt-treated medium at the end of the cultivation, among all the cultures tested (Table 3.7).

Table 3.7: Ethanol production (g/L) of wild type and CI25E during batch cultivation with and witout cobalt.

Time	**Ethanol production g/L**			
(h)	**wt**	**wt 5 mM $CoCl_2$**	**CI25E**	**CI25E 5 mM $CoCl_2$**
0	0.00	0.00	1.43	1.73
2	1.37	1.40	1.58	1.48
4	1.87	1.57	1.97	1.62
6	2.47	1.61	1.97	1.97
8	4.22	1.62	2.54	1.82
10	5.80	1.56	3.65	2.34
12	8.19	1.57	4.57	2.86
14	8.79	1.59	6.61	3.38
16	9.81	1.57	7.75	4.54
24	9.34	1.83	9.70	11.63

Wild type glycerol production started earlier than that of the mutant in the absence of cobalt. CI25E cultivated with cobalt, had a 2 hour of glycerol production delay compared to CI25E incubated without cobalt (Table 3.8).

Table 3.8: Wild type and CI25E extracellular glycerol levels during batch cultivation in shake flasks in the absence and presence of 5 mM $CoCl_2$.

Time	**Glycerol production g/L**			
(h)	**wt**	**wt 5 mM $CoCl_2$**	**CI25E**	**CI25E 5 mM $CoCl_2$**
0	0.00	0.00	0.00	0.00
2	0.00	0.00	0.00	0.00
4	0.00	0.00	0.00	0.00
6	0.00	0.00	0.00	0.00
8	1.49	0.00	0.00	0.00
10	1.16	0.00	0.00	0.00
12	1.25	0.00	1.09	0.00
14	1.65	0.00	1.12	1.04
16	1.57	0.00	1.16	1.11
24	1.63	0.98	1.25	1.37

The growth behaviour of CI25E in the absence of cobalt were slightly different from that of the wild type and their acetate production profiles were also similar (Table 3.9).

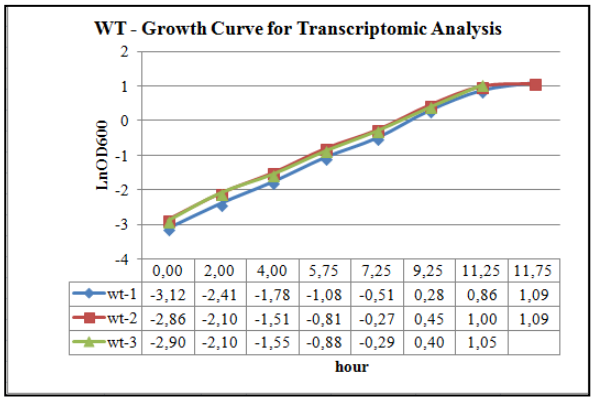
Table 3.9: Wild type and CI25E extracellular acetate levels during batch cultivation in shake flasks in the absence and presence of 5 mM $CoCl_2$.

Time	**Acetate production g/L**			
(h)	**wt**	**wt 5 mM $CoCl_2$**	**CI25E**	**CI25E 5 mM $CoCl_2$**
0	0.00	0.00	0.00	0.00
2	0.00	0.00	0.00	0.00
4	0.00	0.00	0.00	0.00
6	0.00	0.00	0.00	0.00
8	0.00	0.00	0.00	0.00
10	0.69	0.00	0.81	0.00
12	0.54	0.00	---	0.00
14	1.02	0.00	0.74	1.46
16	0.99	0.00	0.69	0.74
24	0.97	0.00	0.77	0.91

3.9 Global Transcriptomic Analyses Revealed Upregulation of the Iron-Regulon in the Evolved Cobalt-resistant Strain

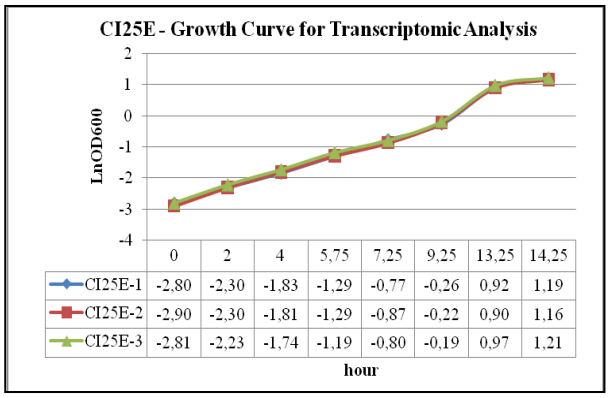
The analysis of gene expression at the level of entire genomes was performed for wild type, CI25E and CI25E defective in the *COT1* gene encoding a vacuolar cobalt transporter. It was previously shown that *COT1* deletion caused cobalt sensitivity both for wild type and CI25E (Figure 3.25). By using this deletion strain in the microarray, it was aimed to eliminate this gene effect on the cobalt resistance phenotypes and determine other potential factors related to cobalt resistance.

For comparison of the expression profile, three independent cultures of wild type CEN.PK, CI25E and CI25E *cot1*Δ cultivated in 500-mL shake-flask containing 100-mL YMM at 30ºC and 150 rpm were performed. Samples were taken in early logarithmic phase, which corresponded to 'balanced growth' and hence should limit effect of 'nutrient' on the gene expression (Figure 3.31-3.33).



	0,00	2,00	4,00	5,75	7,25	9,25	11,25	11,75
wt-1	-3,12	-2,41	-1,78	-1,08	-0,51	0,28	0,86	1,09
wt-2	-2,86	-2,10	-1,51	-0,81	-0,27	0,45	1,00	1,09
wt-3	-2,90	-2,10	-1,55	-0,88	-0,29	0,40	1,05	

Figure 3.31 : Growth curves of three replicate cultures of wild type for transcriptomic analysis.



	0	2	4	5,75	7,25	9,25	13,25	14,25
CI25E-1	-2,80	-2,30	-1,83	-1,29	-0,77	-0,26	0,92	1,19
CI25E-2	-2,90	-2,30	-1,81	-1,29	-0,87	-0,22	0,90	1,16
CI25E-3	-2,81	-2,23	-1,74	-1,19	-0,80	-0,19	0,97	1,21

Figure 3.32 : Growth curves of three replicate cultures of CI25E for transcriptomic analysis.

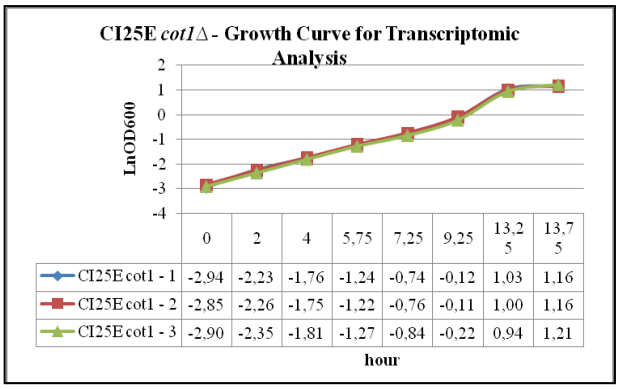


Figure 3.33 : Growth curves of three replicate cultures of CI25E *cot1*Δ strain.

The maximum specific growth rates of the cultures were determined and they were shown as average values with their corresponding standard deviations (Table 3.10).

Table 3.10: μ (h^{-1}) of the cultures used for transcriptomic analysis

Cultures	μ culture1	μ culture2	μ culture3	μ - AVG	ST DEV
wt	0.36	0.35	0.36	0.36	0.005
CI25E	0.28	0.29	0.28	0.29	0.001
CI25E *cot1*Δ	0.30	0.29	0.29	0.29	0.003

Total RNA was extracted as described previously (Section 2.2.13.2), labelled with Cy3 and hybridized to commercial oligonucleotide microarrays (Agilent Technologies) bearing all ORFs of *S. cerevisiae*.

All genes whose expression level are different between CI25E and the wild type were grouped according to their function using FunSpec database (Robinson *et al.*, 2002).

Genes whose expression was upregulated in CI25E and CI25E *cot1*Δ strains at least two fold compared to the wild type with *p* values lower than 0.01 are reported in Table 3.11. Additionally, Figure 3.34 shows a pie chart distribution of upregulated genes according to their biological functions.

Table 3.11: Microarray analysis results (I): CI25E and CI25E *cot1*Δ upregulated genes compared to wild type; The genes whose fold change was lower than 2 are added '*' superscript; ***AFT1*** dependent genes are indicated in bold letters and underlined; Genes regulated by *AFT2* more than *AFT1* are added '•' superscript; Genes whose standard names are not given, are indicates as '---'.

	Systematic name	Gene Name	AVG Fold Change	
			CI25E	CI25E *cot1*Δ
Iron homeostasis	**YLR136C**	***TIS11/CTH2***	18.33	17.20
	YLR136C	***FET3***	12.82	12.19
	YFL041W	***FET5***	2.33	2.35
	YHL040	***ARN1***	10.10	9.91
	YEL065W	***ARN3/SIT1***	7.07	6.67
	YOL158C	***ARN4/ENB1***	5.35	5.52
	YOR382W	***FIT2***	2.90	1.97*
	YOR383C	***FIT3***	7.74	5.83
	YLR214W	***FRE1***	6.10	5.43
	YKL220C	***FRE2***	4.38	4.09
	YOR381W	***FRE3***	2.52	2.28
	YLL051C	***FRE6***	2.84	3.11
	YLR047C	*FRE8*	1.98*	2.10
	YDR270W	***CCC2***	5.59	5.42
	YER145C	***FTR1***	4.49	4.20
	YLR205C	***HMX1***	2.81	2.64
	YLR034C	***SMF3***•	1.97*	2.13
	YBR295W	***PCA1***	2.27	2.15
	YPR124W	*CTR1*	3.58	3.32
	YGL071W	*AFT1*	1.97*	1.97*
Metal Homeostasis	YHR214W-A	---	2.05	2.40
	YOR316C	***COT1***	2.01	0.00
Cell Wall	YKL163W	*PIR3/CCW8*	4.68	4.83
	YNL160W	*YGP1*	4.41	5.19
	YGR189C	*CRH1*	2.33	2.09
	YOL155C	*HPF1*	2.58	2.90
	YDR055W	*PST1/HPF2*	1.86*	2.14
	YBL043W	*ECM13*	2.14	1.99*
Amino Acid Metabolism	YCL025C	*AGP1/YCC5*	3.37	2.77
	YLR121C	*YPS3/YPS4*	3.09	2.85
	YDR046C	*BAP3/PAP1*	2.80	2.30
	YFL030W	*AGX1*	2.40	2.55
	YHR137W	*ARO9*	1.64*	2.29
	YER175C	*TMT1/TAM1*	2.03	2.12
	YPL274W	*SAM3*	2.61	2.63
	YFL056C	*AAD6*	2.48	2.56
	YFL057C	*AAD16*	2.49	2.68

Table 3.11 (contd.): Microarray analysis results (I): CI25E and CI25E *cot1*Δ upregulated genes compared to wild type; The genes whose fold change was lower than 2 are added '*'; ***AFT1*** dependent genes are indicated in bold letters and underlined, Genes regulated by *AFT2* more than *AFT1* are added '•' superscript; Genes whose standard names are not given, are indicated as '---'.

	Systematic name	**Gene Name**	**Average Fold Change**	
			CI25E	**CI25E *cot1*Δ**
Stress Response	YDR059C	*UBC5*	3.10	2.81
	YLR194C	---	2.54	2.41
	YHR048W	*YHK8*	2.12	2.07
	YDL169C	*UGX2*	2.07	2.14
	YIL101C	*XBP1*	2.01	2.26
Functionally Known Genes	YLR134W	*PDC5*	45.81	43.00
	YGR110W	*CLD1*	2.63	2.94
	YPL052W	*OAZ1*	2.32	2.04
	YJL153C	*INO1/APR1*	2.28	2.23
	YMR056C	*AAC1*	2.27	2.11
	YGR043C	*NQM1*	2.23	2.17
	YGL121C	*GPG1*	2.16	2.33
	YDL021W	*GPM2*	1.80*	2.15
	YGL104C	*VPS73*	1.87*	2.08
	YBR046C	*ZTA1*	1.86*	2.05
	YNL202W	*SPS19/SPX19*	2.04	1.93*
	YPR151C	*SUE1*	1.89*	2.02
Functionally Unknown Genes	**YNL237W**	***YTP1***	7.33	8.08
	YJL079C	*PRY1*	4.31	4.12
	YHL035C	***VMR1***	4.19	4.51
	YLR327C	*TMA10*	2.17	3.63
	YGR259C	---	2.93	2.94
	YIL169C	---	2.63	2.92
	YOR072W	---	2.39	2.47
	YHR138C	---	2.27	2.31
	YOL153C	---	1.91*	2.24
	YAR068W	---	2.14	2.44
	YBR071W	---	2.13	2.21
	YBL048W	*RRT1*	2.10	2.07
	YLL058W	---	2.05	1.86*
	YLR030W	---	2.03	1.97*

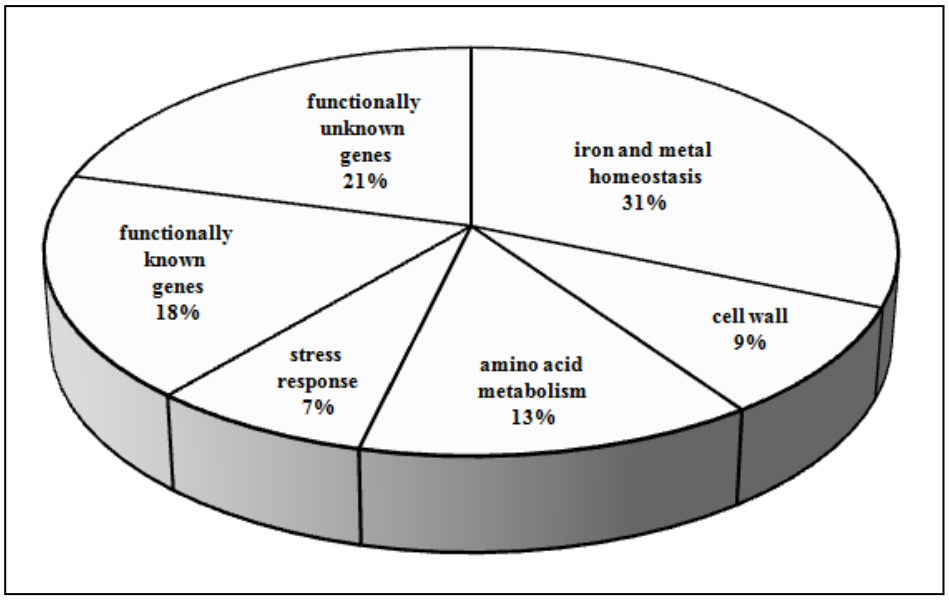


Figure 3.34 : Pie chart representation of upregulated genes on CI25E and CI25E *cot1*Δ compared to wild type, based on their functions.

