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Effect of cobalt on *Escherichia coli* metabolism and metalloporphyrin formation

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Abstract Toxicity in *Escherichia coli* resulting from high concentrations of cobalt has been explained by competition of cobalt with iron in various metabolic processes including Fe–S cluster assembly, sulfur assimilation, production of free radicals and reduction of free thiol pool. Here we present another aspect of increased cobalt concentrations in the culture medium resulting in the production of cobalt protoporphyrin IX (CoPPIX), which was incorporated into heme proteins including membrane-bound cytochromes and an expressed human cystathionine beta-synthase (CBS). The presence of CoPPIX in cytochromes inhibited their electron transport capacity and resulted in a substantially decreased respiration. Bacterial cells adapted to the increased cobalt concentration by inducing a modified mixed acid fermentative pathway under aerobiosis. We capitalized on the ability of *E. coli* to insert cobalt into PPIX to carry out an expression of CoPPIX-substituted heme proteins. The level of CoPPIX-substitution increased with the number of passages of cells in a cobalt-containing medium. This approach is an inexpensive method to prepare cobalt-substituted heme proteins compared to in vitro enzyme reconstitution or in vivo replacement using metalloporphyrin heme analogs and seems to be especially suitable for complex heme proteins with an additional coenzyme, such as human CBS.

Keywords Cobalt toxicity · Cobalt protoporphyrin IX · Protein expression · Heme replacement · Chelatase · Fe–S cluster · Cystathionine beta-synthase · Respiration

Abbreviations

AdoMet	S-adenosyl-L-methionine
δ-ALA	δ-Aminolevulinic acid
AMP	Adenosine monophosphate
BSA	Bovine serum albumin
CBS	Cystathionine β-synthase
Co	Cobalt
ETF	Electron transfer flavoprotein
ETF-QO	ETF ubiquinone oxidoreductase
FAD	Flavin adenine dinucleotide
Fe	Iron
GST	Glutathione S-transferase
ICP-OES	Inductively coupled plasma optical emission spectroscopy.
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
IscS	Cysteine desulfurase
MIC	Minimum inhibitory concentration

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PLP	Pyridoxal-5′-phosphate
PPIX	Protoporphyrin IX
SEM	Standard error of the mean
ThiH	Tyrosine lyase
WT	Wild type

Introduction

Porphyrins, including heme (iron protoporphyrin IX, FePPIX), are abundant and versatile coenzymes utilized in many living organisms. Heme proteins are integral components in a variety of biological processes, in which heme plays crucial roles such as electron transfer (e.g. cytochromes), transport and storage of oxygen (e.g. hemoglobin, myoglobin), detoxification and oxidative damage control (e.g. P450 enzymes, peroxidase, catalase), signal transduction and gas sensing (e.g. nitric oxide synthase, soluble guanylate cyclase) and regulation of specific protein expression (e.g. heme biosynthesis pathway) [reviewed in (Padmanaban et al. 1989; Ponka 1999; Reedy and Gibney 2004; Tong and Guo 2009)]. The catalytic, redox and structural properties of heme in hemeproteins have previously been elucidated by insertion of heme analogs containing modified porphyrins and site directed mutagenesis (Reedy and Gibney 2004).

The properties of the heme can also be modulated by substitution of iron with other metals. The physiochemical and catalytic properties of heme proteins are directed by the heme moiety, thus substitution of the iron or modification of the porphyrin ring offers an additional opportunity to gain new insights into the role of heme or its catalytic mechanism. The process termed "enzyme reconstitution" involves removal of coenzyme (most often porphyrin or flavin derivate), its modification and/or replacement and subsequent reinsertion in order to obtain species with novel properties [reviewed in (Fruk et al. 2009)]. Depending on the localization of heme within the protein structure, the removal of the coenzyme often requires extensive denaturation. Subsequently, successful reconstitution can be complicated or made totally impossible by the complexity of protein folding and oligomerization or by the presence of additional coenzymes. Therefore, novel methods for introduction of heme analogs into heme protein were recently described (Brugna et al. 2010; Woodward et al. 2007). The method of Woodward et al. (Woodward et al. 2007) employs *hemB*-deficient *E. coli* RP523 with an uncharacterized permeability mutation that renders cells heme-permeable. Anaerobic growth permitted cell growth in the absence of heme, while induction of protein expression in the presence of a heme analog under aerobic conditions yielded the substituted heme protein with the porphyrin of interest. However, the protein expression efficiency of the strain, the toxicity and permeability of heme analogs may limit this method. Brugna et al. (Brugna et al. 2010) described similar approach for in vivo porphyrin replacement. Their method utilizes the Gram-positive bacterium, *E. feacalis,* and provides several advantages. *E. faecalis* does not synthesize heme and thus heme is not required for its growth. At the same time, the absence of an outer membrane in this bacterium permits uptake of heme or its analogs from medium. Additionally, *E. faecalis* appears to be resistant to noniron metalloporphyrins. However, all of the latter approaches described so far depend on the exogenous source of expensive heme analogs.

Transition metals such as zinc, copper, cobalt or manganese, often incorporated in heme analogs used in porphyrin replacement studies, are considered toxic at elevated concentrations. Their coordination chemistry and redox properties can lead to non-specific binding to various proteins, displacement of other metals (usually iron) from their natural binding sites and generation of free radicals (reviewed in (Valko et al. 2005)). Recent studies showed that cobalt toxicity in *E. coli* and *S. enterica* is mainly due to its direct competition with iron especially affecting the synthesis of Fe–S clusters (Ranquet et al. 2007; Thorgersen and Downs 2007) or indirectly via cobalt-mediated oxidative depletion of free thiols pool (Thorgersen and Downs 2007). On the other hand, the concentrations of cobalt chloride ranging from 100 μM up to 400 μM were found suitable for optimal *E. coli* growth and expression of cobalt-substituted iron-type nitrile hydratase (Sari et al. 2007).

