Biometals (2010) 23:1105–1112
DOI 10.1007/s10534-010-9356-7

Genome-wide transcriptome analysis of the adaptive response of *Enterococcus faecalis* to copper exposure

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Received: 8 March 2010 / Accepted: 11 June 2010 / Published online: 25 June 2010

Abstract In this work we investigated the adaptive response of *E. faecalis* to Cu and the role of CopY, a Cu-dependent repressor, in the regulation of Cu metabolism. In doing so, we examined the whole-genome transcriptional response of *E. faecalis* wild-type (WT) and a Δ*copY* strain exposed to non-toxic Cu excess. The results indicated that after Cu exposure, most of the genes that displayed a significant change in their expression levels in the WT strain (135 of the 145 up-regulated genes and 115 of the 142 down-regulated genes) were also differentially expressed in the *E. faecalis* Δ*copY* strain. This extensive overlap in the transcriptional response, suggested that additional transcription factors mediate the response of *E. faecalis* to Cu. As a first step to analyze this possibility, we selected among the up-regulated genes five genes encoding putative transcriptional regulators and determined their expression levels at different times after Cu exposure. The temporal expression of these regulators was different from that of *copY*, which reached its maximum at the earliest time measured. Nevertheless, transcription elongation factor GreA, and members of Rrf2, Cro/CI and SorC/DeoR transcription factor families were induced shortly after Cu exposure, suggesting that these proteins are able to complement the role of CopY in the regulatory network activated by Cu. To our knowledge, this is the first report on the global transcriptional response to Cu in a member of this taxonomic group.

Keywords *E. faecalis* · Copper homeostasis · Global gene expression · Real-time RT-PCR · CopY

Electronic supplementary material The online version of this article (doi:10.1007/s10534-010-9356-7) contains supplementary material, which is available to authorized users.

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Introduction

Copper is a transition metal that is able to cycle between two redox states, oxidized Cu^{2+} and reduced

Cu^{1+}, and it is an essential element in most biological systems. Its ability to accept and donate electrons gives Cu the characteristic of being a very efficient cofactor for redox enzymes, but the reactivity of ionic Cu also makes it a toxic metal (Urbanski and Beresewicz 2000; Linder and Hazegh-Azam 1996). As a consequence, cells must have mechanisms to properly handle the intracellular Cu concentration (Rae et al. 1999). In bacterial and eukaryotic cells, Cu homeostasis is controlled principally at three levels: (i) uptake, (ii) management and intracellular storage and (iii) efflux. Different bacterial proteins that bind to and/or transport Cu are known to be involved in this process (Solioz and Stoyanov 2003).

Global analysis of gene expression under different conditions of Cu exposure have been carried out in *Escherichia coli* (Yamamoto and Ishiham 2005; Kershaw et al. 2005), *Pseudomonas aeruginosa* (Teitzel et al. 2006), *Mycobacterium tuberculosis* (Ward et al. 2008), whereas Cu homeostasis has been well described in *Enterococcus hirae* (Solioz and Stoyanov 2003), a Gram-positive member of lactic acid bacteria of the phylum *Firmicutes*. In *E. hirae* Cu homeostasis is regulated by the products of the *cop* operon, which is up-regulated by Cu at concentrations higher than 10 μM (Lu et al. 2003). The *cop* operon is formed by four genes *copA*, *copB*, *copZ* and *copY*, encoding two P-type ATPase Cu transporters, a Cu chaperone protein and a Cu-dependent transcriptional regulator, respectively (Solioz and Stoyanov 2003). The *cop* operon, appears to be conserved in all members of *Lactobacillales* order, and in all cases it includes at least homologues of *copY* and *copA* genes (Reyes et al. 2006). However, the *E. hirae* genome has not been sequenced, and thus it is difficult to examine whether the *cop* operon function is complemented by other proteins of this bacterium.

Considering that *E. faecalis* is the phylogenetically closest species to *E. hirae*, in the present study, our aim was to functionally characterize the *cop* operon in this species. In addition, we analyzed the global transcriptional response of *E. faecalis* to Cu in order to identify a set of genes involved in the cellular response to Cu exposure. Moreover, by using an *E. faecalis* Δ*copY* strain we aimed to determine which part of the transcriptional response was CopY dependent. Our results revealed an extensive overlap between the set of genes differentially expressed in WT and Δ*copY* strains suggesting that additional transcriptional regulators are required to sustain the cellular response to Cu. In addition, we provide information on the temporal expression pattern of a set of regulators, which are induced at early stages after Cu exposure; they might be good candidates to be incorporated within the regulatory network activated by Cu.