3.9.1 Overview of the upregulated genes from the global transcriptomic change in CI25E

Twenty out of the 68 upregulated genes based on the criteria chosen (2-fold change) were found to be related to iron homeostasis (Table 3.11). The most upregulated gene among this group was *TIS11* (*CTH2*). Tis11p degrades mRNA molecules whose function is implicated in Fe-dependent processes in response to iron deficiency (Foury and Talibi, 2001). Puig *et al.* (2005) showed that Tis11p mediates the degradation by binding to the 3'-untranslated regions of the specific mRNAs such as *SDH4* and *ACO1*, which encode TCA-related heme binding and Iron Sulfur Cluster prtoteins, respectively. Actually, all of the upregulated genes related to the iron homeostasis on CI25E and CI25E *cot1*Δ compared to the wild type, are regulated by Aft1p/Aft2p transcription activator with the exception of *PCA1*, which contains a putative Aft1p consensus site and *CTR1* (Yamaguchi-Iwai *et al.*, 1995 and Blaiseau *et al.*, 2001). In this study, a 1.97-fold upregulation of *AFT1* was also observed in CI25E and CI25E *cot1*Δ. This *AFT1* upregulation in CI25E was confirmed by quantitative RT-PCR in triplicates and 1.90±0.06 higher expression than the wild type was found for the same conditions. The error values for *ACT1* and *AFT1* were 0.01 and 0.02, respectively. Additionally, their corresponding efficiency values were 2.01 and 1.90, showing that the results were reproducible (Schmittgen and Livak, 2008).

However, no difference in expression of the ortholog *AFT2* was found between the wild type and the cobalt-resistant mutants CI25E and CI25E *cot1*Δ.

3.9.2 Transcriptomic analysis of cobalt-resistant strain unravels upregulation of *AFT1*-dependent genes

The yeast *S. cerevisiae* expresses high and low affinity iron transport systems. *FET3* and *FTR1* encode proteins that constitute one of the high affinity uptake systems but it needs reduction of iron ions first (Stearman *et al.*, 1996 and Conklin *et al.*, 1992). This is accomplished by proteins encoded by *FRE1*/*FRE2* and *FRE3* iron/cupric and iron reductases localized at the plasma membranes (Dancis *et al.*, 1990; Georgatsou and Alexandraki, 1994; Martins *et al.*, 1998). Actually, iron and copper uptake processes intersect because copper-related genes are induced upon iron deprivation in an Aft1p-dependent manner (Gross *et al.*, 2000). One of these genes is *CCC2* encoding a protein which delivers copper ion to Fet3p. *CCC2* was identified by Yuan *et al.* (1995) as the Menkes/Wilson disease gene homologue in yeast.

The other high affinity iron transport is mediated by siderophore transporters encoded by *ARN1*, *ARN2*/*TAF1*, *ARN3*/*SIT1*, and *ARN4*/*ENB1* genes (Lesuisse *et al.*, 2001 and Yun *et al.*, 2000). In this study, *ARN1*, *ARN3*/*SIT1*, and *ARN4*/*ENB1* were found to be upregulated in the CI25E and CI25E *cot1*Δ strains.

Cell wall mannoproteins encoded by *FIT1*, *FIT2*, *FIT3* are responsible for associating and transporting iron. *FIT2* and *FIT1* are localized on chromosome XV with their transcription taking place on the opposite strands of the chromosome (Protchenko *et al.*, 2001). *FIT2* and *FIT3* were upregulated in this study for the CI25E and CI25E *cot1*Δ, but *FIT1* was slightly downregulated.

At the vacuolar surface, an iron exporter encoded by *FET5*/*FTH1* is localized that is the paralogue of *FET3*/*FTR1* protein complex, which is situated at the cytoplasmic membrane. Both of these protein complexes have the same function, they are transferring reduced iron to the cytoplasm (Urbanowski and Piper, 1999). Iron (II) is provided to *FET5*/*FTH1* by the vacuolar *FRE6* reductase activity (Singh *et al.*, 2007). CI25E and CI25E *cot1*Δ had all these genes upregulated, however the fold change of *FTH1* was less than two. Another protein exporting iron ions out of the vacuole is *SMF3* (Singh *et al.*, 2007). *SMF3* expression level increased in CI25E and CI25E *cot1*Δ cells. Altogether, these genes encoded proteins that are either

responsible for the iron uptake into the cells or iron transport from the vacuole into the cytosol. There is also a heme-degrading protein in this group encoded by *HMX1* (Protchenko and Philpott, 2003). It was upregulated with a fold change higher than two in the CI25E and CI25E *cot1*Δ mutants compared to wild type.

Those results showed that Aft1p dependent iron homeostasis might have direct or indirect effects on the cobalt resistance phenotypes of CI25E and CI25E *cot1*Δ.

Besides iron-deficient conditions, defects in mitochondrial iron-sulfur cluster (ISC) assembly biogenesis could also activate Aft1p (Hausmann *et al.*, 2008). For that reason, genes expression level changes related to this metabolic event are listed in Table 3.12. Nevertheless, the expression level of those genes did not change significantly.

Table 3.12: Expression changes of Fe-S cluster biogenesis-related genes in CI25E and CI25E *cot1*Δ.

	CI25E		**CI25E *cot1*Δ**		**Bound cofactor**
Gene	**p value**	**Fold Change**	**p value**	**Fold Change**	**-**
ISU1	0.02	1.36	0.02	1.41	-
ISU2	0.06	1.27	-	-	[2Fe-2S] Cluster
YAH1	0.04	0.80	0.02	0.75	FAD
MGE1	0.01	0.72	0.02	0.77	-
ISA1	-	-	0.07	0.79	Fe
GSH2	0.01	0.75	0.07	0.81	-
NBP35	0.07	1.21	-	-	ATP or GTP, Fe/S cluster

Similar to iron homeostasis genes, some of the genes related to copper homeostasis were also upregulated. This is the case for *CTR1*, which encodes a member of high affinity copper uptake protein (Dancis *et al.*, 1994). Copper is needed under iron-depleted conditions because the reductive high affinity iron uptake protein, Fet3p, requires copper for oxidation reactions (Askwith *et al.*, 1994). Mrs3p and Mrs4p are responsible for iron transport to the mitochondria. An *mrs3*Δ*mrs4*Δ strain could not accumulate iron in the mitochondria (Li and Kaplan, 2004). In order to balance cytosolic iron level, Ccc1p transports iron into the vacuole, which causes iron depletion in the cytosol. For that reason, *mrs3*Δ*mrs4*Δ strain's high affinity iron uptake system is active due to the low cytosolic iron level. As a result, whenever copper stress is applied to those cells they are more sensitive to copper because high affinity copper uptake system is on. It was shown that the high copper uptake causes

increased copper sensitivity and this new phenotype actually originated from Aft1p upregulation (Li and Kaplan, 2004).

COT1 expression increased in CI25E two-folds in comparison to wild type in the microarray analysis. This result is parallel to those found on the quantitative RT-PCR experiment. Cot1p is another protein related to metal ion transport. It is localized on the vacuolar surface and it takes up cobalt and zinc ions to the vacuole (Li and Kaplan, 1998). Its overexpression was shown to provide cobalt and rubidium resistance to wild type cells (Conklin *et al.*, 1992 and Li and Kaplan, 1998). There are some direct and indirect evidence that *COT1* is also regulated by Aft1 protein (Harbison *et al.*, 2004 and Pagani *et al.*, 2007).

3.9.3 Upregulated-genes related to metabolic genes

One of the striking results of microarray analysis was that *PDC5* was upregulated nearly about 46 and 43 times more than the wild type, for CI25E and CI25E *cot1*Δ, respectively. This is the highest fold difference between wild type and the mutants. It was previously shown that *PDC5* is overexpressed either under thiamine (vitamin B_1) deficiency or in the absence of *PDC1* (Seeboth *et al.* 1990 and Muller *et al.* 1999). The expression profiles of the genes encoding thiamine diphosphate-dependent proteins were therefore investigated to know whether this upregulation of *PDC5* is somehow linked to a thiamine problem (Table 3.13).

Table 3.13: Expression level changes in genes that encode thiamine diphosphate-dependent proteins in CI25E and CI25E *cot1*Δ.

	Gene		**CI25E**		**CI25E*cot1*Δ**	
			p value	**Fold Change**	**p value**	**Fold Change**
PDC related	*PDC5*	YLR134W	0.00	45.81	0.00	43.00
	PDC6	YGR087C	0.01	1.41	0.19	1.21
	ILV2/THI1/SMR1	YMR108W	0.00	0.68	0.02	0.75
		YEL020C	0.00	1.66	0.00	1.80
Transketolase	*TKL2*	YBR117C	0.07	1.24	0.13	1.24

The expression levels of the genes encoding thiamine diphosphate-dependent proteins did not change significantly except *PDC5* (Table 3.13).

Hohmann (1993) stated that the expression of *PDC1* and *PDC5* are positively regulated by *PDC2*. *PDC2*, is also a regulator at thiamine biosynthesis process which links the *PDC5* expression regulation to the thiamine diphosphate production regulation (Mojzita and Hohmann, 2006). However, the upregulation of *PDC5* in the evolved strain does not seem to be linked to *PDC2* since its expression did not change significantly between wild type and evolved CI25E and CI25E *cot1*Δ mutants. Moreover, their *p* values for *PDC2* were higher than the upper limit of acceptance (Data not shown).

The expression level changes in thiamine biosynthesis, uptake and regulation genes were also investigated for CI25E and CI25E *cot1*Δ (Table 3.14). The expression levels of thiamine transporter protein encoded by *THI10*/*THI7* slightly increased in both mutants, CI25E and CI25E *cot1*Δ, which might indicate low intracellular levels of thiamine (Singleton, 1997). Additionally, the expression of *NRT1*, whose nucleotide sequence shows higher similarity with *THI10*/*THI7*, was downregulated in the mutants. The possible role of the protein encoded by *NRT1* could be a low-affinity uptake of thiamine to the cell. When the gene encoding the high affinity thiamine transporter is upregulated, the low affinity system might be downregulated (Enjo *et al.*, 1997). Another significant result for thiamine-related genes was the down-regulation of *RPI1* since *RPI1* over-expression was reported to activate *THI4* in thiamine-grown cells (Hohmann and Meacock, 1998).

Table 3.14: The expression level changes in genes responsible for thiamine biosynthesis, uptake and regulation.

	Gene		**CI25E**		**CI25E*cot1*Δ**	
			p value	**Fold Change**	**p value**	**Fold Change**
Biosynthesis	*THI6*	YPL214c	0.01	1.34	0.01	1.41
	THI4	YGR144w	0.12	0.85	0.05	0.79
High affinity Uptake	*THI10 /THI7*	YLR237w	0.00	1.62	0.00	1.58
Low affinity Uptake	*NRT1*	YOR071C	0.00	0.42	0.00	0.37
Regulation	*RPI1*	YIL119c	0.00	0.48	0.00	0.49

3.9.4 Overview of the downregulated genes from the global transcriptomic change in CI25E and CI25E *cot1*Δ

Functional groups of downregulated genes in CI25E and CI25E *cot1*Δ are shown in Figure 3.35. Table 3.15 provides the list of downregulated genes in evolved strains with a fold change of minus two fold and more, and a *p* value of < 0.01.