Our study was prompted by the limited success in replacing heme with other metalloporphyrins in human cystathionine beta-synthase (CBS) by the method of Woodward et al. (Woodward et al. 2007). However, we were able to obtain small amounts of substituted CBS with cobalt and manganese protoporphyrin (CoPPIX and MnPPIX), respectively. The

enzymatic activities of both substituted enzymes as well as wild type CBS prepared in similar way were substantially reduced (Majtan et al. 2008). Our data described in this communication present new insights into cobalt effects on *E. coli* metabolism and a new approach for preparation of CoPPIX substituted heme proteins. We show that (i) adaptation of *E. coli* to cobalt exposure resulted in the induction of fermentative pathway, (ii) incorporation of in vivo synthesized CoPPIX into heme proteins of the respiratory chain leads to their inactivation and (iii) replacement of heme by the CoPPIX in heme proteins can be used for a preparation of cobalt-substituted heme proteins. Utilizing the method described in here we were able to prepare 92% cobalt-substituted CBS (CoCBS), which yielded a large amount of fully active enzyme indistinguishable from wild type CBS. A detailed characterization of CoCBS will be published elsewhere. In contrast to the previous heme replacement methods, our approach provides an inexpensive alternative for the preparation of in vivo CoPPIX-substituted structurally complex heme proteins, such as human CBS, which could not be achieved by the previous heme replacement procedures.

Materials and methods

Strains and plasmids

E. coli Rosetta 2 (DE3) (Novagen) and *E. coli* C43 (DE3) (Lucigen), both BL21 derivatives, were employed. *E. coli* Rosetta 2 (DE3) cells were transformed with pGEX-6P1 (GE Healthcare) or pGEX-6P1-hCBS WT (Frank et al. 2008) expressing glutathione S-transferase (GST) or CBS, respectively. *E. coli* C43 (DE3) were transformed with pGLG (Griffin et al. 1998) or prEDH (Usselman et al. 2008) expressing human electron transfer flavoprotein (ETF) or *Rhodobacter sphaeroides* ETF ubiquinone oxidoreductase (ETF-QO), respectively. The strains genotypes and important plasmid characteristics are listed in Table 1.

Media and chemicals

The LB medium and M9 minimal medium (Sambrook et al. 1989) were used in the present study. M9 minimal medium (pH 7.4) was supplemented with 0.5% glucose, 0.4% Casamino acids, 2 mM $MgSO_4$, 100 μM $CaCl_2$ and 0.001% thiamine–HCl. $FeCl_3$ or $CoCl_2$ were filter sterilized and added to growth medium at a final concentration of 150 μM from a 150 mM stock in 0.1 M HCl and a 150 mM stock in double distilled H_2O (ddH_2O). For CBS expression, 300 μM δ-aminolevulinic acid and 0.0025% pyridoxine–HCl were added. Chloramphenicol (30 μg/ml) and ampicillin (100 μg/ml) were included when appropriate. Unless stated otherwise, all chemicals were purchased from Sigma or Fisher Scientific. Protoporphyrins were purchased from Frontier Scientific.

Bacterial growth analysis

Bacterial growth was quantified by measuring the absorbance at 600 nm. At least three independent cultures for each strain under each condition were determined. The 5 ml of LB medium supplemented with appropriate antibiotic(s) was inoculated from glycerol stock and cells were grown overnight at 37°C. Starter culture was prepared by overnight growth of 100× diluted LB medium overnight culture in M9 minimal medium with the appropriate antibiotic(s). Sterile 125 ml Erlenmeyer flasks containing 30 ml of M9 medium supplemented with appropriate antibiotic(s) and metal salt were inoculated with starter culture, placed in an air shaker (300 rpm) and incubated at 37°C. Cells were passaged by 50× dilution of previous passage into a fresh medium.

Metabolites analysis and respiration

Metabolites were analyzed by gas chromatography/mass spectrometry (GC/MS) from filter sterilized spent growth media. The analysis essentially followed protocol for organic acids screen from urine as performed by UCD Biochemical Genetics Laboratory.

Oxygen uptake was measured polarographically at 25°C with an YSI model 5300 Clark electrode apparatus. Cells from the last passage were harvested by centrifugation at 4°C, 6500 rpm for 10 min. Medium was used for metabolites analysis and cell pellet was resuspended in phosphate buffered saline (PBS, pH 7.4) and washed twice. Cells were kept in PBS on ice and were equilibrated at 25°C prior the oxygen uptake assay. The oxygen uptake was initiated by adding D-lactate as a substrate from a stock solution

Table 1 Bacterial strains and plasmids used in the present study

Strain	Genotype	References
E. coli Rosetta 2 (DE3)	F^- *ompT* $hsdS_B(r_B^- m_B^-)$ *gal dcm* (DE3) pRARE2 (Cam^R)	Novagen
E. coli C43 (DE3)	F^- *ompT* $hsdS_B(r_B^- m_B^-)$ *gal dcm* (DE3)	Lucigen
Plasmid	**Relevant characteristics**	**References**
pGEX-6P1	pBR322 ori, Amp^R, p_{tac}, GST, PreScission protease site	GE Healthcare
pGEX-6P1-hCBS WT	Human CBS in *Apa*I/*Sal*I-cut pGEX-6P1	Frank et al. (2008)
pGLG	Human ETF in *Nco*I/*Bam*HI-cut pET-23d(+) (Novagen), pBR322 ori, Amp^R, T7 promoter	Griffin et al. (1998)
prEDH	*R. sphaeroides* ETF-QO in *Nde*I/*Hind*III-cut pET-21a(+) (Novagen), pBR322 ori, Amp^R, T7 promoter	Usselman et al. (2008)

at a final concentration of 1.5 mM. The ability of Co-grown cell to utilize oxygen was expressed as a percentage of Fe-grown respiration capability.

Protein expression and purification

CBS was expressed in LB medium, M9 minimal medium supplemented with $FeCl_3$ and from M9 minimal medium supplemented with $CoCl_2$ after first (1×), seventh (7×) and twelfth (12×) passage. CBS expression, purification and activity measurement was essentially performed as described elsewhere (Frank et al. 2008). Preparation of crude extracts containing ETF-QO, ETF-QO purification and activity determination was essentially performed as described elsewhere (Usselman et al. 2008).

Pyridine hemochromogen assays

The pyridine hemochromogen assay was performed as described previously (Majtan et al. 2008) using a HP diode array model 8453 spectrophotometer. For difference pyridine hemochromogen spectra of membrane-bound hemoproteins, the insoluble fractions of the cell lysates were washed twice with 120 volumes of TRIS buffered saline (TBS, pH 8.6). Difference spectra (i.e. reduced minus oxidized) were recorded from 650 to 380 nm with a Shimadzu 2401PC spectrophotometer.

