Journal of Biotechnology 143 (2009) 130–138

Contents lists available at ScienceDirect

Journal of Biotechnology

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

Isolation of cobalt hyper-resistant mutants of *Saccharomyces cerevisiae* by *in vivo* evolutionary engineering approach

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ARTICLE INFO

Article history:
Received 19 October 2008
Received in revised form 21 June 2009
Accepted 25 June 2009

Keywords:
Cobalt resistance
Evolutionary engineering
Population heterogeneity
Saccharomyces cerevisiae

ABSTRACT

Cobalt is an important element with magnetic properties used in various industrial applications, but is also needed for biological activity. Very little is known about the cellular response of living systems to cobalt stress. Towards investigating this mechanism, we isolated individual *Saccharomyces cerevisiae* cells resistant to high cobalt concentrations up to 8 mmol l^{-1}, by employing four different '*in vivo*' evolutionary engineering strategies: selection under constant or gradually increasing stress levels, and selection under continuous or pulse exposure to cobalt stress. Selection under continuous exposure to gradually increasing cobalt stress levels yielded the most resistant cell population to cobalt. However, the resistance was highly heterogeneous within the mutant populations ranging from 3- to 3700-fold survival rate of isolated individuals to 8 mmol l^{-1} $CoCl_2$ in the most resistant population. Moreover, cobalt-resistant individual colonies were associated with 2–4-times lower intracellular cobalt contents as compared to wild-type, and with cross-resistance to metals such as nickel, zinc, manganese, but not to copper and chromium ions. Contrary to mutants evolved under continuous exposure to cobalt, those isolated by pulse exposure strategy also exhibited resistance to heat shock and hydrogen peroxide stress. Taken together, this study reinforced the fact that evolutionary engineering is useful in selecting strains with very specific phenotypes, and further illustrated the importance of the strategy chosen to isolate the best evolved strain.

1. Introduction

Cobalt is an important magnetic element that has a widespread use in several industrial applications such as the production and the refining of alloys, jet engines, gas turbines, electrochemical materials and permanent magnets (Stadler and Schweyen, 2002). Additionally, cobalt is used in varnishes, paints, catalysts, inks, pigments, ceramics, and surgical implants (Beyersmann and Hartwig, 1992; Kazantzis, 1981). Biologically, cobalt is used as a cofactor of vitamin B_{12} and other enzymes in yeast, animals, bacteria, archaea and plants (Kobayashi and Shimizu, 1999). Cobalt can be toxic for living systems when present at high concentrations, but the exact mechanism of this toxicity is still poorly understood. Transcriptomic and toxicogenomic studies have been carried out in *Escherichia coli* and human lung cells to identify potential signature to cobalt exposure. While in bacteria, excess cobalt causes inactivation of some Fe–S proteins and activates iron uptake as a potential compensatory mechanism (Ranquet et al., 2007), in mammalian cells, acute exposure to cobalt induces upregulation of cobalt carriers and stress-responsive genes (Malard et al., 2007). Some yeast studies have shown that cobalt ions are transported into the vacuole and/or mitochondria by a cobalt transporter encoded by *COT1*, and overexpression of *COT1* confers increased tolerance to cobalt and to rhodium (Conklin et al., 1992). Another gene, *COT2*, was also found to be implicated in cobalt resistance as well as to other divalent cations including Zn^{2+}, Mn^{2+}; and Ni^{2+}. It was found that *COT2* is identical to *GRR1* encoding a F-box protein component of the SCF ubiquitin-ligase complex, also implicated in glucose-catabolite repression and expression of high-affinity

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doi:10.1016/j.jbiotec.2009.06.024

glucose transport, morphogenesis, and sulfite detoxification (Flick and Johnston, 1991; Vallier et al., 1994).

To further investigate the mechanism of cobalt resistance, and hence to unravel potential targets of cobalt in yeast, we sought to apply our recent selection procedure for multi-stress resistant phenotype of *Saccharomyces cerevisiae* (Çakar et al., 2005). While classical selection is based on screening in plates of chemically or UV-mutagenized cells for a given phenotype, e.g. growth on certain levels of toxic metals, evolutionary engineering exploits evolutionary principles to enhance microbial properties in a biotechnological context, provided that the desired phenotype is amenable to direct or indirect selection. This methodology is expected to gain relevance both as a complementary strategy to elucidate the molecular basis of desired phenotypes, as well as in metabolic engineering strain performance (reviewed in Sauer, 2001). Many examples of evolutionary engineering have been reported in the literature, such as isolation of ethanol and acetate stress resistant strains (Çakar et al., 2005; Aarnio et al., 1991; Brown and Oliver, 1982), improved strains for xylose utilization (Sonderegger and Sauer, 2003; Sonderegger et al., 2004) and increased pyruvate production (van Maris et al., 2004).

In this report, we have assessed the potential of four different evolutionary engineering strategies to isolate *S. cerevisiae* cells resistant to high-toxic-levels of cobalt. Cobalt resistant yeast populations were obtained upon pulse and continuous selection at gradually increasing cobalt stress levels. The degree of resistance of mutants to high cobalt concentration as monitored by survival rate was significantly different for continuous and pulse selection strategies. Overall, selection under continuous exposure to gradually increasing cobalt stress yielded the most resistant mutants to cobalt stress. Furthermore, for a given selection procedure, the resistant population expressed high heterogeneity in cobalt-resistance when assessed at the levels of single cells.

2. Materials and methods

2.1. Strain, media and growth conditions

The *S. cerevisiae* wild-type strain CEN.PK 113-7D (*MATa*, *MAL2-8^c*, *SUC2*) was used as the initial wild-type strain in this work (P. Kötter, Johann Wolfgang Goethe-University, Frankfurt, Germany) (van Dijken et al., 2000). Unless otherwise stated, yeast cultivations were performed in yeast minimal medium (YMM), containing 6.7 g l^{-1} yeast nitrogen base without amino acids (Difco) and 2.0% (w/v) glucose as the sole carbon source, in test tubes under aerobic conditions at 30 °C and 200 rpm. Cell growth was monitored by determining the optical density at 600 nm (OD_{600}) and by cellular counts with haemocytometer. Aliquots from all selection cultures were taken along the exponential growth phase, and kept at −80 °C in 30% (v/v) glycerol. Ethyl methane sulfonate (EMS, Sigma) mutagenesis was carried out (Lawrence, 1991), under conditions that resulted in 10% of survival rate after the chemical treatment. Briefly, 2.5 ml overnight culture of the wild-type strain grown in yeast peptone dextrose (YPD) medium at 30 °C was washed twice with an equal volume of 50 mM KH_2PO_4 buffer at pH 7.0, when the cell density was $2 \times 10^{+8}$ cells ml^{-1}. It was then resuspended in 10 ml of the KH_2PO_4-buffer and 300 μl EMS was added to the culture in a screw-cap glass tube, upon which it was vortexed and incubated at 30 °C for 30 min. An equal volume of filter sterile fresh 10% (w/v) sodium thiosulfate was added to the solution for neutralization. The solution was well mixed and the cells were collected by centrifugation at 10,000 rpm for 10 min, washed twice with YMM without dextrose and incubated in liquid YPD medium for overnight growth of the surviving population at 30 °C. This cell culture was used directly as the starting population for all selection experiments, as well as screening experiments to determine the cobalt concentration that causes 50% of growth reduction.