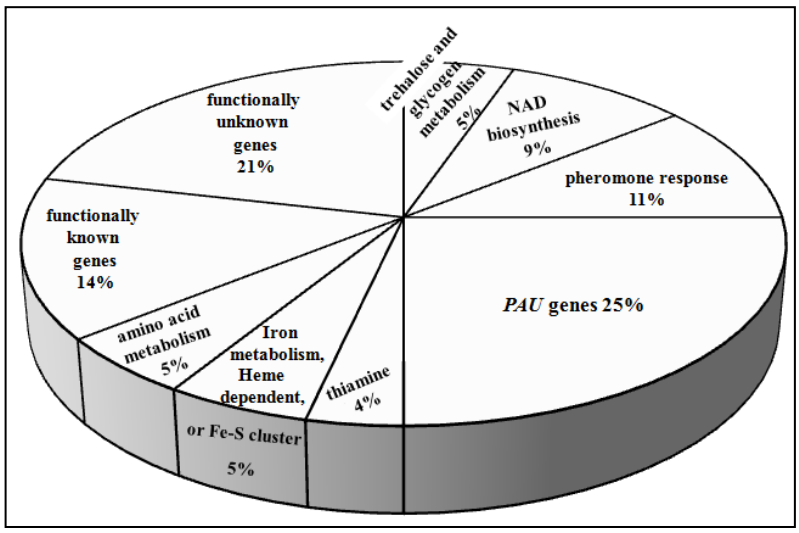


Figure 3.35 : Functional groups of downregulated genes in CI25E and CI25E *cot1*Δ compared to the wild type.

The classification analysis with FunSpec allowed to identify three main classes of genes whose expression was significantly downregulated, namely, genes related to seripauperin multigene family members, NAD biosynthesis, and pheromone response genes (Figure 3.35).

3.9.5 The *PAU* family

PAU genes constitute a multigene family in *Saccharomyces cerevisiae* (Goffeau *et al.*, 1996) which encode seripauperins (Viswanathan *et al.*, 1994). There are 24 *PAU* genes having nearly 85% sequence similarity to each other. They are generally localized in subtelomeric regions (Pryde and Louis, 1997). Rachidi *et al.* (2000) demonstrated that *PAU* genes are induced upon anoxic environmental conditions and are repressed by oxygen in the presence of heme. Based on this previous work, and taking into account that the growth conditions for both wild type and the evolved strain were highly aerobic, it is difficult at this stage to propose an explanation for this potent downregulation of this gene family as a consequence of cobalt resistance. In addition, the fact that the expression of *HMX1* gene which encodes a protein

implicated in heme degradation was upregulated in the CI25E strain is against a role of heme in this process. Thus, some other mechanism in the mutants might be found which enabled the down-regulation of those *PAU* genes.

Table 3.15: Microarray analysis results (II): CI25E and CI25E *cot1*Δ downregulated genes compared to wild type; Genes whose expression was found to have decreased more than 0.5-fold for CI25E and CI25E *cot1*Δ compared to wild type. The genes whose expressions were not found to be downregulated are indicated as "not found".

	Systematic Name	**Gene Name**	**Average Fold Change**	
			CI25E	**CI25E *cot1*Δ**
Trehalose and Glycogen Metabolism	YML100W	*TSL1*	0.23	0.39
	YFR015C	*GSY1*	0.42	0.47
	YMR105C	*PGM2*	0.36	0.57
NAD biosynthetic process	YJR025C	*BNA1/HAT1*	0.28	0.27
	YJR078W	*BNA2*	0.45	0.40
	YBL098W	*BNA4*	0.47	0.46
	YLR231C	*BNA5*	0.38	0.32
	YFR047C	*BNA6/QPT1*	0.72	0.68
Pheromone Response	YIL015W	*SST1/BAR1*	0.32	0.35
	YBR083W	*TEC1/ROC1*	0.44	0.47
	YFL026W	*STE2*	0.44	0.45
	YKL209C	*STE6*	0.46	0.51
	YNL145W	*MFA2*	0.52	0.50
	YLR452C	*SST2*	0.56	0.49
***PAU* genes**	YJL223C	*PAU1*	0.40	0.38
	YEL049W	*PAU2*	0.48	0.46
	YCR104W	*PAU3*	0.52	0.50
	YLR461W	*PAU4*	0.48	0.47
	YNR076W	*PAU6*	0.50	0.52
	YAL068C	*PAU8*	0.35	0.35
	YBL108C-A	*PAU9*	0.43	0.41
	YDR542W	*PAU10*	0.39	0.38
	YGL261C	*PAU11*	0.35	0.33
	YGR294W	*PAU12*	0.39	0.36
	YHL046C	*PAU13*	0.44	0.42
	YIL176C	*PAU14*	0.45	0.41
	YLL064C	*PAU18*	0.50	0.49
	YBR301W	*PAU24/DAN3*	0.44	0.42
Thiamine	YIL119C	*RPI1*	0.48	0.49
	YOR071C	*NRT1*	0.42	0.37
Iron Homeostasis	YMR319C	*FET4*	0.63	0.48

Table 3.15 (contd.): Microarray analysis results (II): CI25E and CI25E *cot1*Δ downregulated genes compared to wild type; Genes whose expression found to have decreased more than 0.5-fold for CI25E and CI25E *cot1*Δ compared to wild type. The genes whose expressions were not found to be downregulated are indicated as "not found".

	Systematic Name	Gene Name	AVG Fold Change	
			CI25E	CI25E *cot1*Δ
Amino acid metabolism	YGR142W	*BTN2*	0.39	not found
	YMR195W	*ICY1*	0.54	0.45
	YJL088W	*ARG3*	0.50	0.44
Heme dependent or Fe-S cluster Proteins	YGR088W	*CTT1*	0.26	0.32
	YLR304C	*ACO1*	0.43	0.52
Functionally known genes	YHR216W	*IMD2/PUR5*	0.51	0.38
	YOR032C	*HMS1*	0.40	0.42
	YAL061W	*BDH2*	0.40	0.44
	YML054C	*CYB2/FCB2*	0.42	0.48
	YGR248W	*SOL4*	0.45	0.67
	YBL042C	*FUI1*	0.52	0.45
	YIR029W	*DAL2/ALC1*	0.52	0.50
	YDR342C	*HXT7*	not found	0.50
Functionally unknown genes	YIR030C	*DCG1*	0.33	0.33
	YCL021W-A	---	0.35	0.32
	YBL107W-A	---	0.36	0.40
	YGR052W	*FMP48*	0.38	0.50
	YNL336W	*COS1*	0.54	0.43
	YNR075W	*COS10*	0.40	0.39
	YNR068C	*BUL3*	0.41	0.47
	YDR516C	*EMI2*	0.44	0.56
	YER138W-A	---	0.45	0.46
	YNR014W	---	0.45	0.55
	YOR161C	*PNS1*	0.43	0.49
	YAR073W	*IMD1*	0.56	0.46

CI25E and CI25E *cot1*Δ had decreased expression of *TSL1,* which is responsible for the trehalose synthesis along with the *TPS1*, *TPS2* and *TPS3* genes (Winderickx *et al.*, 1996). Trehalose and glycogen contents of the wild type and CI25E were determined as described in Section 2.2.12.2. Cobalt caused an increase in the accumulation of both trehalose and glycogen for the wild type (Table 3.16). CI25E trehalose accumulation was similar both in the presence and absence of cobalt. Moreover, cobalt treatement did not increase trehalose concentrations of CI25E unlike the wild type (Table 3.16).

Cobalt-treated CI25E samples glycogen concentrations decreased compared to those of non-treated ones.

Table 3.16: Relative trehalose and glycogen contents of cobalt-treated and non-treated samples of wild type and CI25E.

Time (h)	**Trehalose content (mg glucose equivalents / mg CDW)**			
	wt	wt - cobalt	CI25E	CI25E – cobalt
0	0.01	0.01	0.01	0.01
4.5	0.01	0.01	0.01	0.01
11	0.02	0.01	0.01	0.01
16	0.04	0.03	0.05	0.03
24	0.01	0.07	0.03	0.02
30	0.01	0.07	0.04	0.04
Time (h)	**Glycogen content (mg glucose equivalents / mg CDW)**			
	wt	wt - cobalt	CI25E	CI25E – cobalt
0	0.01	0.01	0.01	0.01
4.5	0.01	0.01	0.01	0.01
11	0.01	0.01	0.01	0.01
16	0.05	0.01	0.03	0.02
24	0.01	0.04	0.02	0.01
30	0.01	0.04	0.04	0.02

The intracellular trehalose levels of cobalt-treated and non-treated samples of CI25E were slightly higher than those of the wild type at 24^{th} and 30^{th} h of incubation (Figure 3.36).

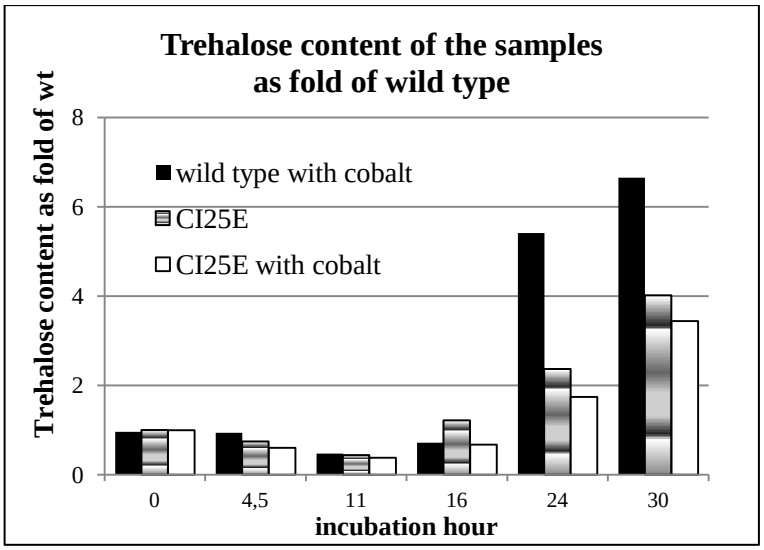


Figure 3.36 : Trehalose levels of cobalt-treated wt, CI25E and non-treated CI25E samples as fold of wild type.

Another group of genes whose expression was downregulated in the CI25E and CI25E *cot1*Δ was related to NAD biosynthesis, namely *BNA1*, *BNA2*, *BNA4* and *BNA5*. *BNA2* is Aft2p-specific which codes for a protein with similarity to indoleamine 2,3-dioxygenases (Rutherford *et al.*, 2003). However, in the present study there is no data related to the expression of *AFT2* upon transcriptomic analysis. In another study by Greenall *et al.* (2008) it was shown that *BNA2* plays a role in chromosome end protection.

3.9.6 Expression profiles comparison of CI25E and CI25E *cot1*Δ

Transcriptomic profiles of CI25E and CI25E *cot1*Δ were compared to each other to investigate if the deletion of *COT1* gene had a significant effect on the whole transcriptome. It was observed that only a few genes had significant differences in expression (Table 3.17). Among these, *ZRT1* was upregulated more than two times in CI25E with a *p*-value lower than 0.01. *ZRT1* is responsible for high affinity plasma membrane zinc uptake in *S.cerevisiae* cells (Zhao and Eide, 1996). Cot1p provides accumulation of zinc ion in the vacuole, and when deleted, the zinc ion may remain in the cytosol (MacDiarmid *et al.*, 2000). As a result, CI25E *cot1*Δ might not need to express high-affinity zinc uptake protein since cytosolic zinc could not be stored inside the vacuole. The other genes upregulated in CI25E are *NSR1*, *AQR1* and *GFD2*. Among these, *NSR1* and *AQR1* are functionally known and *GFD2* is functionally unknown. *NSR1* is a nucleolar protein, which is required for pre-rRNA processing and ribosome biogenesis (Lee *et al.*, 1992) and *AQR1* is a plasma

membrane multidrug transporter (Tenreiro *et al.*, 2002). *AQR1* is also involved in the excretion of excess amino acids (Valesco *et al.*, 2004).

Table 3.17: Microarray analysis results (III): CI25E upregulated genes compared to CI25E *cot1*Δ. Genes whose expression was upregulated at least **1.5**-fold of CI25E *cot1*Δ with a *p* value lower than 0.01 are shown.

	Systematic name	**Gene name**	**Average fold Change**
Zinc Homeostasis	YGL255W	*ZRT1*	2.27
Functionally known genes	YGR159C	*NSR1/SHE5*	1.55
	YNL065W	*AQR1*	1.55
Functionally unknown genes	YCL036W	*GFD2/YCD6*	1.70

Genes whose expression was found to have decreased more than 0.5 fold for CI25E compared to CI25E *cot1*Δ are shown in Table 3.18.

Table 3.18: Microarray analysis results (IV): Downregulated genes of CI25E compared to CI25E *cot1*Δ.

	Systematic name	**Gene name**	**Average fold Change**
Heat-Shock Proteins (HSPs), HSPs related and co-chaperones	YBR072W	*HSP26*	0.39
	YDR258C	*HSP78*	0.53
	YPL240C	*HSP82/90*	0.56
	YLL026W	*HSP104*	0.51
	YER103W	*SSA4*	0.37
	YFL016C	*MDJ1*	0.67
	YNL007C	*SIS1*	0.59
	YNL077W	*APJ1*	0.51
	YPR158W	*CUR1*	0.66
	YDR214W	*AHA1*	0.68
	YOR027W	*STI1*	0.61
	YBR101C	*FES1*	0.64
	YBR169C	*SSE2*	0.52
Functionally known genes	YOL164W	*BDS1*	0.69
	YGR142W	*BTN2*	0.31
	YNL006W	*LST8*	0.59
	YDL020C	*RPN4*	0.60
	YOR298C-A	*MBF1*	0.72
	YLR206W	*ENT2*	0.74
Functionally unknown genes	YGR146C	*ECL1*	0.65

Apart from the genes that could not be classified and grouped as functionally known and unknown, there is only one major group, the expression of which decreased in CI25E compared to CI25E *cot1*Δ. It included genes encoding proteins whose function is related to unfolded or misfolded protein binding, particularly Heat Shock Proteins (HSPs) (Robinson *et al.*, 2002).