Metal content determination

A second derivative visible absorption analysis at 574 nm of reduced protein samples was used for the determination of iron in the purified CoCBS enzymes. Wild type FeCBS in different concentrations served as a standard for a preparation of the calibration curve. The enzyme was reduced under anaerobic conditions by careful titration with 50 mM sodium dithionite in 0.1 M sodium pyrophosphate, pH 9.0. Additions were made with a gas tight syringe and solutions were made anaerobic by 10 cycles of evacuating and purging with argon.

Results

Inhibition of bacterial growth in cobalt-supplemented medium is alleviated by sequential passages

Figures 1 and 2 show the effect of cobalt on bacterial growth, which varied with the number of passages to which the cells were subjected. Two different *E. coli* strains, both BL21 derivates, were used in the absence of an expression vector or transformed with one of four plasmids carrying a different expression cassette (Table 1). Proteins expressed from the gene cassettes differed in the presence and identity of the respective coenzymes: GST expressed from pGEX-6P1 has no coenzyme, ETF expressed from pGLG contains FAD and AMP, ETF-QO expressed from prEDH contains FAD and a 4Fe–4S cluster and finally CBS expressed from pGEX-6P1-hCBS WT has PLP and heme. Figures 1A and 2A show growth curves of the tested strains in M9 minimal medium supplemented with 150 μM $FeCl_3$. The minimal inhibitory concentration (MIC) of cobalt, defined as the lowest $CoCl_2$ concentration that totally prevents bacterial growth after overnight incubation, was

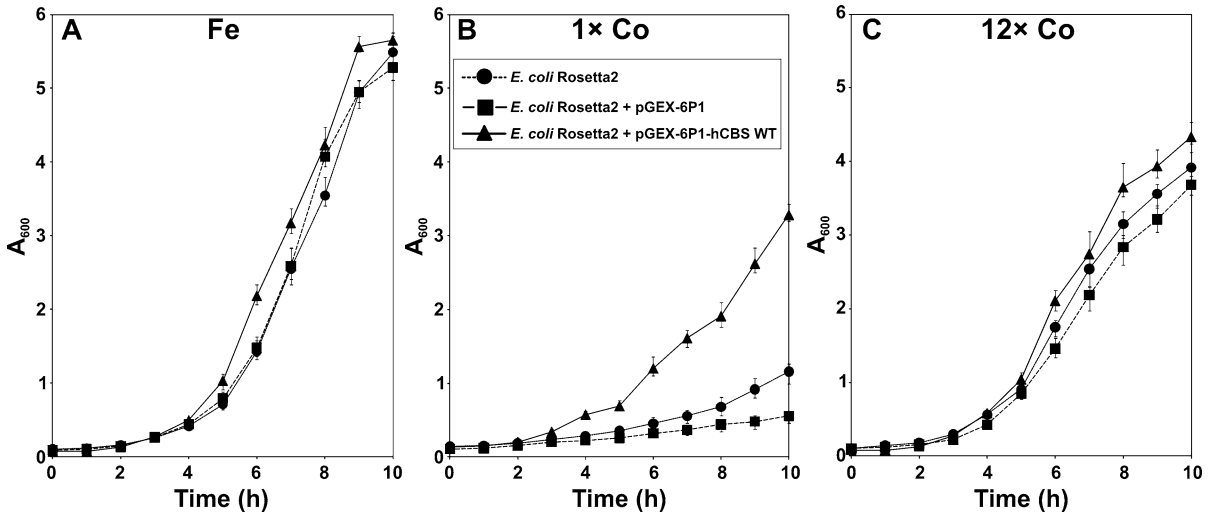

Fig. 1 Effect of cobalt and number of cell passages on *E. coli* Rosetta 2 (DE) growth. Bacterial cultures were grown in M9 minimal medium supplemented with either 150 μM $FeCl_3$ (**A**) or 150 μM $CoCl_2$ (**B**, **C**). Cells were passaged through cobalt-supplemented minimal medium either 1× (**B**) or 12× (**C**). *Dotted line with circles* is the parental strain *E. coli* Rosetta 2 (DE3). *Dashed line with squares* is *E. coli* Rosetta 2 (DE3) strain carrying the GST expression vector (pGEX-6P1). *Solid line with triangles* is *E. coli* Rosetta 2 (DE3) strain carrying the CBS expression vector (pGEX-6P1-hCBS WT). Data are expressed as a mean ± SEM from at least three independent measurements. The inset in the panel **B** applies to all three panels

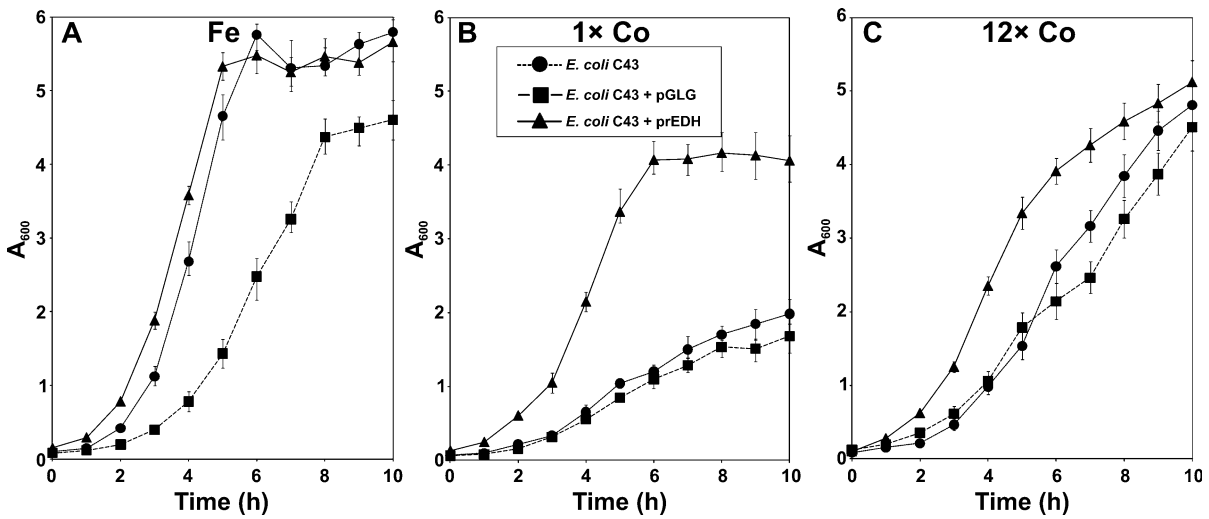

Fig. 2 Effect of cobalt and number of cell passages on *E. coli* C43 (DE) growth. Bacterial cultures were grown in M9 minimal medium supplemented with either 150 μM $FeCl_3$ (**A**) or 150 μM $CoCl_2$ (**B**, **C**). Cells were passaged through cobalt-supplemented minimal medium either 1× (**B**) or 12× (**C**). *Dotted line with circles* is the parental strain *E. coli* C43 (DE3). *Dashed line with squares* is *E. coli* C43 (DE3) strain carrying the ETF expression vector (pGLG). *Solid line with triangles* is *E. coli* C43 (DE3) strain carrying the ETF-QO expression vector (prEDH). Data are expressed as a mean ± SEM from at least three independent measurements. The inset in the panel **B** applies to all three panels