2.2. Evolutionary strategies for selection of cobalt-resistant yeast mutants

The different strategies designed to isolate cobalt-resistant mutants of *S. cerevisiae* are schematically depicted in Fig. 1. For Strategy 1 termed "CONT.INCR.", EMS-mutagenized *S. cerevisiae* was inoculated in 10 ml YMM medium containing 0.5 mmol l^{-1} $CoCl_2$. After 72 h of cultivation, the culture was centrifuged in a benchtop centrifuge at 10,000 rpm for 5 min, washed twice with fresh YMM and inoculated into the same $CoCl_2$-containing YMM at an initial OD_{600} of 0.04. This procedure was repeated 25 times, and at every passage in the fresh medium, the concentration of $CoCl_2$ was increased until no growth occurred. Additionally, at each round of the selection, an aliquot of the yeast culture was centrifuged and stored at −80 °C in 30% (v/v) glycerol (Fig. 1). In the second strategy termed "PULSE.INCR.", a 1 ml culture of EMS-mutagenized yeast cells in YMM at OD_{600} of 0.5–0.6 was chal-

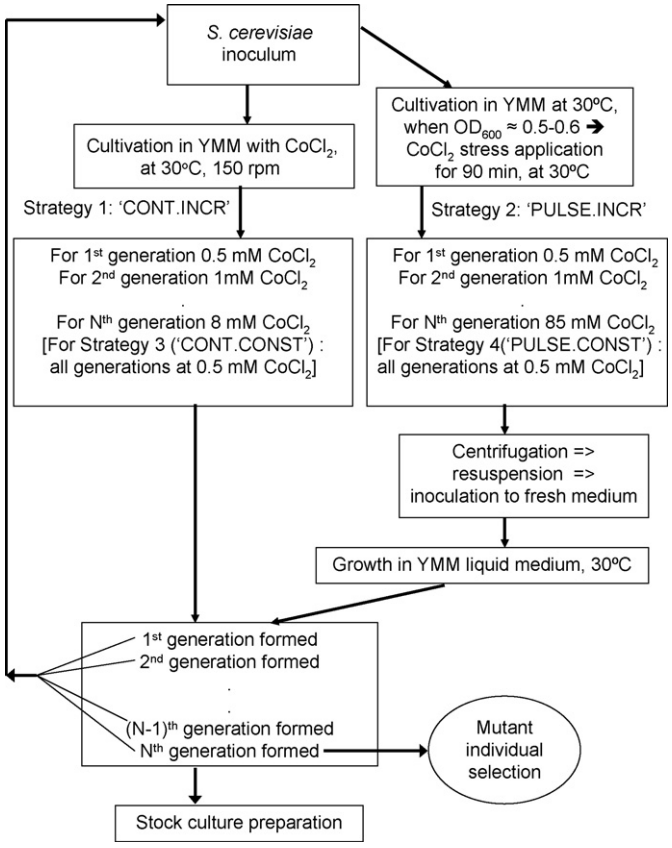

Fig. 1. Experimental protocol for selection under continuous and pulse cobalt stress conditions. After EMS treatment, the mutagenized CEN.PK 113-7D cell population was subjected to serial dilution, transfer and growth in glucose minimal medium containing 0.5 mmol l^{-1} $CoCl_2$, and this procedure was repeated 25 times for Strategy 1 (CONT.INCR) by increasing cobalt concentration at every transfer until no growth was observed. The highest cobalt concentration for Strategy 1 was 8 mmol l^{-1} $CoCl_2$ at the 25th passage. For pulse cobalt stress selection, CEN.PK 113-7D strain was initially mutagenized by EMS and subjected to $CoCl_2$ as a pulse stress for 90 min during early logarithmic phase. In Strategy 2 (PULSE.INCR), the initial $CoCl_2$ concentration was increased at every transfer up to 85 mmol l^{-1} $CoCl_2$ at the 25th passage. Strategies 3 (CONT.CONST) and 4 (PULSE.CONST) represent selection under constant continuous and constant pulse cobalt stress, respectively. In Strategy 3, selection is like Strategy 1, with the exception that the initial $CoCl_2$ concentration of 0.5 mmol l^{-1} was kept constant throughout all repetitions. Similarly, Strategy 4 is like Strategy 2, but in Strategy 4, the initial pulse $CoCl_2$ concentration of 0.5 mmol l^{-1} was applied for 25 passages.

lenged with 0.5 mmol l^{-1} $CoCl_2$ for 90 min. After centrifugation at 3500 rpm for 10 min, and washing the pellet two times with YMM, the cells were resuspended in 5 ml YMM and incubated for 12 h at 30 °C and 200 rpm. After measuring cell density (OD_{600} and cell counting), the culture was diluted to 0.5–0.6 OD_{600} and exposed to increasing cobalt concentrations at each round of repetition to reach 85 mmol l^{-1} at the 25th repetition (Fig. 1). Strategy 3 termed "CONT.CONST" and Strategy 4 termed "PULSE.CONST" were similar to "CONT.INCR" and "PULSE.INCR" strategies, respectively, except that at every repetition, the initial cobalt concentration was kept constant at 0.5 mmol l^{-1} (Fig. 1). Thus, Strategies 3 and 4 were based on selection under constant, mild cobalt stress conditions.

2.3. Estimation of stress resistance

Viable cell numbers were determined by a high-throughput, most-probable number (MPN) assay (Russek and Colwell, 1983), using serial dilutions in 96-well plates containing 180 μl YMM with 2% glucose. Dilutions were made in the range of 10^{-1} to 10^{-8} for five parallel samples. Based on the ability of yeast cells to grow at higher dilutions, the most probable number of survivors was estimated by using published MPN tables (Lindquist, 2008). The MPN assay is a reliable and known method to estimate stress resistance and has also been successfully used before (Çakar et al., 2005).