3.9.7 Molecular and functional analysis of *AFT1* implicated in cobalt resistance

Previous studies have shown that Aft1p and Aft2p differentially activate the expression of a subset of the iron-regulated genes in *S. cerevisiae* (Rutherford *et al.*, 2001). In these current microarray results, a cluster of *AFT1*/*AFT2*-regulated genes was upregulated in cobalt-resistant strain that might indicate that this resistance or a part of it linked to *AFT1*/*AFT2*. For this reason, *AFT1* and *AFT2* were deleted in wild type, CI25E, 9D and ECo2 strains to see their possible role for cobalt resistance phenotypes (Figure 3.37).

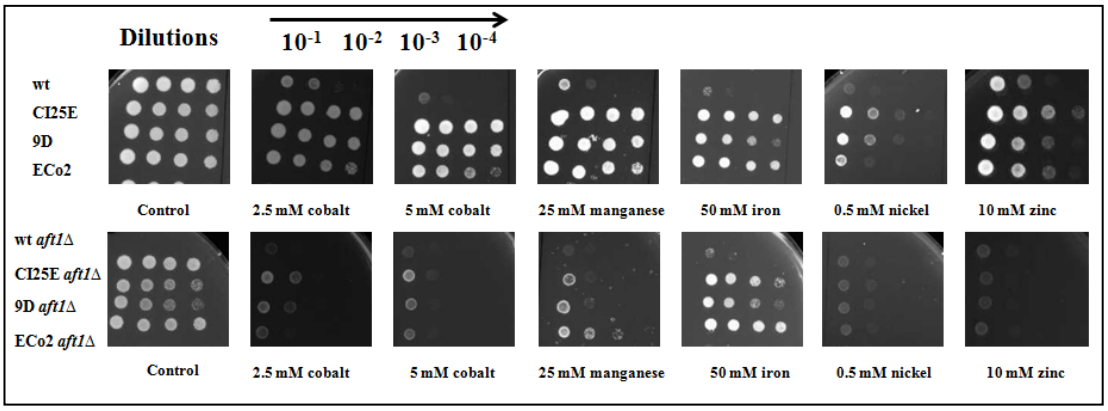


Figure 3.37 : Spot test results of wild type, CI25E, 9D, ECo2 strains and wild type *aft1*Δ, CI25E *aft1*Δ, 9D *aft1*Δ, ECo2 *aft1*Δ mutants grown in YMM containing different concentrations of $CoCl_2$ (2.5 and 5 mM) and other metals (25 mM manganese, 50 mM iron, 0.5 mM nickel, 10 mM zinc).

It is already known that *AFT1* deletion causes a growth defect (Yoshikawa *et al.*, 2011) but this effect was much more pronounced for CI25E and 9D (Figure 3.37). CI25E *aft1*Δ barely grew in the presence of 5 mM $CoCl_2$, which means that *AFT1* has a significant role in the cobalt resistance. Moreover, it was shown that zinc and nickel tolerance were totally lost upon *AFT1* deletion in the CI25E strain. With respect to manganese, loss of *AFT1* also reduced the resistance very strongly, but did not abolish it totally, as for cobalt, suggesting an additional molecular mechanism for tolerance to this ion.

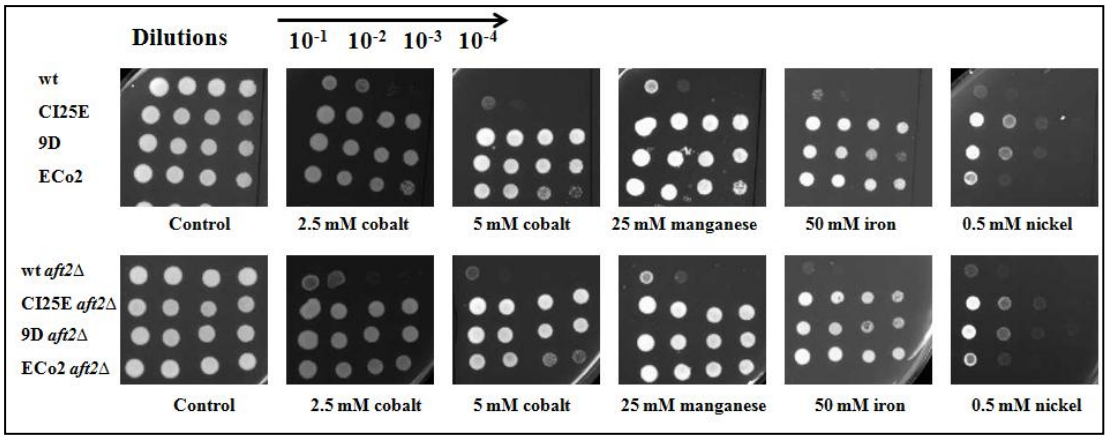


Figure 3.38 : Spot test results of wild type, CI25E, 9D, ECo2 strains and wild type *aft2*Δ, CI25E *aft2*Δ, 9D *aft2*Δ, ECo2 *aft2*Δ mutants grown in YMM containing different concentrations of $CoCl_2$ (2.5 and 5 mM) and other metals (25 mM $MnSO_4H_2O$, 50 mM $(NH_4)_2Fe(SO_4)_26H_2O$, 0.5 mM $NiCl_26H_2O$, 10 mM $ZnSO_47H_2O$).

AFT2 deletion did not cause any changes in cobalt, manganese, iron and nickel resistances of CI25E and 9D. Cobalt resistance of ECo2 *aft2*Δ slightly decreased compared to the ECo2 at 5 mM cobalt stress application (Figure 3.38).

All those results showed that *AFT1* and *COT1* genes were very important for CI25E and 9D. For that reason, *COT1* and *AFT1* were deleted together and their phenotypes were tested to cobalt stress and to other metal stresses. The double deletions diminished the CI25E cobalt resistance. However, they did not abolish it completely (Figure 3.39).

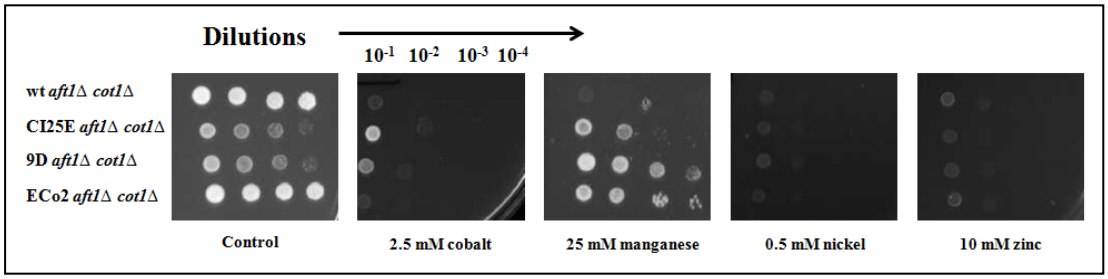


Figure 3.39 : Spot test results of wild type *aft1*Δ *cot1*Δ, CI25E *aft1*Δ *cot1*Δ, 9D *aft1*Δ *cot1*Δ, ECo2 *aft1*Δ *cot1*Δ mutants grown in YMM containing 2.5 mM $CoCl_2$, 25 mM $MnSO_4H_2O$, 0.5 mM $NiCl_26H_2O$, and 10 mM $ZnSO_47H_2O$.

AFT1 deletion caused a decrease in manganese resistance (Figure 3.37) contrary to *COT1* deletion (Figure 3.25). However, *COT1* deletion compensated for the manganese growth inhibition of *aft1*Δ strains for CI25E, 9D and ECo2 (Figure 3.39).

ZRC1 deletion strains of the mutants and the wild type were also constructed to determine the effect of this gene on stress resistance. The lack of *ZRC1* did not diminish cobalt resistance and it did not cause changes in the cross-resistances to manganese and iron (Figure 3.40). However, wild type *zrc1*Δ, CI25E *zrc1*Δ, 9D *zrc1*Δ, and ECo2 *zrc1*Δ completely lost their resistance to 10 mM zinc (Figure 3.40). This result demonstrated once more the importance of *ZRC1* in zinc resistance as other studies in the literature (MaCdiarmid *et al.*, 2003).

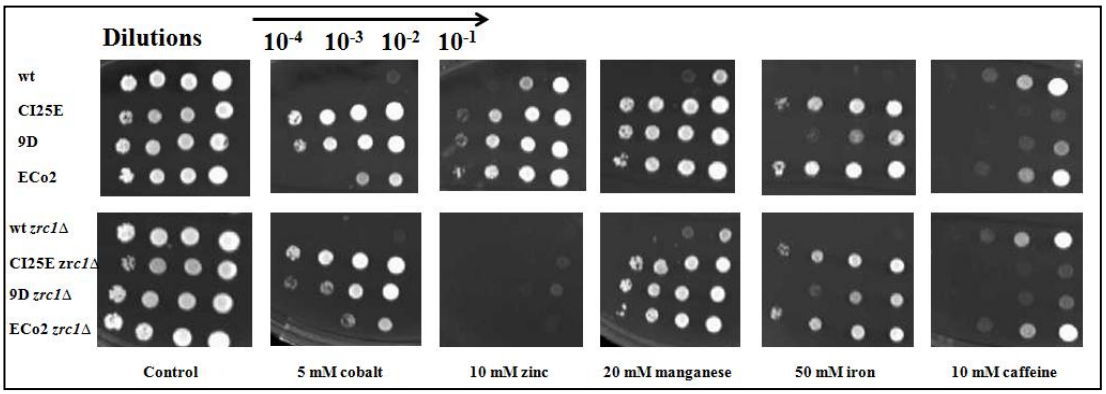


Figure 3.40 : Growth phenotypes of wild type, mutant strains and their *ZRC1*-deletion strains on YMM containing cobalt (5 mM), zinc (10 mM), manganese (20 mM), iron (50 mM) and caffeine (10 mM).

3.9.8 Sequencing of *AFT1, COT1* and *ZRC1* along with up-stream and down-stream regions in the wild type, CI25E, 9D, and ECo2

Results in the previous sections indicated that *AFT1* and *COT1* are important genes in cobalt resistance. Moreover, ECo2 *zrc1*Δ strain slightly lost its cobalt resistance (Figure 3.40). Thus, their sequences of *AFT1, COT1* and *ZRC1* in CI25E, 9D and ECo2 were compared with those of the wild type strain. There might be a mutation or mutations in those gene sequences, which might have caused their upregulation. These genes with their up/down (-1000/+200) stream regions were sequenced according to this hypothesis. The corresponding sequences of CI25E, ECo2, and 9D were aligned with that of the wild type. However, no differences were found neither in nucleotide type nor in nucleotide sequences.

3.9.9 Iron-dependence of wild type, CI25E, 9D and ECo2

Whenever CI25E and 9D were used for spot tests, it was observed that they grew more slowly than the wt and ECo2. Moreover, it was noticed that CI25E *aft1*Δ and 9D *aft1*Δ grew slower than the wild type even on control plates (Figure 3.37). When

AFT1 was deleted, iron transfer into the cells would be blocked or decreased to a low level. Although the wild type and ECo2 could tolerate the lack of this gene, the growth of CI25E and 9D was severely affected by the loss of *AFT1*. In order to observe the growth characteristics of these strains in iron-deficient medium, those cultures were spotted with proper dilutions to solid media containing iron chelator Bathophenanthroline-disulfonic acid 'BPS' (Sigma, USA), cobalt and both 'BPS' and cobalt (Figure 3.41). The growth of CI25E and 9D were inhibited by the presence of 80 µM BPS, but the wild type and ECo2 remained unaffected. Interestingly, cobalt addition (2 mM) to iron-deficient medium restored the growth of the CI25E and 9D. It can be suggested that CI25E and 9D needed iron more than the wild type and ECo2 for growth, because their growth was inhibited when the medium was iron-deficient or when *AFT1* was absent.

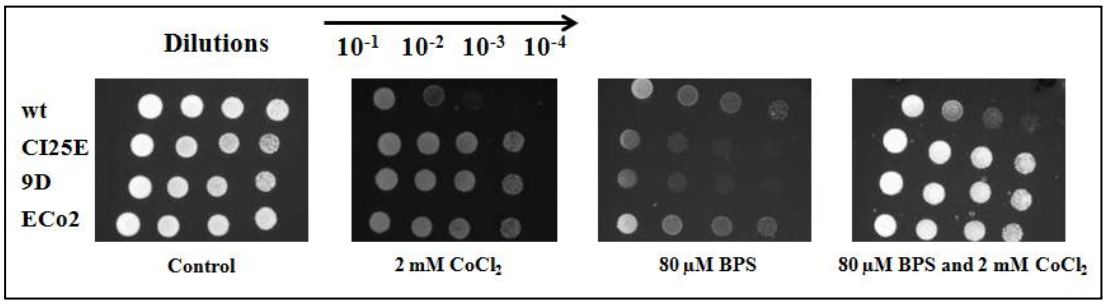


Figure 3.41 : Spot test results of wild type, CI25E, 9D and ECo2 in YMM and YMM containing cobalt, BPS and both cobalt and BPS.