determined to be >1 mM for each strain (data not shown). The cell growth was reduced during the first passage (1×) through M9 medium in the presence of 150 μM $CoCl_2$ when compared to growth in iron-supplemented medium (Figs. 1B, 2B). Interestingly, strains with plasmids carrying gene cassettes for expression of proteins with iron-containing coenzyme (4Fe–4S cluster in ETF-QO and heme in CBS) grew

somewhat faster during the first passage in the cobalt-supplemented M9 medium than the other tested strains. As shown in Figs. 1C and 2C, the bacterial growth significantly improved after twelve passages (12×) through cobalt-supplemented minimal medium. Even though the cobalt-treated cells grew much better after twelve passages, they never reached the total cell density of iron-grown cells after overnight cultivation (Fig. 1A versus C or Fig. 2A versus C; A_{600} of 7.5 ± 0.4 for iron-grown versus 5.1 ± 0.2 for 12× cobalt-grown Rosetta 2 strains or A_{600} of 6.6 ± 0.4 for iron-grown versus 5.4 ± 0.2 for 12× cobalt-grown C43 strains).

These experiments revealed that susceptibility of cells to cobalt toxicity appeared to be strain dependent with *E. coli* Rosetta 2 being more sensitive to 150 μM cobalt chloride than *E. coli* C43. Growth curves also showed variability in growth rate depending on whether the cells carried a metalloprotein expression vector or not. *E. coli* cells carrying the CBS or ETF-QO plasmid grew faster than others even in the absence of IPTG induction of enzyme expression. These data suggest that leaky expression might partially protect the cells against cobalt toxicity. However, Western blot analysis of crude extracts of Rosetta 2 cells carrying CBS plasmid together with CBS activity assays showed negligible CBS antigen or CBS activity (<5% that of IPTG-induced *E. coli* crude extract; data not shown). The growth profiles of at least three independent cultures of six tested cultures essentially followed a similar pattern including initial growth inhibition alleviated by sequential passages. Thus, it is unlikely that the same spontaneous mutation or a genetic change responsible for an increased tolerance of sub-MIC cobalt concentrations occurred in all tested strains. Furthermore, we have provided evidence that the most likely mechanism of survival of *E. coli* cell in sub-MIC cobalt concentrations represents a metabolic adaptation.

Modified mixed acid fermentation rescued bacterial growth

The GC/MS analysis of organic acids in the spent growth media showed significant differences in metabolites profiles between the iron- and cobalt-supplemented cells grown with glucose as the carbon source. The GS/MS chromatogram of the culture media from stationary growth phase shows very high levels of citrate, 2-hydroxyglutarate, succinate, lactate, fumarate, and malate in the cobalt-containing medium (Fig. 3). This pattern, which is different from a classical mixed acid fermentation of *E. coli* on glucose, suggests that adaptation of *E. coli* cells to increased cobalt concentration caused induction of a modified mixed acid fermentation under aerobic conditions.

In support of the modified mixed acid fermentation data, Fig. 4 shows the oxygen uptake analysis of the 12× passaged cells through cobalt-containing M9 medium compared to iron-grown cells. After addition of D-lactate to a final concentration of 1.5 mM, oxygen consumption by cobalt-grown cells showed was virtually unchanged compared to the endogenous rate of oxygen uptake. Analysis of oxygen consumption and comparison with iron-grown cells oxygen uptake revealed the ability of cobalt-grown cells to utilize oxygen as a terminal electron acceptor in aerobic respiration was reduced to ~15% of iron-grown cells. Our data clearly showed that cobalt inhibits the aerobic electron transport system and thus respiration, which in turn forces the cells to a modified mixed acid fermentation.

Increased cobalt concentration yielded degraded Fe–S enzyme

Rhodobacter sphaeroides ETF ubiquinone oxidoreductase (ETF-QQ), containing FAD and a 4Fe–4S cluster, was expressed in cells grown in the presence of 150 μM $FeCl_3$ or $CoCl_2$. The ETF-QO activity measured in crude extracts of cells grown in the presence of cobalt showed virtually no activity. The amount of ETF-QO protein in the soluble fraction of cobalt-grown cells corresponded to activity results: no antigen was found by Western blot analysis compared to the iron-grown crude extracts (data not shown). Interestingly, Western blot analysis of the insoluble fraction showed large amounts of ETF-QO antigen. As Fe–S cluster in ETF-QO functions also in a structural role (Zhang et al. 2006), the incorporation of cobalt-substituted Fe–S clusters into ETF-QO or production of apo-ETF-QO devoid of Fe–S cluster coenzyme ultimately leads to the ETF-QO structural perturbations, misfolding and subsequent degradation and/or aggregation. Inactivation of Fe–S cluster enzymes, such as ETF-QO, as a result of increased