Resistance to cobalt was estimated by growing cells in YMM containing 2–8 mmol l^{-1} $CoCl_2$ for 72 h and determining the viable cell numbers and survival rate by MPN assay. Resistance to nickel, copper, and chromium was determined in a similar manner, using 0.5–2 mmol l^{-1} $NiCl_2$, $CuCl_2$, and $CrCl_3$, respectively. The survival rate was determined after 72 h of growth. Other stress resistance tests were carried out starting with yeast cells cultivated in YMM until late exponential growth phase (OD_{600} = 1.2–1.5), harvested at 3000 rpm in a benchtop centrifuge and resuspended in 1 ml of fresh YMM medium. For pulse oxidative stress, 1 ml of yeast cells at OD_{600} = 1.2–1.5 was treated for 1 h at 30 °C with H_2O_2 at a final concentration of 0.3 mol l^{-1}, then harvested as above and resuspended in 1 ml YMM for 72 h before survival estimation. Alternatively, the cell pellet was resuspended in 1 ml YMM containing 0.8 mmol l^{-1} H_2O_2, and the cell survival was estimated by MPN assay after 72 h of growth in this continuously applied oxidative stress condition. For heat shock resistance test, cells were incubated at 60 °C for 10 min in an Eppendorf heater-shaker, and then cooled on ice. For ethanol resistance, cells were incubated in YMM with 8% (v/v) ethanol for 1 h, then collected by contrifugation as above and resuspended in 1 ml YMM for 72 h before survival rate estimation. Alternatively, for continuous ethanol stress, yeast cells were cultivated in YMM in the presence of 8% (v/v) ethanol for 72 h, and viable cell numbers were determined. Resistance to osmotic stress was tested by growing yeast cells in YMM containing 0.5 M NaCl for 72 h.

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.

2.4. Determination of heavy metal contents

Yeast cells were grown in 50 ml shake flasks containing 10 ml of YMM and 2% glucose in the presence of the heavy metal (cobalt or nickel) for 72 h (Tables 1 and 2). They were harvested in a benchtop centrifuge at 14,000 rpm for 5 min, and washed twice with distilled water. All cell pellets were hydrolyzed in 5 ml of 10 mol l^{-1} nitric acid for 2 h at 90 °C. The heavy metal contents of the cells were determined by a flame atomic absorption spectrometer (Varian AA 280 FS, Australia). The wavelength and slit width values were 240.7 and 0.2 nm for cobalt, and 232.0 and 0.2 nm for nickel, respectively.

Cell dry mass was determined from a 10 ml culture harvested after 72 h of growth on YMM. Cell pellets were dried for 2 h at 90 °C, cooled in a desiccator for 30 min and weighed.

3. Results and discussion

3.1. Determination of cobalt concentration that causes 50% of growth reduction

Before starting the evolutionary engineering experiments, a yeast culture of CEN.PK 113-7D strain was EMS-mutagenized in conditions resulting in 10% survival following the procedure described previously (Lawrence, 1991). The minimum inhibitory concentration (MIC) for cobalt was then measured for both wild-type and the mutagenized strain, termed S101. The method was to cultivate the two types of cell population in the presence of $CoCl_2$ varying from 0 to 8 mmol l^{-1} and measure final OD_{600} after 72 h of cultivation. About 2.5 mmol l^{-1} of $CoCl_2$ was sufficient to reduce the cell density by 50% both for the wild-type culture and the mutagenized yeast population S101 (Fig. 2). This result indicated that at this stage of the procedure, the EMS-treatment did not significantly increase the sensitivity or resistance of the cells to cobalt ions in the population.

Table 1
Cobalt contents and cell dry weights of selected mutant individuals under 2, 5 and 8 mmol l^{-1} $CoCl_2$ stress conditions.

Name of individual cells	$CoCl_2$ stress (mmol l^{-1}) (initial concentration in the medium)	Cell dry weight (g l^{-1})[a]	Co content (Co per cell dry weight)[b] (mg g^{-1})
100 (wild-type)		0.77	1.39
CONT.INCR25C		0.91	0.56
CONT.INCR25D	2	0.99	0.58
CONT.INCR25E		0.97	0.90
CONT.INCR25H		0.82	0.69
100 (wild-type)		0.39	4.10
CONT.INCR25C		0.70	1.31
CONT.INCR25D	5	0.85	1.21
CONT.INCR25E		0.95	1.48
CONT.INCR25H		0.76	1.21
100 (wild-type)		0.29	3.33
CONT.INCR25C		0.71	2.04
CONT.INCR25D	8	0.73	2.04
CONT.INCR25E		1.01	2.02
CONT.INCR25H		0.77	2.00

The data are reported as mean values of three experiments, with standard deviations ≤5%.

[a] Biomass density was determined after 72 h of growth in YMM containing 2% glucose and supplemented with either 2, 5 or 8 mmol l^{-1} $CoCl_2$.

[b] No cobalt was detected in cell samples grown in the absence of $CoCl_2$.

Table 2
Nickel contents in selected cobalt-resistant mutant individuals cultivated in the presence of 0.5 mmol l^{-1} $NiCl_2$.

Name of individual cells	$NiCl_2$ stress (mmol l^{-1}) (initial concentration in the medium)	Cell dry weight (g l^{-1})[a]	Ni content (Ni per cell dry weight)[b] (mg g^{-1})
100 (wild-type)	0.5	0.49	1.34
CONT.INCR25E		1.13	0.63
CONT.INCR25H		0.84	0.76

The data are reported as mean values of three experiments, with standard deviations ≤5%.
[a] Biomass density was determined after 72 h of growth in YMM containing 2% glucose and supplemented with 0.5 mmol l^{-1} $NiCl_2$.
[b] No nickel was detected in cell samples grown in the absence of $NiCl_2$.

3.2. Selection of cobalt-resistant populations and characterization of cobalt resistance at the single cell level

In order to reveal the most suitable evolution scheme for selecting the most cobalt-resistant yeast population, four different evolutionary engineering strategies were employed as described in Section 2 and illustrated in Fig. 1. For Strategy 1, the EMS-mutagenized culture of CEN.PK113-7D was subjected to serial dilution, transferred and grown in a glucose minimal medium supplemented initially with 0.5 mmol l^{-1} $CoCl_2$ and with increasing cobalt concentrations at every transfer until no growth occurred (25 passages). In this case, we found that 8 mmol l^{-1} was the highest concentration of $CoCl_2$ allowing growth of the evolved strain (Fig. 1). In the case of Strategy 2, the selection was based on challenging the cells initially with 0.5 mmol l^{-1} $CoCl_2$ and by increasing concentrations of cobalt as a pulse stress for 90 min at each repetition (Fig. 1). Following this latter procedure, we were able to reach evolved yeast cells that could survive after a pulse of 85 mmol l^{-1} $CoCl_2$ (25 passages). The maximum cobalt concentrations at which the populations could survive were determined and verified by three repetitive cultivations at the maximum concentrations. Strategies 3 and 4 represent selection under constant continuous and constant pulse stress, respectively. In these strategies, the initial mild $CoCl_2$ stress level of 0.5 mmol l^{-1} was kept constant throughout all 25 passages (Fig. 1).