3.9.10 The Aft1 protein was found essentially in the nucleus of the evolved cobalt resistant-strain

Yamaguchi-Iwai *et al.* (2002) demonstrated that Aft1p shuttles between the cytosol and the nucleus depending on the cytosolic iron concentration. Aft1p could sense the intracellular iron levels and it contains a nuclear export signal (NES)-like sequence on its Open Reading Frame. When iron levels are high, it translocates to the cytoplasm and low iron concentration causes nuclear retention of Aft1p. Since *AFT1* in CI25E was upregulated independent of the iron concentration, Aft1p translocations were studied both in wild type and CI25E. To this end, wild type *ura3*Δ and CI25E *ura3*Δ strains were transformed with pRS416-*AFT1*-HA12x centromere vector that expressed Aft1 protein tagged with HA epitope. Cellular localisation of the HA-tagged Aft1p was visualized by immunofluorescene microscopy upon cultivation of wild type and CI25E in the absence and presence of

2 mM $CoCl_2$. In wild type cells, Aft1p was dispersed throughout the cell (Figure 3.42), whereas in CI25E most of the Aft1p was permanently located in the nucleus even in the absence of cobalt. This finding might explain the constitutive upregulation of iron regulon genes detected in CI25E.

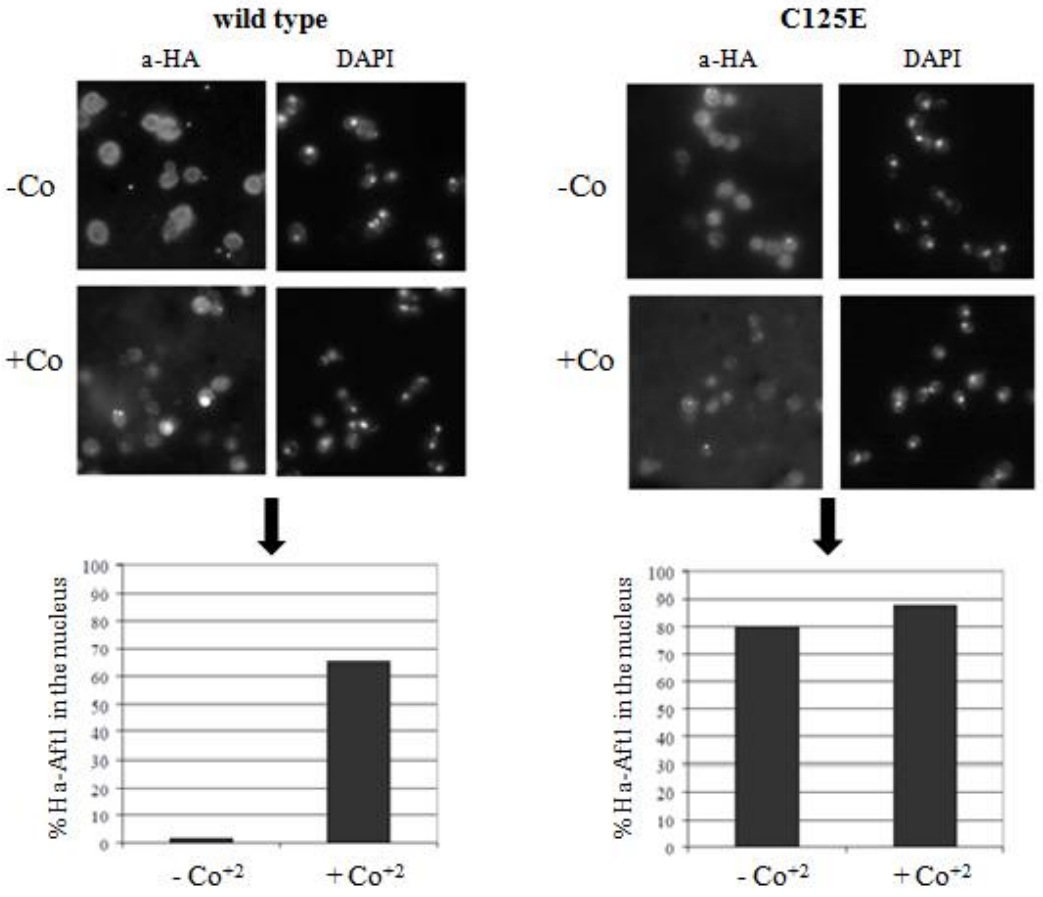


Figure 3.42 : Subcellular localization of Aft1p in wild type and CI25E both in the absence and presence of $CoCl_2$.

Stadler and Schweyen (2002) already showed that cobalt causes relocation of Aft1p into the nucleus. In this study, cobalt causes a similar increase of Aft1p in wild type nucleus and its presence increased slightly the localization of Aft1p in CI25E cells.

3.9.11 Cellular cobalt and iron accumulation upon pulse metal stress application

To determine whether the cellular cobalt and iron contents were altered in response to cobalt (2.5 mM), iron (2 mM) and both cobalt and iron (2.5 and 2 mM, respectively) stress, cells were grown in YMM at 30ºC to an OD_{600} of 3. The corresponding metal stresses were then applied to the cells as pulse stresses for 90

min. Flame atomic absorption spectrophotometry was used to quantify the related cobalt, iron, cobalt and iron-treated cells and untreated control cells (Figure 3.43).

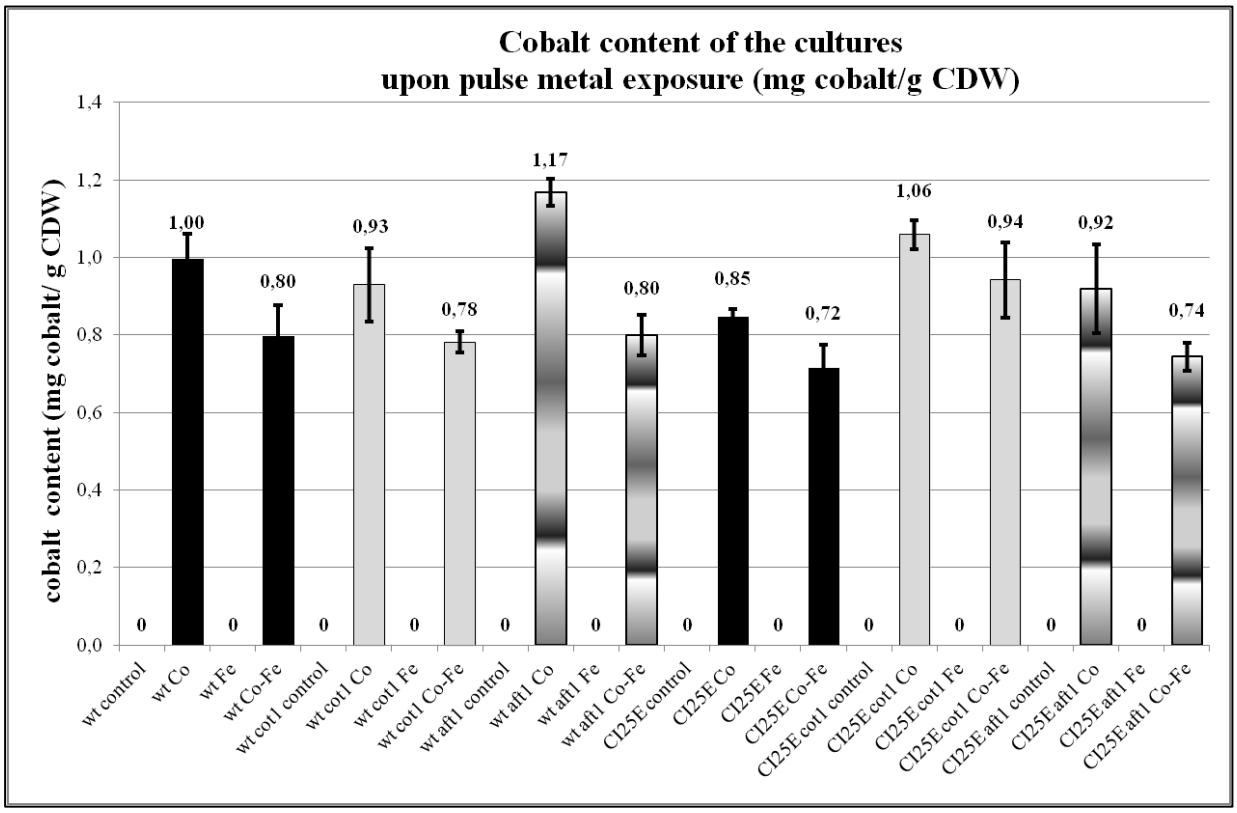


Figure 3.43 : Cobalt contents in wt, wt *cot1*Δ, wt *aft1*Δ, CI25E, CI25E *cot1*Δ and CI25E *aft*1Δ cells cultivated in the presence of 2.5 mM $CoCl_2$, 2 mM iron and both 2.5 mM $CoCl_2$ and 2 mM iron (No cobalt was detected in cell samples grown in the absence of $CoCl_2$).

The results showed that when there was only cobalt in the medium, the cobalt contents of all strains were higher than when there were both cobalt and iron present in the medium (Figure 3.43). Wild type and its *COT1* and *AFT1* deletion mutants had very similar cobalt holding patterns both in the presence of cobalt only and in the co-presence of cobalt and iron. Wild type *aft1*Δ mutant seemed to hold cobalt more than the wild type and wt *cot1*Δ in the presence of cobalt. However, the difference was not significant. Among all the strains tested, the lowest cobalt content was found in CI25E that was incubated in the presence of both cobalt and iron.

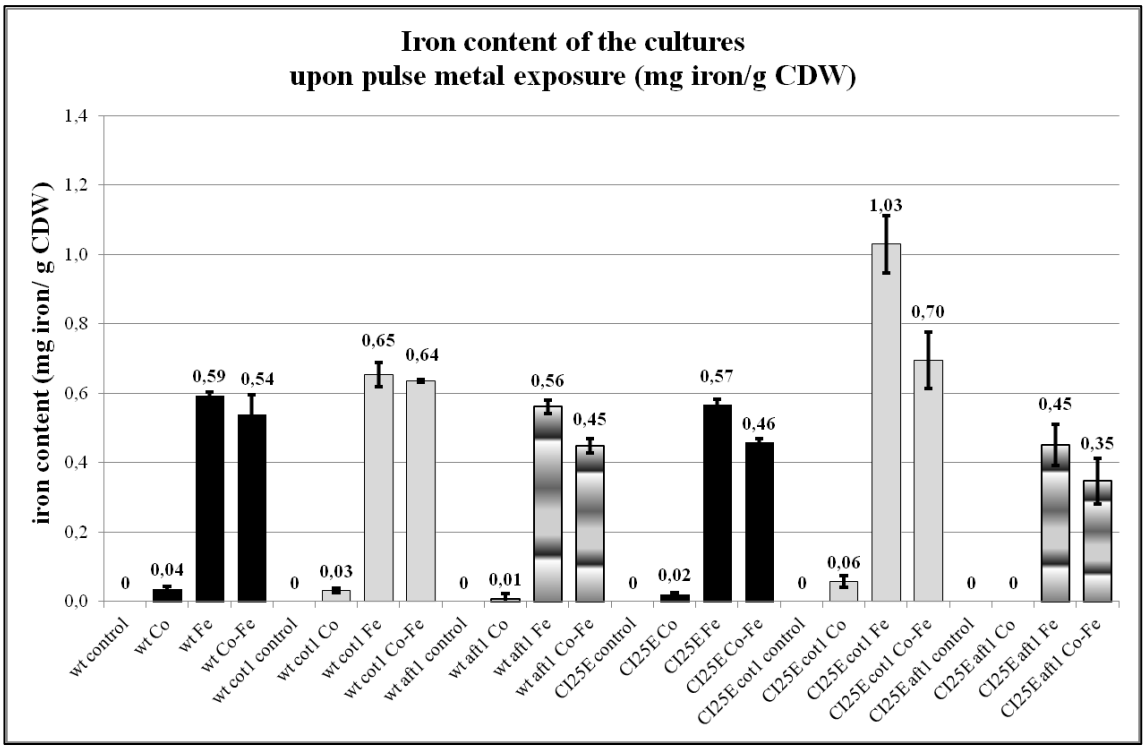


Figure 3.44 : Iron contents of wt, wt *cot1*Δ, wt *aft1*Δ, CI25E, CI25E *cot1*Δ and CI25E *aft*1Δ cells cultivated in the presence of 2.5 mM $CoCl_2$, 2 mM iron and both 2.5 mM $CoCl_2$, 2 mM iron.

CI25E *cot1*Δ deletion mutant had significantly higher iron content than all the strains tested, when grown in the presence of iron only. For all the strains tested, when grown in the presence of iron, iron contents were slightly less than or equal to the iron contents of the same strains grown in the presence of both cobalt and iron (Figure 3.44).

3.9.12 Cellular cobalt and iron accumulation upon continuous metal stress application

It was aimed to determine cobalt and iron-holding patterns of the wild type, wild type *cot1*Δ, wild type *aft1*Δ, CI25E, CI25E *cot1*Δ and CI25E *aft*1Δ cells in response to addition of 2.5 mM cobalt, 2 mM iron or both 2.5 mM cobalt and 2 mM iron.

In the presence of either iron or cobalt, CI25E accumulated less iron or cobalt than the wild type. Moreover, in the co-presence of both iron and cobalt, CI25E accumulated significantly less iron and cobalt than the wild type. At this condition, CI25E had three times lower iron content than the wild type, which is a significant difference (Figure 3.45).