Materials and methods

Strains, growth conditions and plasmids

The bacterial strains and plasmids used in this study are listed in supplementary Table 1. *E. faecalis* OG1RF and its derivatives were grown in brain heart infusion (BHI) medium (Difco-USA) at 37°C. *E. coli* TG1 was used for routine cloning and Ec1000 was used for pCJK47 construct (Leenhouts et al. 1996) both were grown in Luria–Bertani (LB) (Difco-USA) broth at 37°C. Cu exposure assays were carried out in N medium (Odermatt and Solioz 1995) (Peptone 1% (Difco), yeast extract 0.5% (Difco), Na_2HPO_4 1% (Merck) and glucose 1% (Oxoid)) supplemented as indicated with $CuSO_4 \cdot 7\ H_2O$ (Merck). The pGEM-T Easy (Promega) and pCJK47 (Chen et al. 2007) plasmid were used for PCR cloning and genetic construction, respectively. Erythromycin (Em), when required, was added at 10 μg/ml for *E. faecalis* and 200 μg/ml for *E. coli*.

Deletion of the copY gene

copY mutants were constructed using the $PheS^*$ system, resulting in non-polar deletion mutants (Kristich et al. 2007). Briefly, fragments of ca. 1300 bp located downstream and upstream of target gene were amplified by PCR using the primers shown in supplementary Table 2. The amplicon was first cloned in pGEM-T Easy (Promega) and then assembled in pCJK47. The resulting constructs were transferred to CK111 by electroporation and finally to OG1RF by conjugation (single cross over insertions). Loss of the plasmid was then selected on medium MM9YEG agar supplemented with 10 mM p-Cl-Phe and X-gal 200 μg/ml. Possible mutants were first screened by PCR, then the junction area was sequenced, and the strain background confirmed by pulsed field gel electrophoresis.

Minimum inhibitory concentration (MIC) of Cu

The agar dilution method was used to determinate the effect of Cu on growth (Bissig et al. 2001). N medium agar plates were supplemented with 0, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0, 3.0, 4.0, 5.0 and 6.0 mM of $CuSO_4$. All experiments were repeated at least six times. The MIC of Cu was defined as the lowest concentration of Cu at which no growth was observed following overnight incubation at 37°C.

Growth curves

WT and Δ*copY* strains were pre-cultured overnight in N medium broth at 37°C. The next day, the cells were diluted in 50 ml N medium broth to a starting OD_{600nm} of 0.05, and grown at 37°C with 160 rpm. To assess Cu effect on expression of the *cop* operon, N medium broth was supplemented with 0 and 0.5 mM of $CuSO_4$. Growth was monitored each hour from 0 to 12 h and at 24 h by OD_{600nm}. Colony forming units (CFU) was assessed at 0, 3, 5, 7 and 9 h post inoculation. Each strain was tested in the same conditions at least three times, and the mean generation time was calculated.

Cellular Cu content

The Cu content was determined in WT and Δ*copY* mutant strains after exposure to copper sulfate. The culture was initiated as above with N medium broth supplemented with 0 or with 0.5 mM of $CuSO_4$. After 3 h of incubation, 6 ml of each culture were collected by centrifugation and washed with phosphate-buffered saline (PBS: 0.15 M NaCl, 50 mM glycine (pH 3.5) and 1.0 mM EDTA) pH 6.8. Cells were resuspended in 1 ml PBS, a 10 µl aliquot was removed to assess CFU, and the rest of the cells were disrupted by sonication. Supernatants and cell debris were separated by ultracentrifugation at 100,000×*g*, 30 min. 100 µl of supernatant were treated with 250 µl ultra-pure nitric acid at 65°C for 24 h. The Cu content was determined in triplicate by atomic absorption spectrometry (AAS) as previously described (Gonzalez et al. 1999). The Cu concentration was expressed as Cu atoms per colony forming units. The statistical analysis for cellular Cu content was done by Mann–Whitney U test, and $P < 0.05$ were considered statistically significant.

RNA extraction, cDNA synthesis and quantitative real-time RT-PCR

WT and Δ*copY* strains were grown in basal medium or in medium supplemented with 0.5 mM of $CuSO_4$ for 3 h prior to harvesting (at mid-log phase, $OD_{600nm} \sim 1.0$). The RNA was extracted using a Qiagen RNeasy mini kit (Qiagen). Residual contaminating genomic DNA was removed with RQ1 RNase-free DNase (Promega) treatment according to the manufacturer's protocol. The integrity of the RNA was assessed by gel electrophoresis. Two micrograms of total RNA were reverse transcribed with Moloney Murine Leukemia Virus Reverse (Promega, USA) using random primers (Invitrogen). PCR primers were designed with Primer3 plus software using *E. faecalis V583* genome sequence (supplementary Table 2). Real-time RT-PCR reactions were carried out in triplicate using two independent RNA samples. The experiments were performed and data were analyzed as described in (del Pozo et al. 2010), except that transcript levels of genes were normalized to the *gdh* (EF1004) housekeeping gene (Ruiz-Garbajosa et al. 2006). Data was expressed as fold-change between Cu-treated and untreated cells. Significant differences in fold-change values were assessed by REST 2008 algorithm and ANOVA test.