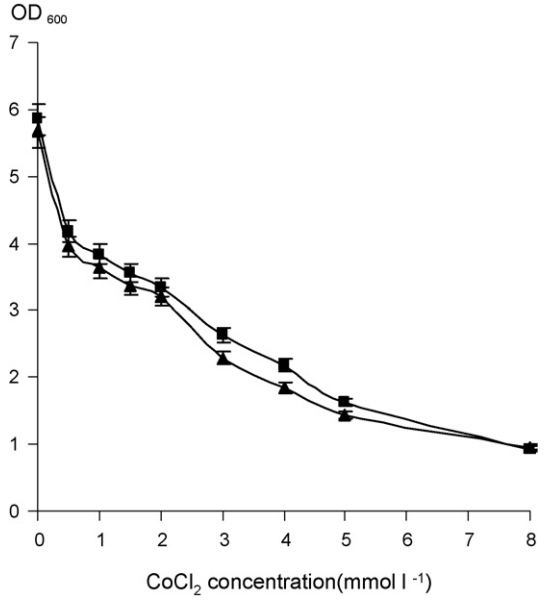

Fig. 2. The cobalt concentration for 50% inhibition of growth of wild-type and EMS-mutagenized *S. cerevisiae*. Triangles indicate OD_{600} values of the wild-type, and squares indicate OD_{600} values of the EMS-mutagenized culture (S101). The results are the mean values of three independent experiments with the same inoculum and initial OD_{600} for each culture.

The gain in strain evolution was monitored by measuring the survival rates of intermediate cell populations of Strategies 1 and 2 to 5 and 8 mmol l^{-1} $CoCl_2$. Survival rates were determined with MPN analysis after 72 h of growth. In general, the resistance to cobalt stress was a singular event, where a resistant cell population appeared suddenly. In our case and whatever the strategy employed, the resistant trait was obtained at around the 10th cycle of the selection procedure (data not shown). Afterwards, this trait did not evolve significantly for the next 15 serial transfers. Inter-

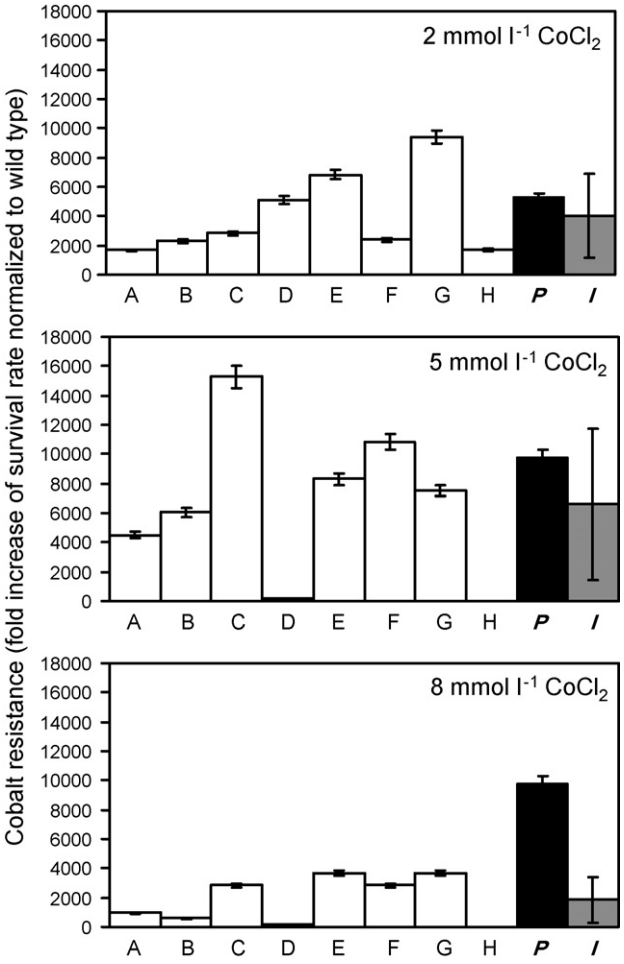

Fig. 3. Determination of survival rate of yeast population and individuals isolated from continuous, increasing $CoCl_2$ stress selection (CONT.INCR) strategy at 2, 5 and 8 mmol l^{-1} $CoCl_2$. A–H are selected individual mutant clones of final evolved population *p* from 'CONT.INCR' selection strategy. *P* and *I* correspond to the final population and the mean resistance values for the 8 individual mutants tested, respectively. The survival rates of each individual and wild-type cells were determined by the MPN in the presence of 2, 5 and 8 mmol l^{-1} $CoCl_2$. The cobalt resistance was then expressed as the fold increase in survival rate normalized to that of wild-type. The results are the mean values of five completely independent experiments. The error bars indicate standard deviations in those five experiments.

estingly, a similar behaviour with no gradual evolution has been observed in a previous study, while looking for multi-stress resistance in selections under various stress conditions (Çakar et al., 2005).

The next step involved the detailed investigation of cobalt resistance at the single cell level. To this end, the evolved populations obtained for each evolutionary strategy were diluted and spread onto YMM agar plates to isolate colonies. Eight colonies randomly selected were grown in 96-well plates in YMM containing 2, 5 or 8 mmol l^{-1} $CoCl_2$ and their survival rate was compared to that of the wild-type using MPN-assay. The individuals selected at a constant, mild cobalt stress level (Strategies 3 and 4) had no significant improvement in their cobalt stress resistance as compared to the wild-type (data not shown). Individuals selected at gradually increasing cobalt stress levels, however, had highly improved cobalt stress resistance. Thus, our further investigations focused on mutants selected by Strategies 1 and 2, based on selections at increasing stress levels. Significant differences of individual colonies for resistance to cobalt concentrations were found for both Strategies 1 and 2 (Figs. 3 and 4, respectively), which revealed a high heterogeneity in the evolved populations. As for instance, while the survival rate to 5 mmol l^{-1} cobalt of the evolved population obtained according to Strategy 1 was about 9800-fold greater than that of the wild-type, the survival rate of single cells isolated from this population ranged from 27 to about 15,000-fold (Fig. 3). Similarly, isolated cells from evolved populations obtained according to Strategy 2 (termed PULSE.INCR) harbored survival rates to cobalt that were ranging from about 4–22-fold, while the survival rate of the evolved population was about 15-fold of that of the wild-type (Fig. 4).