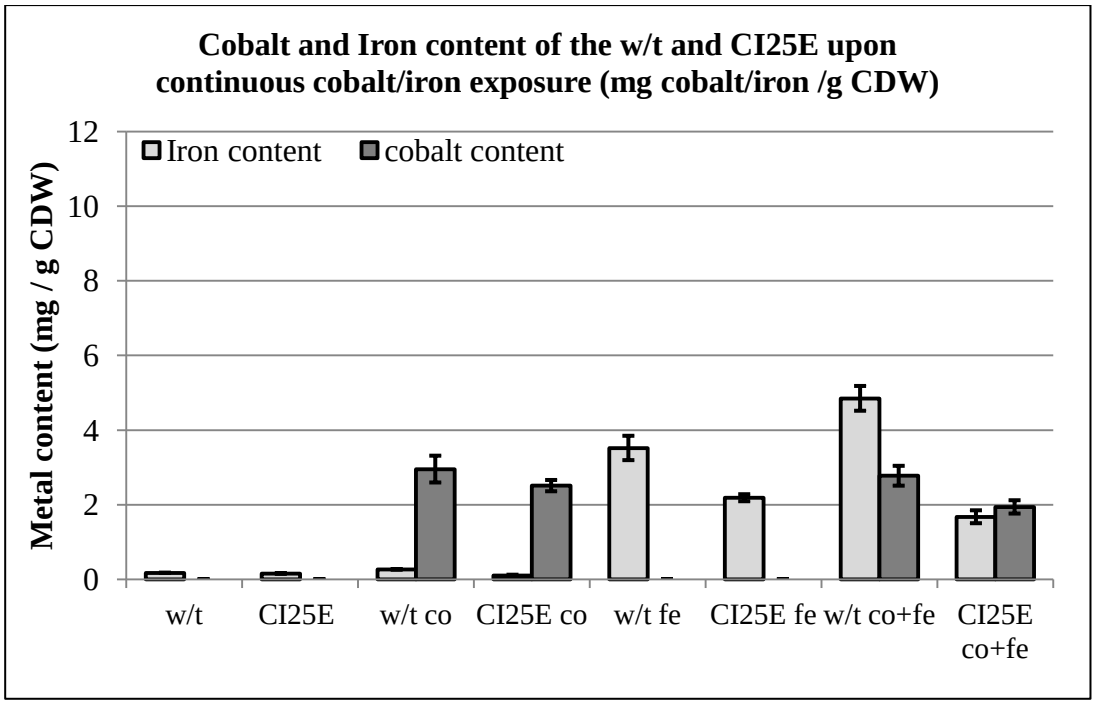


Figure 3.45 : Cobalt and iron contents of wt and CI25E cells under 2.5 mM cobalt, 2 mM iron and both 2.5 mM cobalt/2 mM iron stress conditions (Cobalt accumulation was not observed for the samples at which no cobalt stress applied).

AFT1 deletion caused an increase in iron uptake of the wild type and CI25E in the presence of iron. The lack of *AFT1* did not cause a significant change in cobalt uptake in the presence of cobalt. In the co-presence of both iron and cobalt, *AFT1* deletion of wild type caused a significant increase in iron uptake and this is the highest amount of iron hold by the cells among all the cultures and conditions tested. The iron uptake of CI25E *aft1*Δ increased in the co-presence of cobalt and iron, as compared to CI25E, whereas the cobalt uptake did not change upon *AFT1* deletion (Figure 3.46).

Moreover, *COT1* deletion did not significantly affect iron and cobalt uptake in CI25E and it did not significantly affect iron uptake in wild type in the presence of iron. In the co-presence of cobalt and iron, the iron uptake of wt *cot1*Δ and CI25E *cot1*Δ increased but cobalt uptake slightly decreased (Figure 3.47).

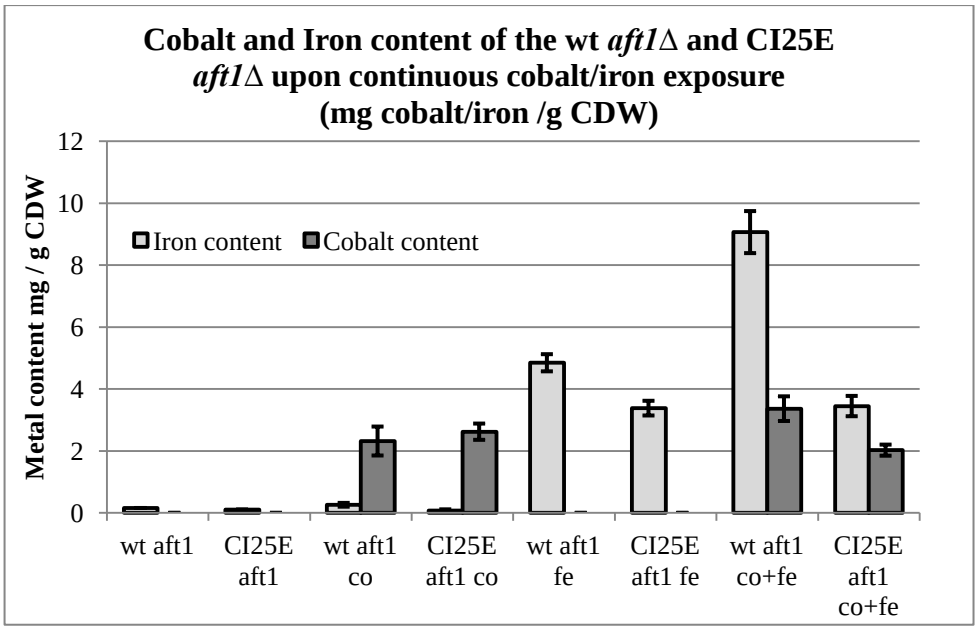


Figure 3.46 : Iron and cobalt contents of wt *aft1*Δ and CI25E *aft*1Δ cells under 2.5 mM cobalt, 2 mM iron and both 2.5 mM cobalt/2 mM iron stress conditions.

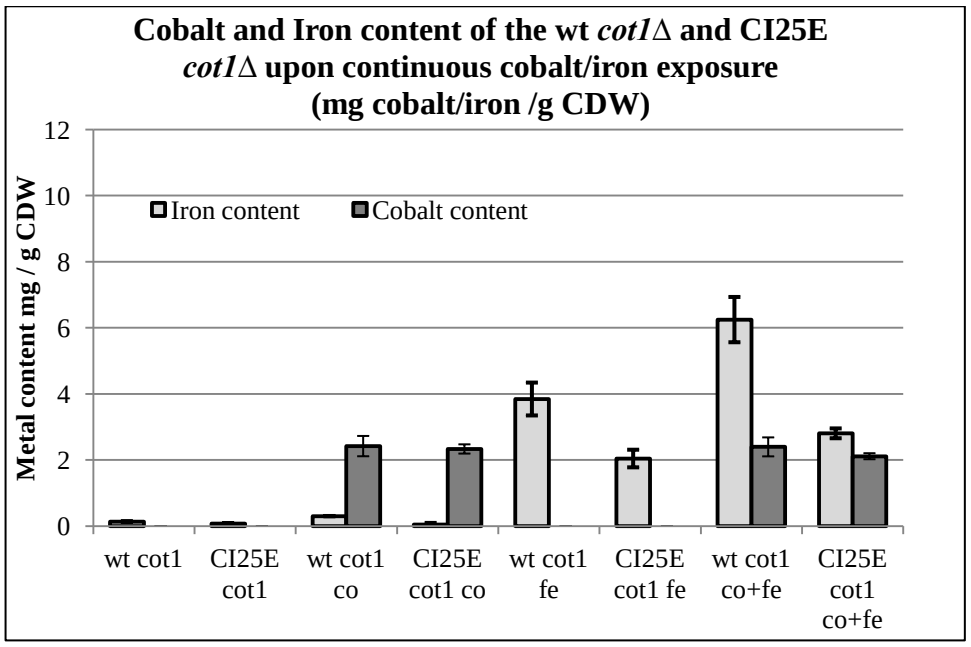


Figure 3.47 : Cobalt and iron contents of wt *cot1*Δ and CI25E *cot1*Δ cells under 2.5 mM cobalt, 2 mM iron and both 2.5 mM cobalt/2 mM iron stress conditions.

From these results, it can be suggested that cobalt uptake/efflux is similar to the iron uptake/efflux in *S. cerevisiae*. Yeast cells apparently use the existing transport/resistance mechanism for iron when encountered with cobalt ions. This mechanism and/or system seems to have a much higher affinity for iron than for cobalt, based on the significantly higher iron levels detected in iron/cobalt co-presence samples.

The deletion of *AFT1* caused an increase in iron uptake but the deletion of *COT1* did not significantly change iron and cobalt uptake except for the increase in iron content of wt *cot1*Δ in the co-presence of iron and cobalt. Thus, *AFT1*-related iron transport system seems to be very important in cobalt and iron resistance. For both iron and cobalt resistance mechanisms, it can be suggested that yeast cells reduce their uptake and therefore become resistant, as in the case of CI25E. The increase in iron uptake in the co-presence of cobalt and iron for *cot1*Δ deletion mutant of the wild type is consistent with the previous study of Stadler and Schweyen (2002). It can be suggested that *COT1*-deleted wild type cells might have increased their iron uptake in order to resist against excess cobalt (Figure 3.47).

3.9.13 Growth characteristics of wild type, wt *aft1*Δ, CI25E and CI25E *aft1*Δ in aerobic respiration conditions

The slow growth phenotype of CI25E, ethanol-sensitive phenotype and the possible defects in its iron metabolism suggested a research strategy to control the aerobic respiration ability of CI25E and CI25E *aft1*Δ. In order to investigate the ability of wt, wt *aft1*Δ, CI25E and CI25E *aft1*Δ to utilize glycerol and ethanol as the sole carbon source instead of glucose, their growth in glycerol and ethanol-containing minimal medium was analyzed by OD_{600} measurements through 20 h of incubation at 30°C and 150 rpm (Figure 3.48). During aerobic growth, maximum specific growth rates of wild type (0.20 h^{-1}) and CI25E (0.17 h^{-1}) did not significantly differ from each other. Moreover, *AFT1* deletion mutants' specific growth rate for wild type (0.16 h^{-1}) and CI25E (0.17 h^{-1}) were nearly the same. For that reason, all the strains studied are not respiratory deficient.

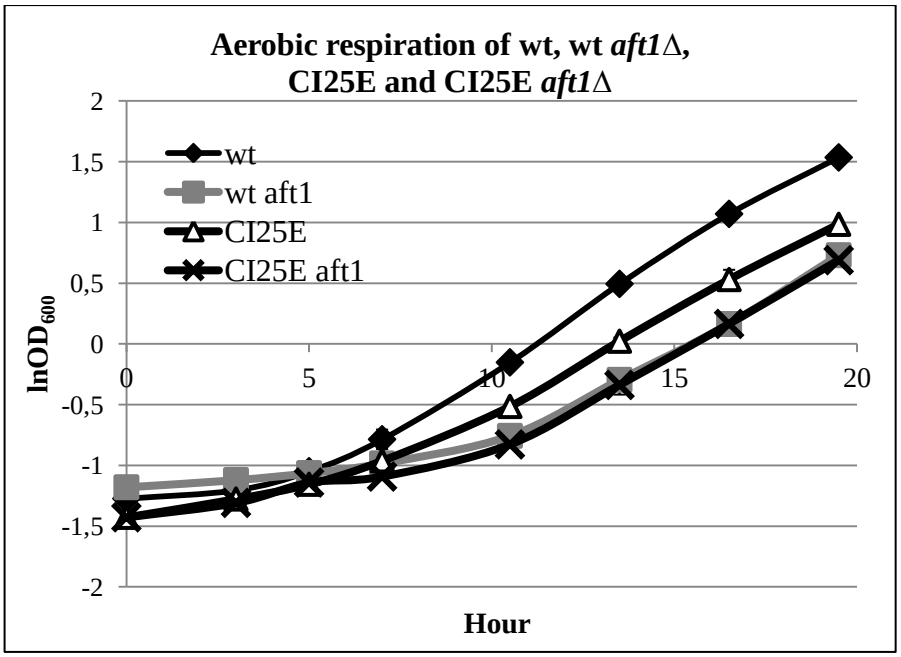


Figure 3.48 : Growth of wt, wt *aft1*Δ, CI25E and CI25E *aft1*Δ during aerobic respiration.

4. DISCUSSION

In this study, we employed two different strategies to obtain cobalt-resistant *S.cerevisiae* mutant cells. In the first one, cobalt resistance of EMS-mutagenized *S.cerevisiae* cell population was developed by mutagenesis and direct selection on solid media supplemented with cobalt. In the second strategy, *in vivo* evolutionary engineering based on batch selection under continuous exposure to gradually increasing cobalt stress levels was applied to EMS-mutagenized *S.cerevisiae* cell population. The results showed that evolutionary engineering is more powerful strategy to obtain a desired phenotype, compared to mutagenesis and direct selection techniques (Figure 3.24).