Microarray experiments

The overall expression of genes was performed using a chip of four arrays of 72 K (catalog number A7980-00-01) from NimbleGen Systems, Inc. Each array contains 72,000 oligonucleotides 60-mer, with an average of 11 oligos designed for the 3,114 open reading frames of *E. faecalis* V583 that represent twice the genome of the bacterium (two technical replicates per arrays). For WT and *copY* mutant four independent hybridizations (two biological replicates per treatment paired with their respective controls) were performed by the manufacturer (Nimblegen) on equal conditions in a single glass, thus reducing variability between hybridizations (pre-hybridization, hybridization and washing steps). Scanning of the slides and data analysis also were carried out by Nimblegen. Gene expression data from Cu-treated WT cells were compared to that of untreated cells; and data from Cu-treated *copY* mutant cells were

compared to untreated *copY* cells. Student's t-test statistics were used to identify significantly different gene expression levels with a $P < 0.05$ and a 4-fold magnitude of change between the average value of each gene and its corresponding reference, using the DNASTAR software Array star 3.0. The GEO accession number for the microarray data reported in this paper is GSE20453.

Sequence alignments of CopY

The amino acid sequences of CopY from *Lactobacillales* species with complete genome sequence available were obtained from the NCBI website (November 2008). Sequences alignments were performed using Clustal W (BioEdit).

Results and discussion

In order to evaluate how *E. faecalis* responds to extracellular Cu, we first determined the minimum inhibitory concentration of Cu for WT strain using the agar dilution method. Under our experimental conditions, inhibition of growth by Cu was detected in a concentration range of 2.0–4.0 mM. Next, we used AAS to determine changes in the cellular Cu content in response to an increase in metal exposure. The results showed that when WT cells were exposed to 0.5 mM of $CuSO_4$, the cellular metal content increased 8-fold, compared to the cells grown without Cu (Fig. 1a). In addition, the results showed that the generation times of cells growing in control medium (46 ± 3 min) was not significantly altered compared to cells grown in presence of 0.5 mM Cu (48 ± 5 min), indicating that this Cu concentration did not affect the growth of WT *E. faecalis* and suggesting that Cu homeostasis mechanisms are encoded in the genome of this bacteria.

Within Gram positive bacteria, one of the most extensively studied models for Cu homeostasis is *Enterococcus hirae*. The regulation of intracellular Cu in this species is mediated by the *cop* operon: *copA, copB, copY,* and *copZ* (Solioz and Stoyanov 2003). In a previous work we found that *copA, copY,* and *copZ* genes were conserved in *E. faecalis*, in particular a putative CopY repressor was conserved among the *Lactobacillales* order, suggesting that this group has a common transcriptional regulator that is activated by Cu (Reyes et al. 2006). In the current work, we determined the effect of Cu exposure on the expression levels of *cop* genes of *E. faecalis*. In doing so, WT OG1RF strain was grown in N medium broth supplemented with 0.5 mM $CuSO_4$ or in basal medium and the expression levels of *copY*, *copA* and *copZ* genes were quantified by real-time RT-PCR. We determined that the relative expression levels of these three genes rose noticeably (>5-fold-change) in the presence of Cu (Fig. 1b). Considering the genome organization of the *copYAZ* locus and the similar level of expression that was observed for each of the *cop* genes, the results indicated that during adaptation to non toxic extracellular Cu excess, *E. faecalis* induced *cop* genes as a transcriptional unit. The results indicated that Cop proteins might be responsible for the regulation of cellular Cu levels (Pecou et al. 2006).

Next, we investigated the role of CopY in the regulation of the *cop* operon in *E. faecalis*; we constructed a Δ*copY* strain and examined the transcriptional pattern of *copA–copZ* genes. The results indicated that *copA–copZ* genes were over-expressed in the *E. faecalis* Δ*copY* strain grown in basal medium (Fig. 1c), in agreement with a role of CopY as a repressor. The deletion of *copY* gene also affected cellular Cu content (Fig. 1d), thus Δ*copY* strain grown in the presence of 0.5 mM of $CuSO_4$ showed a significantly lower Cu content that WT cells, with only 65 μg Cu/μg protein compared to 117 μg Cu/μg protein in WT cells exposed to Cu ($P < 0.05$). Under these conditions, the generation times of Δ*copY* cells growing with or without Cu were no affected and they maintained similar levels than WT cells.