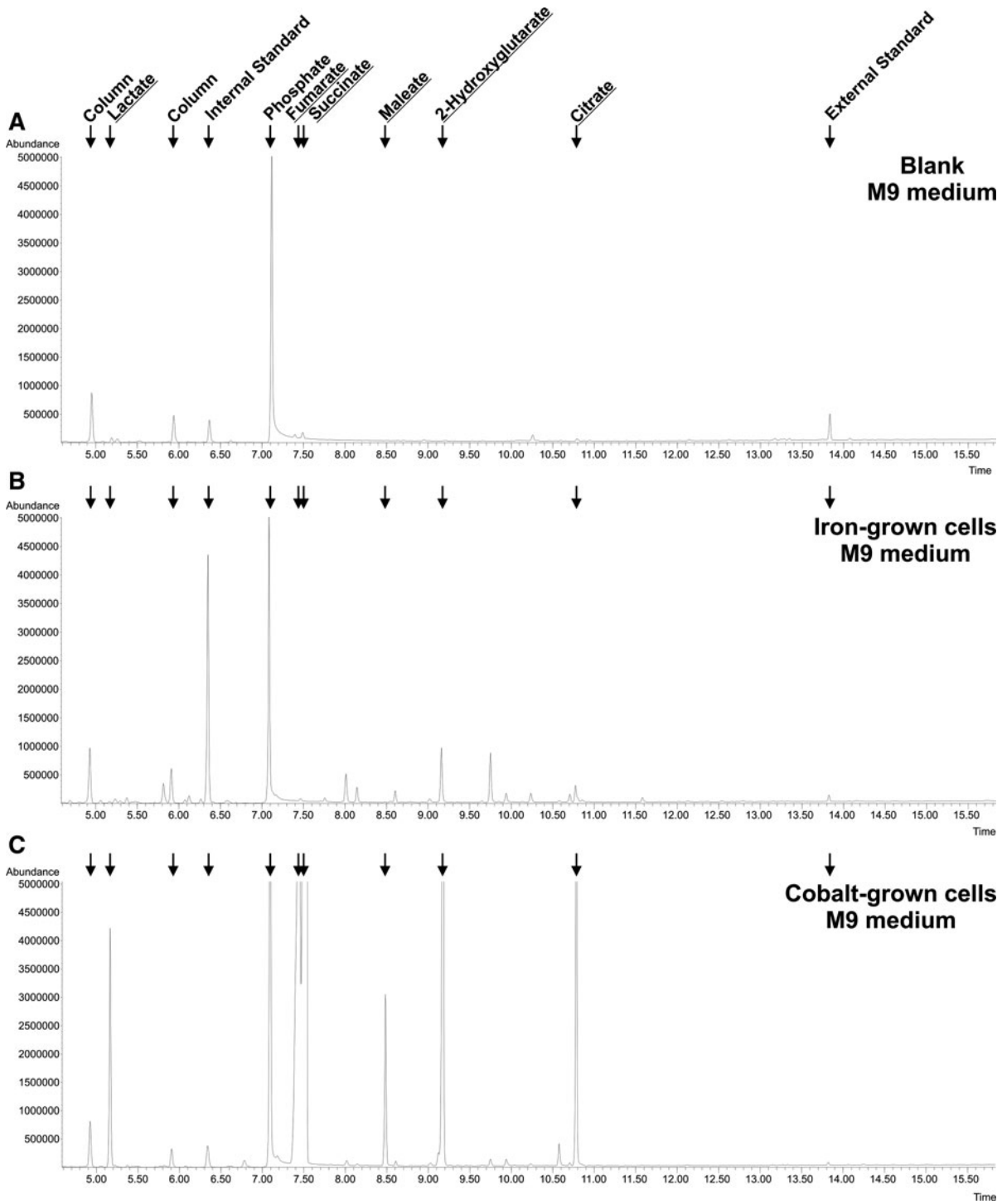

Fig. 3 The GC/MS analysis of the metabolites in the spent cultivation media. The GC/MS chromatograms show profiles of selected organic acids in the various media: **A** fresh M9 minimal medium as a blank, **B** spent M9 minimal medium by cells grown in the presence of 150 μM $FeCl_3$ and **C** spent M9 minimal medium by cells grown in the presence of 150 μM $CoCl_2$

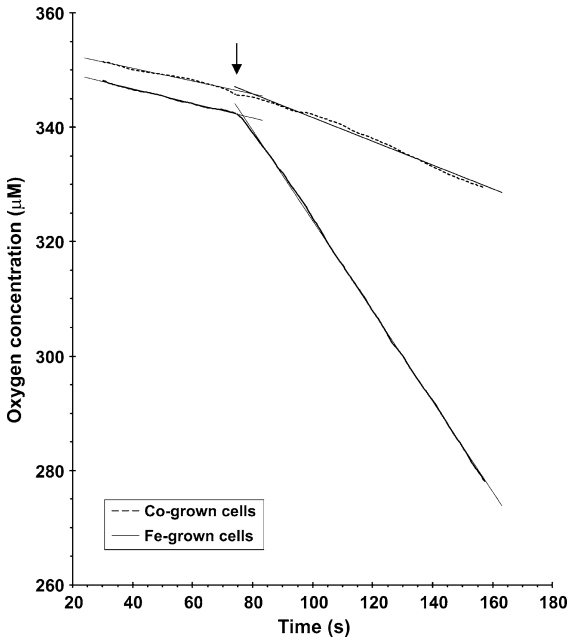

Fig. 4 Respiration of bacterial cells grown in the presence of iron and cobalt, respectively. Oxygen uptake was recorded by using Clark oxygen electrode. The chamber contained 2.7 ml of 50 mM sodium phosphate buffer, pH 7.6, and 0.3 ml washed bacterial suspension grown in M9 minimal medium supplemented with 150 μM $FeCl_3$ or 150 μM $CoCl_2$. The substrate D-lactate was added after 1 min to final concentration of 1.5 mM (indicated by an arrow) and a change in oxygen uptake was recorded

cobalt concentrations is consistent with previously published studies (Ranquet et al. 2007; Thorgersen and Downs 2007).

Increased cobalt content results in the production of CoPPIX

Pyridine hemochromogen analysis on washed membranes from bacterial cells grown in minimal medium supplemented with either 150 μM $FeCl_3$ or $CoCl_2$ after twelve passages indicated the presence of FePPIX or CoPPIX, respectively. Figure 5 shows the difference pyridine hemochromogen spectra of washed bacterial membranes from cobalt- and iron-grown cells. The positions of peaks obtained for FePPIX and CoPPIX standards corresponded to the same values released from purified wild type FeCBS and CoCBS enzymes (Majtan et al. 2008). These results support two conclusions. First, the increased cobalt concentration results in a production of CoPPIX instead of heme (FePPIX). Second, in vivo biosynthesized CoPPIX is incorporated into heme proteins such as membrane-bound cytochromes, which in turn resulted in the inhibition of their electron transport capacity and subsequent respiration. We performed pyridine hemochromogen assays on all washed membrane samples including those from cells not expressing any recombinant protein. We detected similar spectral pattern as the one presented in Fig. 5 for all tested strains suggesting that the formation of CoPPIX in cells grown in the presence of $CoCl_2$ is a general phenomenon and does not depend on the presence of any plasmid carrying gene cassette for expression of recombinant protein.