Individual mutants obtained from evolutionary engineering showed cross-resistance to nickel, iron, manganese, and zinc (Figure 3.10). Manganese, iron, cobalt, and nickel are placed in the Periodic table sequentially on the same row and zinc follows these ions only one metal (copper) after (Petrucci, 2002). There are a large number of yeast enzymes, which can substitute zinc/iron/nickel/manganese cofactor for cobalt and are able to perform their corresponding metabolic activities. The replacement of zinc by cobalt for alcohol dehydrogenase (Vanni *et al.*, 2000) and major apurinic/apyrimidinic endonuclease (Levin *et al.*, 1991) did not preclude those enzymes from functioning. Cobalt can replace and sometimes attack the iron/manganese/nickel atoms in their catalytic sites. The common properties of those metal ions for yeast cells are their storage localization, which is vacuole (Ramsey and Gadd, 1997). The resistance phenotype of the evolved strain to those ions could be related to the high capacity to accumulate them on its vacuole. To directly understand vacuolar role(s) in individual mutants, metal contents of each organelle might be determined by atomic absorption spectrophotometer.

When in excess, cytosolic cobalt and zinc accumulated in the vacuole (MacDiarmid *et al.*, 2000) by Cot1 and Zrc1 proteins, respectively. *COT1* identified by Conklin *et al.* (1992) was shown to be implicated in cobalt tolerance in yeast and Kamizono *et al.* (1989) identified *ZRC1* gene to be responsible for zinc resistance. Li and Kaplan

(1998) reported that Cot1p and Zrc1p are localized at the vacuolar membrane inside the cell. Lack of *ZRC1* could tolerate only mild zinc stress. Deletion of *COT1* results in a weak effect on sensitivity for the mutant to zinc ions, but double mutant *cot1*Δ *zrc1*Δ could not even tolerate very low concentrations of zinc. There is a possibility of some random mutations happened either during EMS treatment step or selection procedures of the evolutionary engineering strategy, which may have caused cross-resistance to zinc in the individual mutants. Lin and co-workers (2008) showed that a missense mutation in the vacuolar zinc transporter *ZRC1* gene resulted in a change in metal specifity of the protein encoded by this gene. This permitted the mutant cells to grow under high iron conditions. A similar phenomenon might have occured in CI25E that possibly made this mutant resistant to cobalt, nickel, iron and zinc.

Conklin *et al.* (1993) isolated cobalt-resistant mutant of *S.cerevisiae* strains. It was reported that the locus with the mutation, which confers resistance to cobalt, was *GRR1*/*COT2* in *S. cerevisiae* cells. *cot2*Δ mutants were also capable of growth in media containing zinc, manganese and nickel. This result is in parallel with those in this study, where CI25E was also found to have cross-resistance to zinc, manganese and nickel.

A link between cobalt and nickel co-resistance was demonstrated in *Escherichia coli*. *E. coli* yohM ('rcna', resistant to cobalt and nickel) gene is responsible for transportation of cobalt and nickel out of the cell. Moreover, when this gene is inactivated, cells become sensitive to cobalt and nickel (Rodrigue *et al.*, 2005). Additionally, ferric uptake regulator (Fur) of *E. coli* controls both cobalt and nickel transport out of the cells (Rodrigue *et al.*, 2005 and Koch *et al.*, 2007).

Another study was conducted by using a *S. cerevisiae* mutant that displayed resistance to both cobalt and nickel with reduced accumulation of magnesium (Joho *et al.*, 1991). However, it was sensitive to zinc and manganese, contrary to the mutant 'CI25E' obtained by evolutionary engineering in this study.

SMF2 encodes a protein responsible for manganese homeostasis and Smf2p belongs to NRAMP family of metal transport proteins (Luk and Culotta, 2001). Among the divalent cations, it can transport both cobalt and manganese. *SMF1* is another NRAMP family member protein in yeast cells and it is responsible for manganese, as well as iron ion transport (Liu *et al.*, 1997). *PHO84* is another gene involved in

manganese homeostasis in yeast cells that encodes high affinity inorganic phosphate transporter like *SMF1/2*. *PHO84*-disrupted mutant was shown to be resistant to manganese, cobalt, zinc and it was slightly resistant to copper (Jensen *et al.*, 2003). Those genes are examples for the possible relation between iron/cobalt/manganese resistances.

In this study, it was also shown that the individual cobalt-resistant mutants were sensitive to H_2O_2, sodium chloride, magnesium, ethanol, and caffeine (Figure 3.11). Lindsay and Dawis (1992) demonstrated that *S. cerevisiae* shows sensitivities to both H_2O_2 and superoxide-generating agents. It was stated that reactive oxygen species (ROS) could be formed via metal catalyzed "Fenton reactions" and peroxidation of lipid subsequently (Stohs and Bagchi, 1995). For that reason, it was expected that cobalt-resistant individual mutants should be resistant to H_2O_2, which was not the case. However, it should be considered that *CTT1*, which encodes cytosolic catalase T (Traczyk *et al.*, 1985), was significantly downregulated in CI25E (Table 3.15). Ctt1 protein protects cells from the oxidative damage by H_2O_2. The reason for H_2O_2 sensitivity could be related to the low levels of Ctt1p.

Individual mutants and the final population were slightly sensitive to NaCl stress (Figure 3.11). Hamilton *et al.* (2002) stated "Salt tolerance is a complex trait involving responses to both osmotic and ionic stresses". It was thought that cobalt resistance could provide NaCl resistance to the individual mutants. However, those mutants were not as resistant as the wild type, regarding NaCl stress.

Magnesium sensitivity of individual mutants seems to be reasonable because it was previously suggested that the existence of magnesium might prevent cobalt and nickel toxicity and those three ions are transported into the cell using the same mechanisms (Joho *et al.*, 1991). It may be possible to some extent that individual mutants are protected from the inhibitory effects of cobalt by increasing magnesium uptake when high concentrations of magnesium is applied to the culture as in the case of 1 M exposure (Figure 3.11), they might accumulate higher amounts of magnesium, which could possibly impair their growth. However, cellular magnesium contents of the wild type and CI25E should be determined to clarify that behavior during cobalt exposure.

Individual mutants in this study also demonstrated sensitivity to caffeine (Figure 3.11). Cell wall integrity signaling is induced upon caffeine treatment because this agent creates cell wall stress (Martin *et al.*, 2000). It was previously demonstrated that MAP kinase cascade genes are required for cell wall integrity. The mutations in one of these cascade elements showed sensitivity to caffeine (Jacoby *et al.*, 1998). Maybe, individual mutants were defective in the maintenance of cell wall stability and for that reason they were sensitive to this cell-wall perturbing agent. Ethanol sensitivity of the individual mutants could be another indicator for the mutants' improper cell wall integrity (Takahashi *et al.*, 2001).

Although phenotypic characterization was performed for all individual mutants and their corresponding population (CI25A to H and 25th population), classic genetic analysis was performed with only one individual mutant, CI25E. In the course of genetic characterization, it was observed that cobalt hyper-resistance is a genetically complex trait that cannot be ascribed to a single gene. It was also shown that genes related to cobalt-resistance in CI25E seem to have semi-dominance (Figure 3.15 and 3.16).

COT1 has been reported to be the unique gene directly implicated in cobalt tolerance. For that reason, *cot1*Δ null mutants of wild type and CI25E were constructed. It was demonstrated that at mild cobalt stress levels (2.5 mM), the growth of CI25E *cot1*Δ slightly diminished, contrary to wild type that totally lost its resistance. At high cobalt concentrations, which correspond to 5 mM $CoCl_2$, wild type did not grow as expected, however; CI25E lost only its partial tolerance to cobalt stress (Figure 3.25). Moreover, in this study, it was found that even in the absence of cobalt ion treatment, evolved strain overexpressed *COT1* upon quantitative RT-PCR (Figure 3.26). All these results demonstrated that *COT1* is an important gene for cobalt resistance phenotype of CI25E. There could be some mutations on the ORF as well as up/down-stream regions of *COT1*, which makes it upregulated even in the absence of cobalt. Thus, *COT1* gene was isolated from both wild type and CI25E and sequenced. It turned out that both genes had the same sequence, which also included 1000 bp upstream and 200 bp downstream of the coding sequence. This result indicated the presence of other factors, which possibly influence *COT1* expression rather than its ORF or its regulation sites.

In order to gain insight into cellular/molecular resistance mechanisms to cobalt stress in detail, DNA microarray analysis was used to study the global pattern of transcription difference between the wild type *S.cerevisiae*, evolved strain 'CI25E' and CI25E *cot1*Δ mutants. CI25E *cot1*Δ was included in the transcriptomic analysis in order to eliminate *COT1* effects on cobalt resistance and to find out other factors implicated in cobalt resistance mechanisms. Genome expression profiles were compared between the wild type and CI25E, as well as CI25E and CI25E *cot1*Δ. In this comparison, ~70 genes (~1.2% of the genome) were overexpressed in CI25E and CI25E *cot1*Δ by a factor of two or more (Table 3.11) and 55 genes (0.9 % of the genome) were underexpressed with a fold change lower than and equal to 0.5, compared to wild type (Table 3.15).

Upregulated genes in the mutants were associated with iron homeostasis, amino acid metabolism, cell wall and some functionally known and unknown genes.

Nineteen of the 68 most highly overexpressed genes in Table 3.11 are involved in iron homeostasis. All those genes are under the control of a transcription regulator, Aft1p (Yamaguchi-Iwai *et al.*, 1995). *AFT1* encodes a protein, which binds to iron regulon to induce the genes responsible for iron transfer into the yeast cell. Stadler and Schweyen (2002) demonstrated that cobalt has the ability to induce yeast iron regulon. They applied sub- and mild-toxic cobalt stress to wild type and *aft1*Δ cells for different sets of experiment. They also created an iron-depleted condition and checked again the expression profile of the wild type cells. The highest upregulation was observed for the genes related to iron homeostasis, similar to this study. *PRY1*, *TAM1*/*TMT1, ARO9, YGP1*, *COT1* were the genes which were upregulated in both studies, and which had different functions than iron homeostasis. It can be concluded that there are not too many genes, which intersect for both of these studies apart from the iron homeostasis cluster and other few genes. A possible explanation for that would be the different experimental conditions; they performed the transcriptomic analyses of the wild type samples grown in the presence and absence of cobalt ion in complex medium (YPD). In this study, however, samples of wild type and evolved strain were grown in synthetic defined medium (YMM) under non-stress conditions, prior to transcriptomic analyses. Nonetheless, it was interesting to observe that iron homeostasis was affected in both studies.

In another microarray study, wild type BY4741 and a copper-responsive transcription factor, *MAC1*-deleted strain expression levels were compared (Pagani *et al.*, 2007). Interestingly, one of the major groups of genes that showed high expression levels were involved in iron homeostasis and regulated again by Aft1p in *mac1*Δ strain. Fourteen of 18 upregulated genes in that study were also upregulated in the present study. *FRE8* and Mac1p/Aft1p regulated *FRE1* expression levels decreased in the *mac1*Δ strain, different from the present study (Jungmann *et al.*, 1993). Vacuolar iron transporter *FTH1*/*FRE6* were upregulated in CI25E and CI25E *cot1*Δ in contrast to *mac1*Δ strain, as well (De Freitas *et al.*, 2004). In that study, they found five new genes containing Aft1p consensus sequences. Four of them were upregulated in the present study as well, and three of them (*PCA1*, *YTP1*, *VMR1*) with more than two times fold change are indicated in the Table 3.11.

In the microarray analysis of the present study, *UBC5*, *VPS73*, *SPS19*, *GTT1*, *GTT2*, and *GPG1* were found to be upregulated in both mutants. These genes contain one or more than one Aft1p binding motif in their promoter region, according to YEASTRACT tool. However, so far, they have not been implicated in cobalt resistance phenotype in yeast and this makes these gene products new candidates for cellular cobalt homeostasis.

Chen *et al.* (2004) showed that Aft1p could be active even if the cytosolic iron level is high. They explained that iron regulon is induced by the mitochondrial Fe-S cluster synthesis. A microarray analysis was carried out for mitochondrial Iron Sulfur Cluster (ISC) assembly (*YAH1*) and export (*ATM1*) components depleted cells to compare global transcription responses of those mutants. The expression response was partially similar to that of iron-deprivation conditions. More than 200 genes showed at least 2-fold expression changes compared to wild type cells in this condition (Hausmann *et al.*, 2008). In the present study, there were no data for *ATM1* expression level, but *YAH1* was found to be slightly downregulated in CI25E and CI25E *cot1*Δ mutants with a fold change of 0.80 and 0.75, respectively (Table 3.12). Apart from *YAH1* and *ATM1*, genes related to Fe-S protein biogenesis in yeast were investigated in the CI25E and CI25E *cot1*Δ transcriptome data (Lill and Mühlenhoff, 2006). Out of 22 genes related to Fe-S cluster biogenesis, six and five of them were differentially regulated on the CI25E and CI25E *cot1*Δ with a p value < 0.08 (0.08 is

chosen to increase the number of the genes). The expression levels of those genes did not change significantly (Table 3.12).

Another gene in *S.cerevisiae* related to Fe-S cluster assembly and iron homeostasis in mitochondria is *YFH1* that encodes frataxin. A mutation in frataxin gene in human causes a neurodegenerative disorder, Friedrich ataxia (Campuzano *et al.*, 1996). Foury and Talibi (2001) performed a transcriptomic analysis by using wild type and *S. cerevisiae yfh1*Δ strain. There are 13 upregulated genes, which overlapped for *yfh1*Δ and CI25E/CI25E *cot1*Δ mutants of the present study. All those genes are known to encode proteins that are involved in iron homeostasis. However, *FET4* was upregulated in *yfh1*Δ strain, unlike the CI25E/CI25E *cot1*Δ mutants. The high number of intersected genes for both studies might be a sign for iron homeostasis defect in CI25E.