To summarize, the majority of the mutants selected from Strategy 1 had cobalt resistance levels ranging between 1000 and 15,000-fold of the wild-type level, whereas those from Strategy 2 were only to 2–5-fold less sensitive than the wild-type to 2 or 5 mmol l^{-1} $CoCl_2$. These results indicated the importance of the selection procedure design, and particularly the stress conditions. Strategy 1 was based on selection under continuous cobalt stress, whereas Strategy 2 involved selection under pulse cobalt stress exposure. Apparently, selection under continuous exposure to increasing levels of cobalt stress results in the hyper-resistant mutants, and is the best selection strategy among all strategies tested in this study.

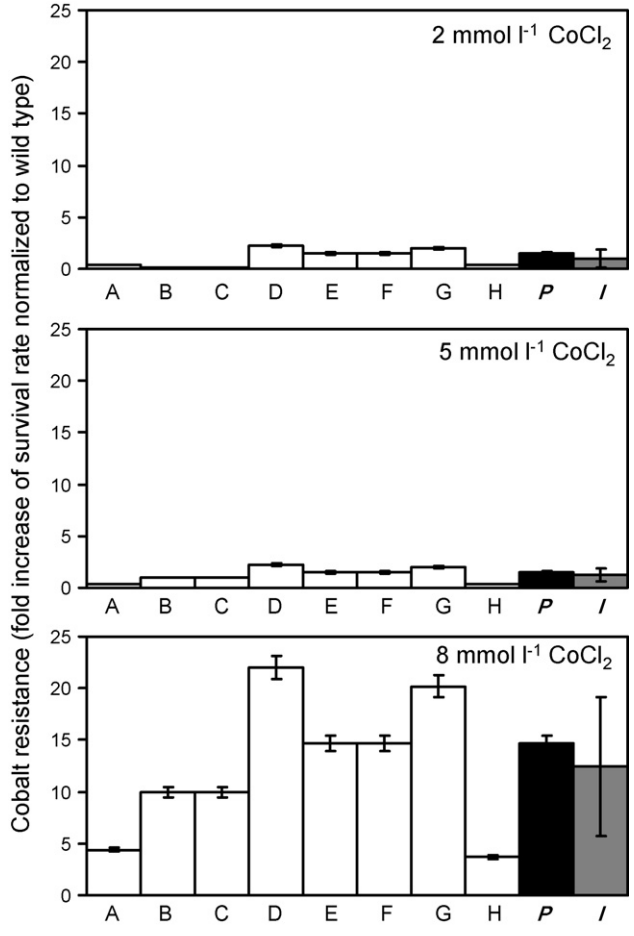

Fig. 4. Determination of survival rate of yeast population and isolated individuals from pulse increasing $CoCl_2$ stress selection (PULSE.INCR) strategy at 2, 5 and 8 mmol l^{-1} $CoCl_2$. A–H are selected individual mutant clones of final evolved population ***p*** from 'PULSE.INCR' selection strategy. ***P*** and ***I*** correspond to the final population and the mean resistance values for the 8 individual mutants tested, respectively. The survival rates and cobalt resistance were determined as in Fig. 3. The results are the mean values of five completely independent experiments. The error bars indicate standard deviations in those five experiments.

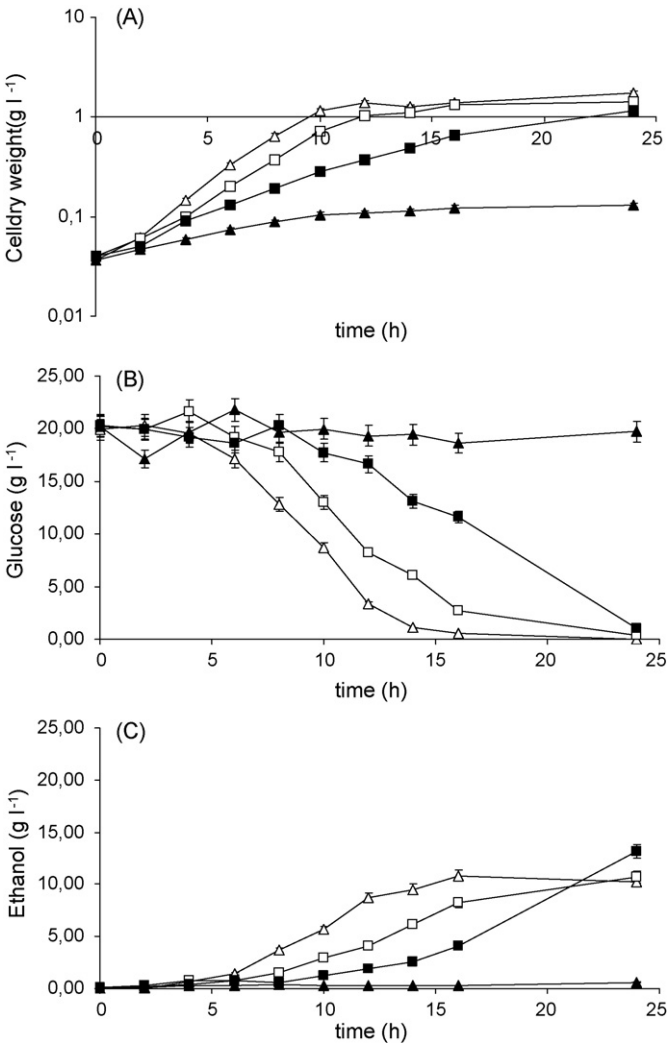

Fig. 5. Growth kinetics of wild-type and the cobalt resistant mutant CONT.INCR25E in minimal medium in the absence or presence of 5 mmol l^{-1} $CoCl_2$. (A) Cell dry weight (g l^{-1}) versus time as a semi-log plot. (B) Residual glucose concentration versus time. (C) Ethanol concentration versus time. For (A, B and C); wild-type grown in the absence and presence of 5 mmol l^{-1} $CoCl_2$ are indicated by △ and ▲, respectively. Similarly, the mutant CONT.INCR25E grown in the absence and presence of 5 mmol l^{-1} $CoCl_2$ are indicated by □ and ■, respectively. The results are the mean values of three independent experiments. The error bars indicate standard deviations.

3.3. Physiological analysis of the cobalt-resistant mutants

The isolated colony termed "CONT.INCR25E mutant" that showed the highest survival rate in the presence of 8 mmol l^{-1} $CoCl_2$ was further examined for some relevant physiological traits. In the absence of cobalt, the growth pattern on minimal medium and the maximal growth rate (μ^{max}) of the hyper-resistant clone to cobalt was identical to that of the wild-type. In the presence of 5 mmol l^{-1} $CoCl_2$ in the medium, the cobalt resistant strain showed about 40% decrease in the maximal growth rate, whereas the wild-type strain was unable to grow under this condition (Fig. 5). By-products of fermentation were also determined in the mutant in the absence and presence of cobalt. Results shown in Fig. 5 indicated that the cobalt-resistant mutant produced more ethanol when grown in the presence of 5 mmol l^{-1} $CoCl_2$ and less glycerol and acetate (data not shown).