CoPPIX replaces heme in CBS

The finding that growth in cobalt-supplemented medium results in the production of CoPPIX and its subsequent incorporation into *E. coli* heme proteins led us to develop an alternative approach for in vivo preparation of CoPPIX-substituted heme proteins. We used human CBS as an example of a complex heme protein, where a previous method yielded CoPPIX-substituted enzyme. However, as Table 2 shows, total protein amount as well as activity were substantially decreased even for wild type FeCBS prepared in a similar manner (Majtan et al. 2008). The successful application of the proposed heme replacement approach for CoPPIX resulted in the purification and characterization of fully active, 92% CoPPIX-substituted CoCBS (detailed biochemical and biophysical study on the purified CoCBS enzyme will be described in full elsewhere). The extent of heme replacement for CoPPIX of presented alternative approach depends on the number of cells passages through the cobalt-supplemented M9 medium prior protein expression. We expressed and purified CoCBS from cells grown in cobalt-containing minimal medium after first (1×), seventh (7×) and twelfth (12×) passage. Wild type FeCBS expressed in cells grown in rich LB medium or minimal medium supplemented with 150 μM $FeCl_3$ served as a control and standard. Spectral analysis of the purified enzymes showed distinct α peak at 574 nm of the reduced FeCBS, which allowed us to estimate the content of iron in various CoCBS preparations using a second derivative analysis of the visible absorption spectrum of reduced enzymes (Fig. 6). The data shows that first passage of cells in

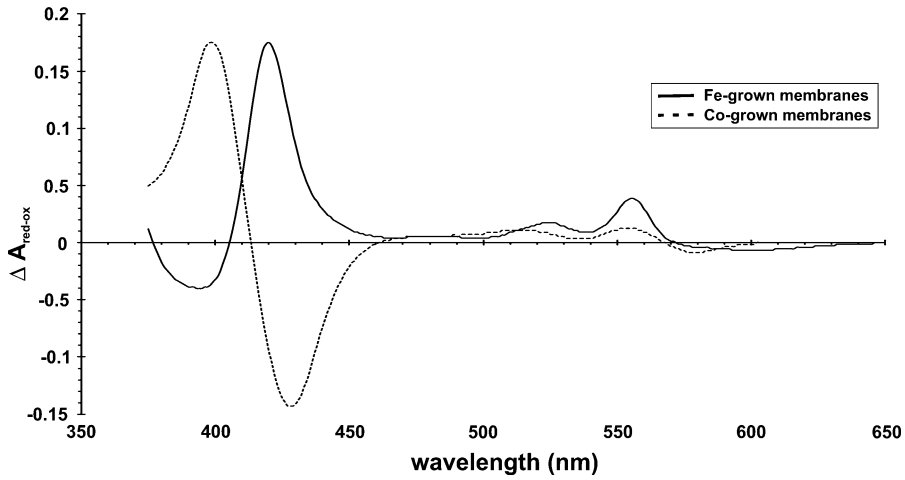

Fig. 5 Difference pyridine hemochromogen spectra of protoporphyrins released from washed membranes of cells grown in M9 minimal medium supplemented with 150 μM $FeCl_3$ and 150 μM $CoCl_2$, respectively. Solid line is the difference spectrum (reduced–oxidized) of metalloporphyrins released from iron-grown cell membranes. Dashed line is the difference spectrum (reduced–oxidized) of metalloporphyrins released from cobalt-grown cell membranes. Reduction was achieved by addition of traces of solid sodium dithionite and spectra were recorded immediately

Table 2 Specific activity, yield and porphyrin/protein ratio of wild type FeCBS and CoCBS prepared by two different approaches

Enzyme	Specific activity (U/mg of protein)	Yield (mg/l of culture)	Soret/280 ratio	References
Wild type FeCBS	101.0	2.8–11.7	0.94–1.40	This study
CoCBS	95.0	1.6–4.2	0.93–1.00	This study
Anaerobic FeCBS	10.5	0.07	0.71	Majtan et al. (2008)
Anaerobic CoCBS	6.0	0.05	0.74	Majtan et al. (2008)

cobalt-containing M9 medium results in 64% substitution of CoPPIX for heme (FePPIX). The following passages further increased content of cobalt in the purified CoCBS to 79% substitution after seventh passage and up to 88% of CoPPIX content after twelfth passage. The cobalt content of CoCBS 12× correlates well with the analytical determination of metal content to 92% of cobalt by using ICP-OES (unpublished). The yield of CoCBS enzyme, its catalytic activity and metalloporphyrin content are very similar to the wild type FeCBS (Table 2).

Discussion

High intracellular concentrations of cobalt as well as any other transition metal are toxic; however, molecular basis of cobalt toxicity has not been well documented until recently. Several studies have explored the deleterious effects of cobalt on bacterial metabolism revealing the cobalt competition with iron at several metabolic pathways and adaptive changes that occur in response to elevated Co concentration in the growth medium (Ranquet et al. 2007; Skovran et al. 2004; Thorgersen and Downs 2007, 2009). The studies concluded that cobalt affected the Fe–S cluster assembly process during de novo synthesis or repair. A mutant lacking cysteine desulfurase (IscS), which provides sulfur for Fe–S cluster synthesis, was reported with thiamine requirement likely due to impaired sulfur insertion into thiazole moiety of thiamine (Skovran and Downs 2000) in addition to impaired synthesis or repair of Fe–S cluster in tyrosine lyase ThiH (Schwartz et al. 2000). Indeed, thiamine synthesis was found to be strongly affected by cobalt toxicity, which can be alleviated by the supplementation of growth medium with either thiamine or iron, again