PDC5 attracted attention with the highest expression level among other genes for both CI25E and CI25E *cot1*Δ. This gene product is involved in alcoholic fermentation. In the literature, it was stated that *PDC5* might be upregulated either in the absence of *PDC1* or in the thiamin deficiency (Seeboth *et al.*, 1990). Thiamine pyrophosphate is the cofactor for both *PDC1* and *PDC5* encoding enzymes, pyruvate decarboxylase isozyme 1 and 2, respectively. There is neither up- nor down regulation for *PDC1* expression levels in the transcriptomic data of the present study. In addition, so far there is no link between cobalt - iron homeostasis and thiamin in yeast cells. However, there are studies made on the prokaryotes. In *Salmonella enterica*; *gshA*, *apbC*, *apbE*, and *rseC* are responsible for Fe-S metabolism. The strains having mutations on those loci along with an *yggX* lacking background became conditional thiamine auxotrophs. This was explained by the inability to deliver or fix Fe-S cluster in the ThiH enzyme. When a 91-amino-acid protein encoded by *yggX* which is related to oxidative stress resistance and also iron orientation was expressed, it suppressed thiamine auxotrophy in the *gshA*, *apbC*, *apbE*, and *rseC* mutants. Interestingly, growth of *gshA*, *apbC*, *apbE*, and *rseC* mutants lacking *yggX* was inhibited when cobalt was added into the medium. The inhibition of growth was impaired by adding thiamine or iron into the medium. Cobalt induced thiamine auxotrophy was eliminated by adding iron to the media or by expression of *yggX*. It showed that cobalt has an effect on thiamine synthesis in

bacteria (Skovran *et al.*, 2004). Further studies must be performed to enlighten the possible link between iron-cobalt and thiamine metabolism in yeast cells.

AFT1 was deleted in wild type and CI25E to elucidate possible relationship between iron homeostasis and cobalt resistance (Figure 3.37). As expected, cobalt resistance decreased both for the wild type and CI25E, but it did not abolish completely. *COT1* was already deleted in both strains and double deletion mutants were prepared (Figure 3.39). However, CI25E *cot1*Δ *aft1*Δ double mutant preserved its cobalt resistance against low level treatment. Possible sequence differences in those genes in CI25E were also investigated, but no difference was observed compared to those of the wild type.

Aft1p localization demonstrated that even in the absence of cobalt treatment, Aft1p were found in the nucleus for CI25E, but in the wild type, it was located in the cytosol (Figure 3.42). Two hypotheses were proposed for CI25E, related to Aft1p constitutive localization in the nucleus:

1. CI25E could be defective in iron uptake into the cells possibly the lack and/or malfunctioning of high affinity uptake proteins.
2. Iron Sulfur protein biosynthesis might be impaired.

Iron and cobalt content of the cells were determined by atomic absorption spectrophotometer in the presence of cobalt, iron and both cobalt and iron. CI25E seems to hold less metal ions both for separate presence and co-presence of cobalt and iron. However, in the co-presence of cobalt and iron, CI25E accumulated significantly less iron than the wild type. This might be the sign of defective iron uptake system in CI25E (Figure 3.45, 3.46 and 3.47).

To understand the possible interactions and overlaps between cobalt resistance phenotype and iron homeostasis controlled by Aft1p, another transcriptomic analysis of the wild type and CI25E lacking *AFT1* gene could be proposed.

Down-regulated genes were found to be associated with *PAU* genes, trehalose and glycogen metabolism, NAD biosynthetic process, pheromone response, thiamine homeostasis, iron regulation and amino acid metabolism.

The family of seripauperin (*PAU*) genes was the most significant functional category among the repressed genes. *PAU* genes are comprised of 24 genes, which are situated

generally in the subtelomeric regions of chromosomes. Their protein products have approximately 120-124 amino acids with a high degree of similarity. It was reported that *PAU* genes were expressed upon exposure to different environmental stress conditions and most apparently adaptations to stress during alcoholic fermentations (Luo and Vuuren, 2009).

DAN/*TIR* genes were expressed during anaerobic growth and they were found to encode cell wall mannoproteins (Abramova *et al.*, 2001). Luo and Vuuren (2009) suggested renaming of *DAN2* and *DAN3* as *PAU23* and *PAU24*, respectively. Possible functions of *PAU* genes might be attributed to the protection of cell wall integrity.

Pagani and co-workers (2007) conducted a genome-wide transcriptomic analysis for *aft1*Δ and wild type *S.cerevisiae* strain in the presence and absence of 5 mM zinc. Genes that were upregulated in *aft1*Δ versus wild type strain growing in the presence of zinc consisted of 10 (*PAU1, PAU2, PAU5, PAU6, PAU11, PAU12, PAU14, PAU20, PAU21, PAU24*) out of the 24 *PAU* genes. They found only *PAU19* with an increased expression between *aft1*Δ versus wild type in the absence of zinc ions. In order to assess different cellular processes affected by Aft1p, Berthelet *et al.*, (2010) performed functional genomics analysis and they found again *PAU* genes to be upregulated in *aft1*Δ, compared to wild type cells (*PAU3, PAU4, PAU5, PAU7, PAU9, PAU13, PAU14, PAU15, PAU16, PAU20* and *PAU23*). It was stated in YEASTRACT database that Aft1p is a transcription factor for all *PAU* genes stated in both studies. Since *AFT1* was upregulated in the transcriptomic and quantitative RT-PCR analyses throughout the present study, the downregulation of *PAU* genes could be considered as a significant result. Three additional *PAU* genes (*PAU8, PAU10*, and *PAU18*) were also found in the present study, which were not indicated so far as a target for Aft1p. Thus, they are now candidates to be *AFT1*-regulated *PAU* genes. This is an outcome of the present microrray experiment in this study. Actually, downregulation of *PAU* genes could be a side effect of CI25E evolution because the upregulation of those genes was expected in the course of stress exposure, contrary to the present study. However, *AFT1* upregulation downregulated many *PAU* genes in CI25E. CI25E is cobalt hyper-resistant with this genetic background and all possible drawbacks like downregulation of this gene group was ignored by CI25E.

Nicotinamide adenine dinucleotide (NAD) is involved in energy metabolism, cellular redox reactions and many different biological processes (Lin and Guarente, 2003). Two major NAD biosynthesis pathways are *de novo* synthesis of NAD from tryptophan via kynurenine and NAD salvage pathways, the latter of which occurs by degradation products of nicotinamide. The key enzymes in *de novo* synthesis of NAD are Bna1p, Bna2p, Bna4p, Bna5p, Bna6p and Bna7p (Bedalov *et al.*, 2003). Interestingly, Table 3.15 and Figure 3.35, show a group of repressed genes involved in NAD biosynthesis including *BNA1*, *BNA2*, *BNA4* and *BNA5* with a decrease in expression less than 0.5-fold in CI25E and CI25E *cot1*Δ. Moreover, *BNA6* expression, which could not be included in this group because of the fold-change criteria, also decreased in CI25E and CI25E *cot1*Δ with a 0.72 and 0.68-fold change, respectively compared to wild type. The reason for the downregulation of these genes might be to keep tryptophan concentration constant inside the cell. Regarding the upregulated genes in Table 3.11, a major functional group consists of 9 genes coding for proteins involved in amino acid metabolism. Among this group, *ARO9* expression was slightly higher than in wild type. Aro9p catalyzes the first step of tryptophan, phenylalanine, and tyrosine catabolism (Iraqui *et al*., 1998). This seems to be conflicting with the downregulation of NAD biosynthesis genes with the potential aim of keeping tryptophan levels constant. However, one of the induction parameters for *ARO9* is the presence of aromatic amino acids in the growth medium (Iraqui *et al*., 1998). It is likely that, CI25E might try to keep tryptophan levels inside the cell within certain limits but could not prevent the sensing mechanisms for these amino acids which results in *ARO9* upregulation.

Additionally, almost 11% of the annotated genes that showed decreased transcript levels were found to be involved in pheromone response. *SST2* was found to be responsible for negative regulation of pheromone response and *sst2*Δ mutants developed self-shmooing phenotype (Chan and Otte, 1982). Another gene from this group is *SST1* which encodes an extracellular protease that cleaves α-factor pheromone (Ballensiefen and Schmitt, 1997). Additionally, "a-specific" *STE* genes (*STE2/6*) which are needed for *MATa* cells to mate were downregulated in both mutants (Wilson and Herskowitz, 1984). It is likely that *SST1* and *SST2* downregulation facilitates/helps mating processes but this seems to be conflicting with the downregulation of *STE2* and *STE6* which encode a receptor for α-factor

pheromone and a transporter of a-factor, respectively. The common factors associated with these genes are two transcription factors; Ste12p and Yhp1p which are implicated in the regulation of these genes. It is difficult to explain why these genes were downregulated both in the CI25E and CI25E *cot1*Δ. Nevertheless, it was stated that pheromone-induced morphogenesis leads to cell lysis for the mutants with defects in the cell wall integrity signalling genes (Heather *et al.*, 2004). If caffeine and salt stress sensitivity of CI25E are assumed as indicators of the possible cell wall defects, a possible link between this phenotype and the downregulation of the pheromone response gene could then be suggested.

CI25E expression profile was compared along with that of CI25E *cot1*Δ. Genes whose expression were found to be increased with a *p* value lower than 0.01 are shown in Table 3.17. There were only 4 genes with the appropriate *p* value criteria. *ZRT1* encodes a member of high-affinity zinc transporter (Zhao and Eide, 1996). In CI25E *cot1*Δ mutant cytosolic zinc could be transferred into the cytosol only by the function of Zrc1p, but in CI25E, both Cot1p and Zrc1p facilitate the zinc ion transfer. For that reason, CI25E did possibly not hesitate to take up more zinc contrary to CI25E *cot1*Δ which is defective in zinc storage. Apart from these genes, it is difficult to comment on the upregulation of *NSR1*, *AQR1* and *GFD2* in CI25E, compared to CI25E *cot1*Δ.

An important group of genes showing repression in CI25E versus CI25E *cot1*Δ code for Heat-Shock Proteins (HSPs), HSP-related proteins and chaperons which mediate protein folding (Table 3.18). For example, *HSP26*, *HSP78*, *HSP82/90*, and *HSP104* are classified in this group. The genes encoding co-chaperone proteins like *AHA1* and *STI1* have been included in this group. Aha1p regulates Hsp90p (*HSP90* is alias for *HSP82*) by binding to its middle domain (Lotz et al., 2003). Since the expression of *AHA1* decreased, it is expectable that *HSP82*/*90* was downregulated. *STI1* is another co-chaperone that interacts with *HSP82*/*90* in order to inhibit its ATPase (Prodromou *et al.*, 1999). In addition, *SSA4* and *SSE2* were found to be downregulated in CI25E compared to CI25E *cot1*Δ. The proteins encoded by these two genes were reported to be the elements of nine Hsp70 proteins of yeast. It would be interesting to investigate further if *COT1* is mainly responsible for this significant downregulation of HSPs.

5. CONCLUSIONS

In this study, in vivo evolutionary engineering strategy was used to gain a very specific characteristic, cobalt-resistance, in *S.cerevisiae* cells. With this aim, cobalt hyper-resistant *S.cerevisiae* mutants were obtained which could resist up to 8 mM $CoCl_2$ in the medium. This study showed once more that evolutionary engineering is a powerful method to select and characterize mutant yeasts with specific phenotypes.

Genetic and transcriptomic analyses revealed that cobalt resistance is strongly associated with iron metabolism / homeostasis in *S. cerevisiae*. Further analysis of this cobalt-resistant phenotype promises to be of great interest in clarifying the molecular connections between iron homeostasis pathways and cobalt. Moreover, *S.cerevisiae* studies for transition metal metabolism have revealed many genes, some of which are highly conserved in humans. For that reason, the cobalt hyper-resistant mutant obtained in this study could also provide valuable information for iron- and/or cobalt-related responses and/or diseases of human metabolism.

This is the first study which investigated the genomic basis of cobalt resistance in a cobalt-resistant yeast strain. Although some factors were found in this study that seem to constitute the basis of the cobalt hyper-resistant phenotype, it is still not possible to identify the names of specific genes that are mainly responsible for this specific phenotype. It could be concluded that comprehensive understanding of metabolic networks are still very challenging, considering particularly the complex interactions between genes and the regulation of gene expression. For that reason, proteomic analysis, along with whole genome sequencing could be suggested as future strategies to clarify the molecular mechanisms of cobalt resistance in yeast.

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1. Alkim C., Çakar Z.P., İşeri Ö.D., **2012**. Transkriptom ve Mikroarrayler, Moleküler Biyoloji, 303-323, Nobel Yayınevi, Ankara.

2. Çakar Z.P., Turanlı-Yıldız B., **Alkim C.**, Yılmaz U., **2011**. Evolutionary engineering of *Saccharomyces cerevisiae* for improved industrially important properties, *FEMS Yeast Research,* 12, 171-182.

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▪ Çakar Z.P., **Alkim C.**, Turanlı B., Tokman N., Akman S., Sarıkaya M., Tamerler C., Benbadis L., François J.M., **2009**. Isolation of cobalt hyper-resistant mutants of *Saccharomyces cerevisiae* by in vivo evolutionary engineering approach. *J. Biotechnol.* 143: 130-138.

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