Since the toxicity by cobalt was associated with cobalt uptake, we measured the cobalt contents in some of the selected cobalt-resistant mutant clones after 72 h of growth in YMM, in the absence and in the presence of 2, 5 or 8 mmol l^{-1} $CoCl_2$. Results of this experiment are summarized in Table 1. No cobalt was detected in cell samples grown in the absence of $CoCl_2$ (data not shown). It can be seen that for all cobalt concentrations tested, the selected mutant clones grew to higher cell densities than the wild-type, with the CONT.INCR25E clone reaching the highest biomass yield at 8 mmol l^{-1} $CoCl_2$. Moreover, the growth efficiency of the isolated colonies was associated with lower intracellular cobalt content for all mutants, as compared with the wild-type cobalt content. These results suggested that the resistance to cobalt may be in part due to a mechanism that prevents its uptake or stimulates its expulsion.

3.4. Cross-resistance of cobalt-resistant mutants to other metal stresses

It is usually observed that acquisition of a resistance of the yeast *S. cerevisiae* to a given stress protects the yeast against other stresses (Lewis et al., 1995; Park et al., 1997; Trollmo et al., 1988; Hohmann, 2002). Therefore, with respect to cobalt resistance, we evaluated the cross-resistance of the cobalt evolved clones isolated from Strategies 1 and 2 to other metals (all added as chloride salts) that are in the vicinity of cobalt in the Periodic Table of the Elements, including manganese, zinc, iron, nickel, chromium and copper, as well as some cations that are quite distant from cobalt such as lithium and aluminum. Cross-resistance was found with Mn^{2+}, Zn^{2+}, Fe^{2+} and Ni^{2+} ions (Fig. 6), whereas resistance to Cu^{2+} was indistinguishable to that of the wild-type strain, and the same sensitivity as the wild-type was found with Al^{3+} and Li^{+} (data not shown). We then investigated in more detail the nickel resistance added at concentrations ranging between 0.5 and 2.0 mmol l^{-1} to cell populations and individual mutants isolated from Strategies 1 and 2. Interestingly, there were significant differences in nickel resistances of the mutants selected by the two strategies. While mutant individuals of Strategy 1 were between 6000 and 900,000-fold resistant to 0.5 mmol l^{-1} $NiCl_2$ (Fig. 7), the resistance to this metal ions of individuals from Strategy 2 was only 2–52-fold higher than wild-type (data not shown). As compared to cobalt resistance, the isolated individuals were apparently more sensitive to this metal since only one of these isolated colonies could grow in the presence of 2 mmol l^{-1} $NiCl_2$. While cross-resistance was found with Ni^{2+} and Co^{2+} ions, there was no direct quantitative correlation between the resistance levels to these cations, i.e. mutants with the highest cobalt resistance could have lower nickel resistance

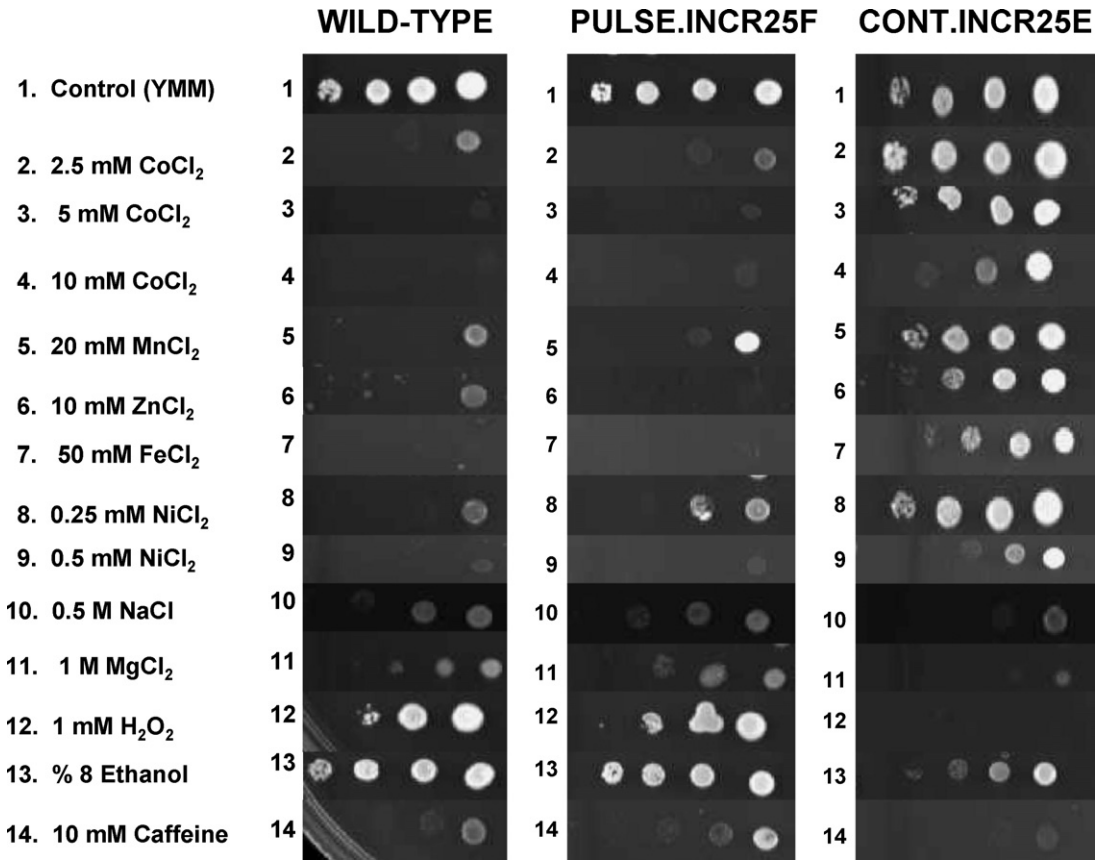

Fig. 6. Cross-resistance test results of wild-type and cobalt-evolved mutants PULSE.INCR25F and CONT.INCR25E to metal ions and other stress factors. The results are based on serial dilution (from right to left: 10^{-1} to 10^{-4}) and growth on solid YMM plates at 30 °C for 72 h in the presence of stress factors.