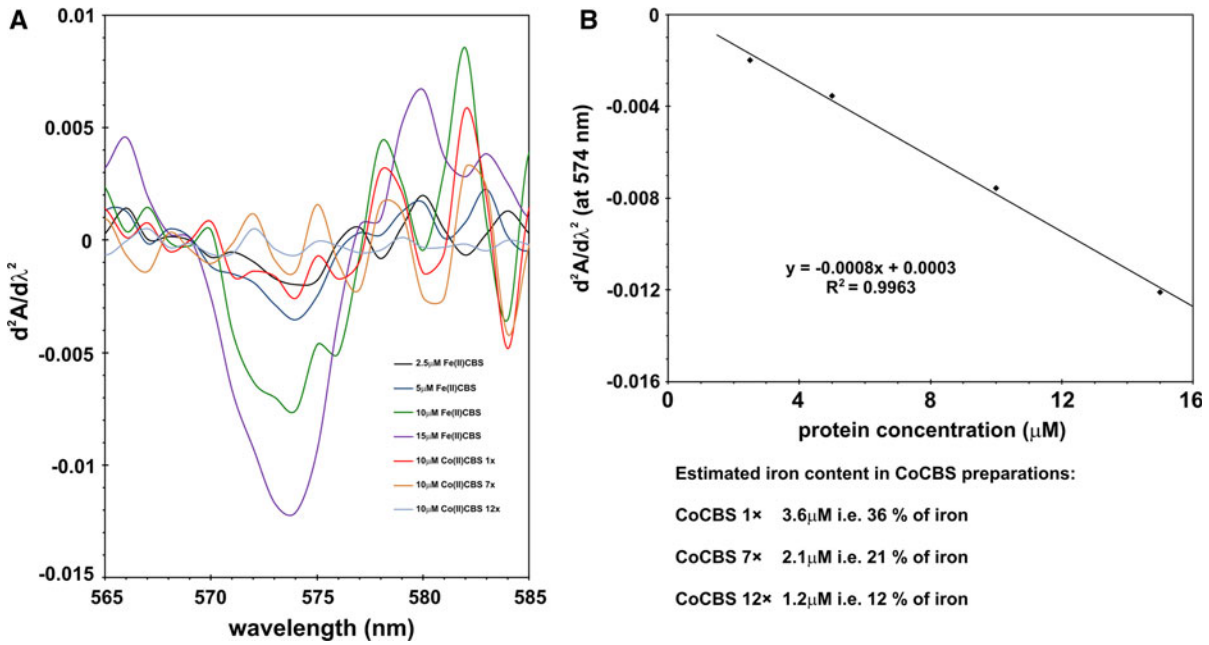

Fig. 6 Estimation of iron content in various CoCBS preparations using 2nd derivative analysis of the visible adsorption spectra of reduced proteins. **A** Overlay of 2nd derivative spectra of four reduced Fe(II)CBS standards (2.5 μM, 5 μM, 10 μM and 15 μM) and three Co(II)CBS proteins varying in the number of passages in minimal medium supplemented with 150 μM $CoCl_2$ prior to protein expression. **B** Calibration curve from Fe(II)CBS standards and iron content estimates in analyzed CoCBS preparations. The CoCBS 1×, 7× and 12× denotes number of passages in cobalt-containing minimal medium prior protein expression

suggesting the competitive relationship between iron and cobalt (Skovran et al. 2004). Cobalt is well known to generate reactive oxygen species (Valko et al. 2005). Interestingly, the thiamine requirement of cysteine desulfurase IscS mutant or of cobalt-grown cells was rescued by anaerobic growth, which is consistent with the role of oxidative stress caused by cobalt on Fe–S cluster proteins (Ranquet et al. 2007; Thorgersen and Downs 2007, 2009). The elevated concentrations of cobalt resulted in perturbation of iron homeostasis and the competition between iron and cobalt during Fe–S cluster assembly or repair. Ranquet et al. (Ranquet et al. 2007) showed that incomplete or incorrectly assembled Fe–S clusters containing cobalt ions were incorporated into Fe–S proteins, which resulted in their inactivation. The activity of Fe–S enzymes aconitase and succinate dehydrogenase of the Krebs cycle in cobalt-grown cells was substantially reduced to 20–40% of activity compared to iron-grown cells (Ranquet et al. 2007; Thorgersen and Downs 2007). Indeed, no antigen of the expressed ETF-QO, a 4Fe–4S cluster enzyme, was found in the soluble fraction of cobalt-grown cells, which correlated with lack of activity compared to ETF-QO from the iron-grown cells. Our metabolite analysis is also consistent with the previously published data. The accumulation of citrate and complete absence of isocitrate points to the inactivation of aconitase. High levels of other metabolites such as fumarate and succinate are also consistent with inactivation of Fe–S clusters by cobalt insertion since succinate dehydrogenase and fumarate reductase contain Fe–S clusters and succinate dehydrogenase also contains a heme *b* (Horsefield et al. 2004; Hudson et al. 2005; Ranquet et al. 2007; Tran et al. 2007). Both metabolites have most likely been replenished by anaplerotic pathways utilizing amino acids supplemented in the growth medium. Accumulation of fumarate and succinate supports the contention that both enzymes are inactivated by cobalt and succinate is a metabolic dead end. Fumarate could be metabolized through malate up to citrate. Both were found in the spent medium from cobalt-grown cells. The 2-hydroxyglutarate likely results from the reduction of 2-ketoglutarate that could be produced by deamination of glutamate in the culture medium.

The variability in growth rate during the first passage in cobalt-supplemented medium and the significantly faster rate observed in the presence of a metalloprotein expression vector is puzzling. The ~5% leaky expression of CBS or ETF-QO in uninduced cultures might have provided some level of protection against cobalt toxicity: redirection of CoPPIX to CoCBS or cobalt-containing Fe–S clusters to ETF-QO may serve as a cobalt "detoxification" route. The overall better growth of *E. coli* C43 strains compared to the Rosetta 2 strains may be explained by two observations. First, *E. coli* Rosetta 2 cells carry an additional plasmid (pRARE2 with Cam^R, see Table 1), for whose maintenance the cultures were supplemented with an additional antibiotic (30 μg/ml chloramphenicol). Second, *E. coli* C43 strains carry at least one uncharacterized mutation, which prevents the cell death associated with the expression of toxic recombinant protein. Such strain resistance against expressed toxic proteins may also be involved in better cell viability in increased cobalt concentration.