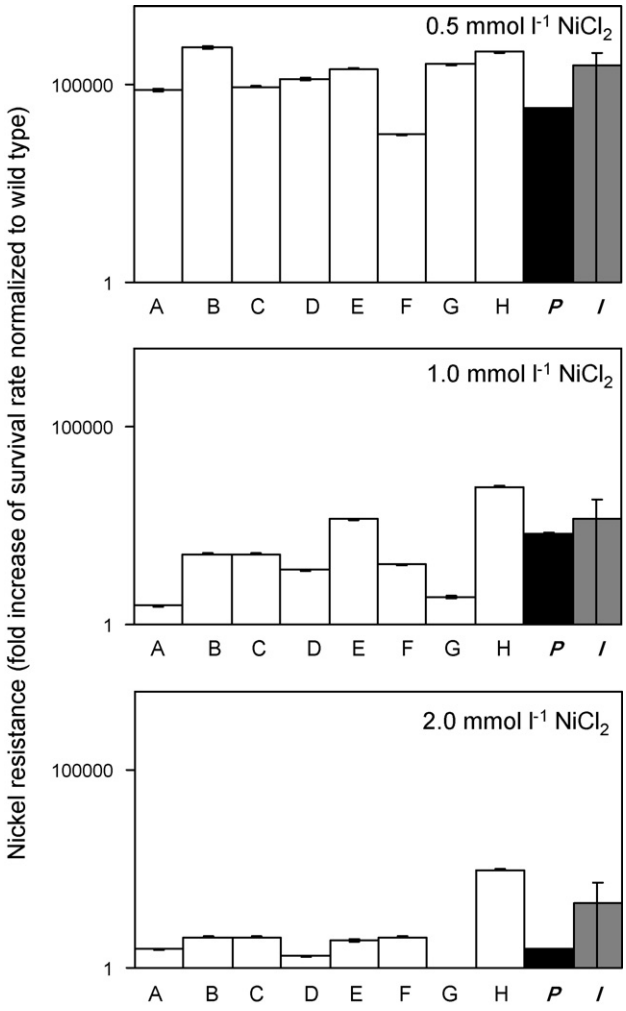

Fig. 7. Nickel cross-resistance of cobalt-resistant mutants obtained by 'CONT.INCR' selection strategy. A–H are selected individual mutant clones of final evolved population *p* from 'CONT.INCR' selection strategy. *P* and *I* correspond to the final population and the mean resistance values for the 8 individual mutants tested, respectively. The survival rates and nickel resistance were determined as in Fig. 3, except that the experiment was conducted with 0.5, 1.0 and 2.0 mmol l^{-1} $NiCl_2$ instead of $CoCl_2$. Please note that the scale on *y*-axis of the figures indicating nickel resistance is logarithmic, indicating significantly higher resistances normalized to those of the wild-type at 0.5 mmol l^{-1} $NiCl_2$ concentration than at 1.0 and 2.0 mmol l^{-1} $NiCl_2$. The results are the mean values of five completely independent experiments. The error bars indicate standard deviations in those five experiments.

than a mutant with moderate cobalt resistance and reciprocally (Figs. 3 and 7). As for instance, the mutant clone CONT.INCR.25E showing about 3700-fold resistance to 8 mmol l^{-1} $CoCl_2$ (Fig. 3) exhibited 500-fold resistance to 1.0 mmol l^{-1} nickel, whereas the CONT.INCR.25H that was only 3-fold more resistant to 8 mmol l^{-1} $CoCl_2$ than the wild-type showed about 3000-fold increase of survival rate to nickel. However, as for the cobalt-resistant individuals, intracellular content of nickel was lower in the mutants than in the wild-type (Table 2). Overall, these results indicated that there may be a common genetic mechanism for cobalt and nickel resistance, as well as to other metals that are in the vicinity of cobalt in the Periodic Table of the Elements, with the exception of Cu^{2+} ions in *S. cerevisiae*. There are previous reports in the literature on bacterial ion transporters like CorA family (Niegowski and Eshaghi, 2007), which can transport both nickel and cobalt as divalent cations. Studies with *E. coli* revealed that when the *yohM* gene (also named as *rcnA* = resistance to cobalt and nickel) was inactivated, cells became sensitive to both cobalt and nickel (Rodrigue et al., 2005). Other studies report on vacuolar accumulation of cobalt, nickel, iron, manganese and zinc, mediated by *ZRC1* and *COT1* genes, whose overexpression have been reported to increase resistance to these ions (e.g. MacDiarmid et al., 2000). It is therefore possible that our cobalt-evolved mutants are somehow altered in this process. This hypothesis is currently under investigation.

3.5. Cross-resistance of cobalt-resistant mutants to oxidative, osmotic, heat and ethanol stresses

We investigated whether the cobalt-resistant mutants could show cross-resistance to other more commonly studied stresses. Interestingly, we did not find any cross-resistance to osmotic and ethanol stresses with cobalt-evolved resistant cells isolated from Strategy 1 (CONT.INCR) and 2 (PULSE.INCR). In addition, the cobalt-resistant mutants from Strategy 1 did not exhibit resistance to oxidative stress induced by H_2O_2 (data not shown). In contrast, individuals isolated from Strategy 2 showed significant cross-resistance to this oxidative stress, which was applied either as a continuous stressor at 0.8 mmol l^{-1} or as a pulse stress at 0.3 mol l^{-1} (Fig. 8). The survival rate to a permanent presence of H_2O_2 was only 50-fold higher than the wild-type for the best individual clone, whereas survival rates to a pulse of H_2O_2 could reach up to 3000–64,000-fold with some individuals as compared to wild-type cells (Fig. 8). The lack of cross-resistance of cobalt mutants isolated by Strategy 1 to H_2O_2 may be explained by the difference between the strategies such that the 'pulse' application of cobalt stress in Strategy 2 might have improved the resistance of those mutants against general oxidative stress, compared to mutants selected upon con-

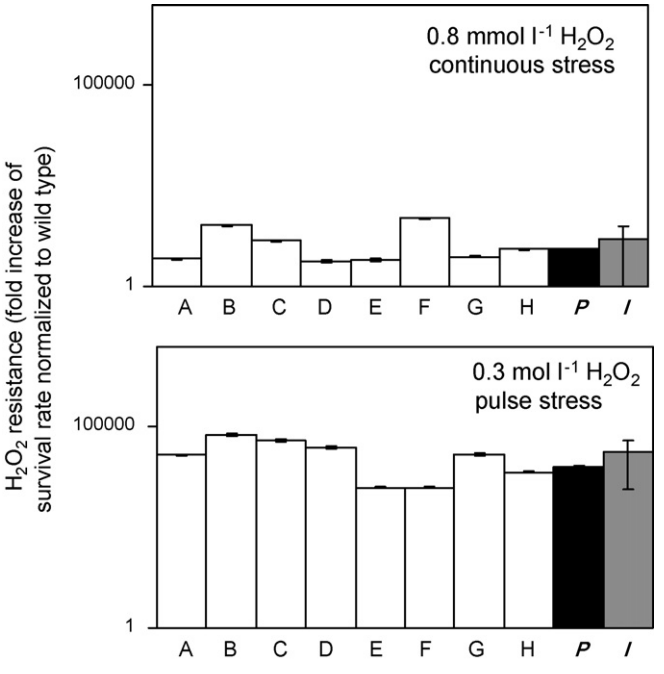

Fig. 8. Hydrogen peroxide cross-resistance of cobalt-resistant mutants obtained by 'PULSE.INCR' selection strategy. A–H are selected individual mutant clones of final evolved population ***p*** from 'PULSE.INCR' selection strategy. *P* and *I* correspond to the final population and the mean resistance values for the 8 individual mutants tested, respectively. The survival rates and hydrogen peroxide (H_2O_2) resistance were determined as in Fig. 3, except that the experiment was conducted with either 0.8 mmol l^{-1} H_2O_2 stress that was applied continuously, or 0.3 mol l^{-1} H_2O_2 pulse stress applied for one h, instead of $CoCl_2$ stress. Please note that the scale on *y*-axis of the figures indicating H_2O_2 resistance is logarithmic, indicating significantly higher resistances normalized to those of the wild-type under pulse H_2O_2 stress conditions than under continuous H_2O_2 stress conditions. The results are the mean values of five completely independent experiments. The error bars indicate standard deviations in those five experiments.

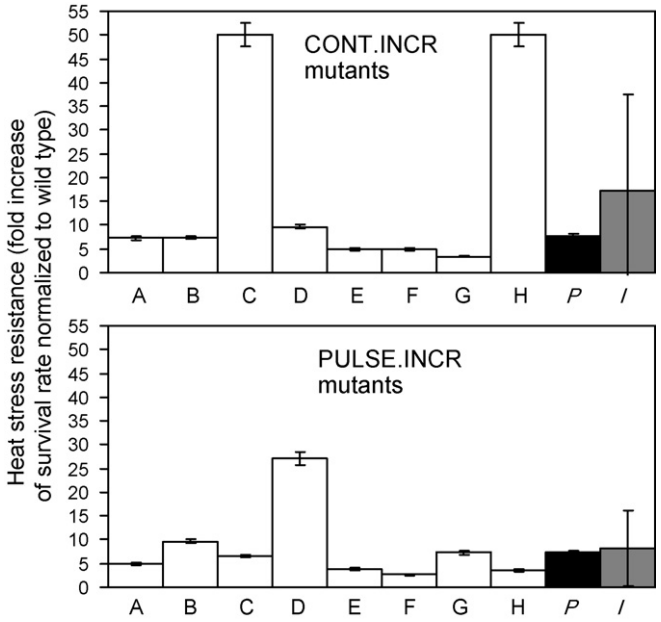

Fig. 9. Cross-resistance of cobalt-resistant mutants to heat shock. Populations and individual mutant clones of final evolved populations from 'CONT.INCR' and 'PULSE.INCR' selection strategies were tested for cross-resistance against heat shock applied at 60 °C for 10 min. The survival rates and heat shock resistance were determined as in Fig. 3, using heat shock stress, instead of $CoCl_2$. The results are the mean values of five completely independent experiments. The error bars indicate standard deviations in those five experiments.

tinuous exposure to cobalt in Strategy 1. There are also some reports on the similarities between the yeast response to oxidative stress caused by hydrogen peroxide and cadmium stress (Dickinson and Schweizer, 2004; Penninckx, 2002).

In the case of cross-resistance to heat shock stress, however, all cobalt-resistant mutants from both Strategies 1 and 2 were cross-resistant (Fig. 9). There is a higher level of variation between the heat shock resistance levels of the Strategy 1 (CONT. INCR) mutants, compared with those of Strategy 2 (PULSE.INCR). The resistance levels reached up to 50-fold and 27-fold of the wild-type level for the mutants of Strategies 1 and 2, respectively (Fig. 7). These results may indicate a common mechanism between resistance to cobalt and heat shock stresses, independent of the stress type (e.g. pulse or continuous) applied during the selection strategy.

4. Conclusions

In this study, four different strategies of *in vivo* evolutionary engineering have been employed to generate cobalt-resistant yeast cells. The two main conclusions that could be made from this evolutionary engineering approach were, on the one hand, that each strategy led to significant differences in resistance to cobalt among the final evolved yeast populations, and on the other hand, the cobalt-resistance of single cells within the evolved population was highly heterogeneous, suggesting that the mechanism of resistance is complex and involves many genes. Among all strategies tested in this study, selection at increasing levels of continuously applied cobalt stress (CONT.INCR) was the most successful strategy to yield mutants with the highest cobalt resistance. In addition, the cobalt resistant individual mutants exhibited lower intracellular contents of cobalt than the wild-type, when cultivated in the presence of high concentration of this metal. This finding suggests that one possible mechanism of resistance is to prevent the uptake of cobalt and this mechanism would be similar to the detoxification mechanism identified in mammalian cells (Malard et al., 2007) and in yeast (MacDiarmid et al., 2000). Finally, the cobalt-resistant mutants exhibited cross-resistance to Fe^{2+}, Mn^{2+}, Zn^{2+} and Ni^{2+} that are in the vicinity of cobalt in the Periodic Table of the Elements, but not to copper or chromium. These results suggest a common mechanism of resistance of the yeast cell with respect to these former metal ions, while the resistance mechanism to copper ions, which is located between nickel and zinc in the Periodic table is different and perhaps specific to this cation (Cervantes and Gutierrezcorona, 1994; Field et al., 2002). Similarly, cross-resistance of the mutants to heat shock stress suggests that the genetic factors involved in heat stress resistance (e.g. Lindquist and Craig, 1988; Wiemken, 1990; Dickinson and Schweizer, 2004) may also be useful for studying cobalt resistance mechanism. Genetic and genomic studies are currently underway to identify the molecular mechanism that accounts for the hyper-resistance of the evolved yeast mutant strains to cobalt and to explain the cross-resistance with the cations that belong to the same category in the elementary classification.

Acknowledgements

We thank Aslı Baysal, Ülkü Yılmaz, Hüseyin Tayran, Çeşminaz Karabulut and Esma Nur Özkan for technical assistance. This work was supported in part by the Turkish State Planning Organization DPT (Advanced Technologies and ITU-ARINANOTEK 2008K120710), TUBITAK (105T314, to Z.P. Çakar), TUBITAK-EGIDE (PIA-BOSPHORUS 107T284, to Z.P. Çakar and J.M. François) and by NSF-MRSEC and ARO-DURINT projects at the University of Washington, Seattle (M. Sarikaya and C. Tamerler).

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