The recent study of Fantino et al. (Fantino et al. 2010) compared transcription pattern of *E. coli* treated with 250 μM $CoCl_2$ for 30 min with untreated cells. Only 23 genes were found to be differentially expressed. Immediate upregulation of genes involved in cobalt efflux (*rcnA*) and Fe–S cluster biogenesis, such as *iscS, iscU, nfuA* or *hscA*, suggests the ability of the cell to preserve iron pool and redirect all available iron into production of Fe–S clusters. Downregulation of iron (*feoB*) and nickel (*nikA*) uptake systems should prevent additional cobalt uptake as cobalt can compete out iron and nickel to enter the cell. Finally, the downregulation of Fe–S cluster-containing enzymes, such as *nirB, hybO, nark, grcA* or *cysP* suggests that upregulation of compromised Fe–S cluster biogenesis pathway is insufficient and adaptation of cells exposed to cobalt treatment by turning down the dispensable metabolic pathways requiring Fe–S cluster enzymes takes place. Taken together, our data and data from the microarray transcription analysis (Fantino et al. 2010) suggest that the mechanism responsible for the cell survival in increased cobalt concentrations is most likely represented by a metabolic adaptation including transition from severely impaired respiration to a modified mixed acid fermentation.

In addition, cobalt has been shown to affect (i) the homeostasis of the labile iron pool (Kruszewski 2003) by oxidative stress and its competition with iron and also (ii) the assimilatory pathway for sulfur by direct competition with iron at uroporhyrinogen III methylase CysG (Thorgersen and Downs 2007, 2008, 2009). Thorgersen and Downs (Thorgersen and Downs 2007) showed that the catalytic activity of sulfite reductase and nitrate reductase, the only *E. coli* enzymes requiring siroheme, was strongly inhibited when cells were grown in the presence of 160 μM $CoCl_2$. Here we show the similar effect of cobalt on heme proteins involved in electron transport and respiration. The most likely candidate for insertion of cobalt into protoporphyrin IX (PPIX) is *E. coli* ferrochelatase HemH. Among ferrochelatases, the two best characterized are those from *B. subtilis* and *H. sapiens*. Both ferrochelatases utilize Fe, Ni and Zn in vivo and in vitro. In addition, human ferrochelatase can incorporate Co and *B. subtilis* ferrochelatase can insert Cu into PPIX (Dailey 1987; Medlock et al. 2009). Unfortunately, the metal specificity of *E. coli* ferrochelatase is not known (Frustaci and O'Brian 1993). Production of CoPPIX and its successful incorporation into e.g. membrane-bound cytochromes resulted in the inhibition of cellular oxygen uptake capacity up to 85%. The inactivation of cytochromes by the presence of CoPPIX is in agreement with the 2–3% of enzymatic activity of cytochrome P450*cam* reconstituted with CoPPIX relative to the native enzyme (Wagner et al. 1981).

The present study and the fact that *E. coli* is able to synthesize in vivo CoPPIX and incorporate it into heme proteins lead us to devise a procedure for preparing Co-substituted CBS. The human enzyme was expressed in *E. coli* Rosetta 2 cells in the presence of 150 μM $CoCl_2$, purified and characterized (unpublished). The activity of CoCBS was essentially identical to that of wild type FeCBS. Also, the yield of CoCBS was similar to that of the expressed FeCBS (Table 2). Previously, we purified two CBS enzymes, where heme was substituted with either MnPPIX or CoPPIX (Majtan et al. 2008). However, the yields of these enzymes from the heme-biosynthesis mutant strain grown anaerobically were low, which precluded their detailed biochemical, spectroscopic and functional studies. Activities of both substituted CBS as well as of FeCBS prepared by following a similar procedure were substantially reduced compared to the wild type FeCBS activity (Table 2) (Majtan et al. 2008). In the present

approach, the extent of CoPPIX incorporation into CBS has been increasing with the number of passages of bacterial cells through cobalt-containing medium prior to the induction of enzyme expression. The 88% or 92% CoPPIX saturation of CoCBS determined by the 2nd derivative visible spectrum analysis (Fig. 6) or analytical ICP-OES determination (unpublished), respectively, were found comparable with ≤5% contamination of MnPPIX-substituted $iNOS_{heme}$ prepared by the method of Woodward et al. (Woodward et al. 2007) utilizing the expensive, pure MnPPIX heme analog. The method of Brugna et al. (Brugna et al. 2010) utilizing *E. faecalis* for in vivo production of heme-substituted variant of *E. faecalis* KatA catalase was found to be more specific for incorporation of proper substituted metalloporphyrin with very low heme contamination. However, CuPPIX- and MgPPIX-substituted catalases were devoid of copper and magnesium, respectively. The presence of empty PPIX in KatA suggests that even in vivo incorporation of metalloporphyrins available in growth medium in place of heme can fail due to the removal of metal from the porphyrin during transport and thus represents a possible drawback of this method (Brugna et al. 2010).

Our approach to preparation of the CoPPIX-substituted heme protein provides significant improvements over the methods used so far (Brugna et al. 2010; Fruk et al. 2009; Woodward et al. 2007). Using an *E. coli* strain such as Rosetta 2 aerobically grown and passaged in the presence of cobalt, one can obtain high yield of a highly CoPPIX-enriched heme protein. In particular, the present method appears to be useful for complex heme proteins requiring a specific folding and/or with additional coenzyme(s), such as CBS. After all, coenzymes have been shown to participate in protein folding by binding to an intermediate in the folding pathway, limiting the ensemble of intermediates in the folding to the native state (Wittung-Stafshede 2002). Moreover, the use of inexpensive metal salt instead of expensive metalloporphyrin represents the advantage of this approach over the previous methods of heme replacement (Brugna et al. 2010; Fruk et al. 2009; Woodward et al. 2007). The limitation of our method stems from the metal used for the substitution. Here we described the successful use of cobalt for preparation of CoPPIX-substituted heme proteins such as membrane-bound cytochromes and CBS. For the utilization of other transition metals using a similar approach, one would have to consider their toxicity and the specificity of the involved chelatase. Additionally, the in vivo stability and folding of heme proteins substituted with metalloporphyrins other than FePPIX or CoPPIX would have to be taken into account. The activity of the substituted heme proteins can be seriously affected depending whether the heme plays a catalytic role such as in cytochromes or not as in the case of CBS. As cobalt replaces iron in the Fe–S clusters as well, this method could be potentially useful for preparation of cobalt-substituted Fe–S cluster proteins. However, the stability of cobalt-substituted Fe–S clusters and their incorporation into Fe–S proteins needs to be investigated in detail. Thus far, our study significantly contributes to the general knowledge about cobalt toxicity. Replacement of iron for cobalt as a substrate for *E. coli* chelatase, production of CoPPIX and its incorporation into heme proteins resulting in the inhibition of electron transport and respiration due to Co-substituted cytochromes represents an additional mode of action of cobalt on cellular metabolic processes.

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