Recently, it has been shown that CopY homologues from different species of genera *Enterococcus*, *Lactococcus*, and *Streptococcus* exhibit similar affinity not only for their native promoters, but also for heterologous *cop* promoters (Portmann et al. 2006). Interestingly, amino acid sequence comparisons revealed that 11 out 21 CopY proteins of *Lactobacillales* bacteria, including CopY of *E. faecalis*, had a complete version of $CxCx_{4-6}CxC$ domain at the C-terminus (supplementary Fig. 1), which is a conserved domain of Cu response transcription factors, such as Ace1, Amt1 and Mac1 that are known to undergo copper metalloregulation in yeast (Rutherford and Bird 2004; Keller et al. 2005). Thus, this conserved structural domain of CopY seems to be important for copper sensing within the *Lactobacillales* order. This idea was also

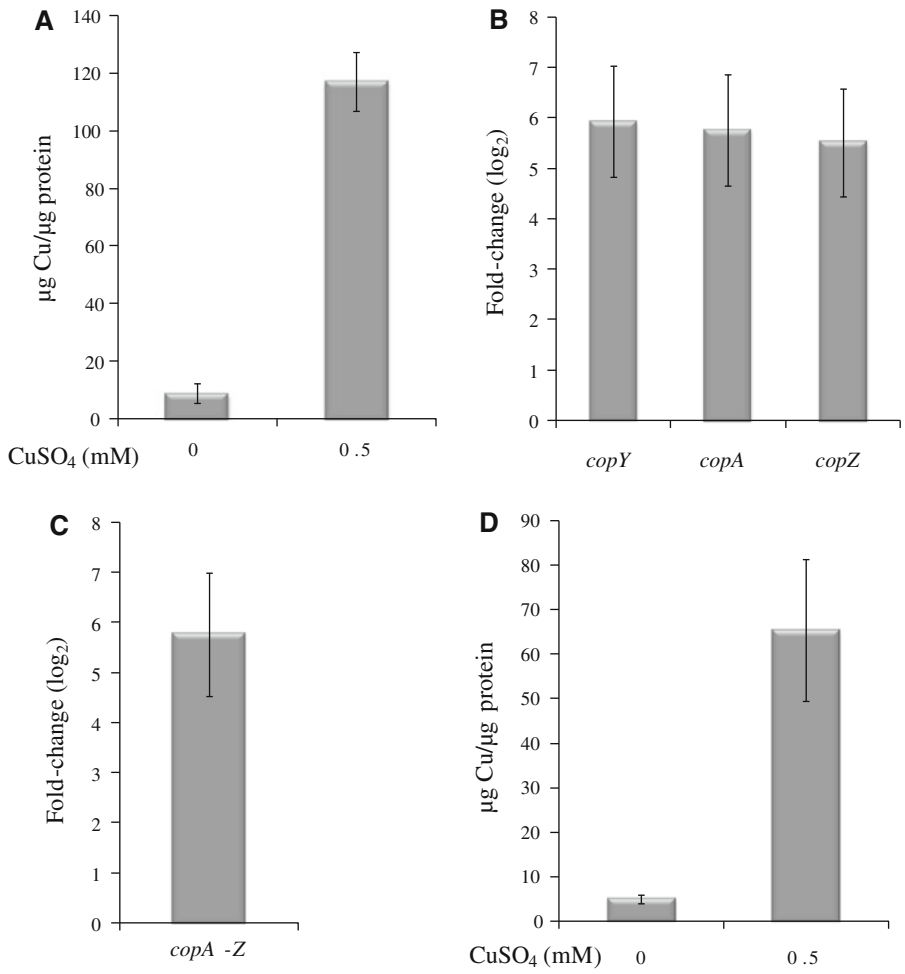

Fig. 1 Copper content and *cop* operon expression in WT and Δ*copY* strains (**a**–**b**) WT *E. faecalis* was grown in basal medium or in medium supplemented with 0.5 mM of $CuSO_4$ for 3 h before harvesting. **a** AAS was used to determine Cu content. **b** Transcripts of *copY*, *copA* and *copZ* genes were quantified by real-time RT-PCR. Data are expressed as fold-change between Cu-treated and untreated cDNA samples. **c** Transcripts of *copA-copZ* genes were quantified by real-time RT-PCR in *E. faecalis* Δ*copY* strain growing in basal medium, data are expressed as fold-change between Δ*copY* and WT cDNA samples. **d** Cu content was measured in Δ*copY* strain grown in basal medium or in medium supplemented with 0.5 mM of $CuSO_4$. In all cases, the values correspond to the mean and SD of triplicate determinations

supported by the presence of at least one CopY binding site in the promoter region of the *cop* operon of all *Lactobacillales* species analyzed (Reyes et al. 2006).

To further elucidate the response of WT *E. faecalis* strain to Cu and to identify Cu-responsive genes other than the genes in the *cop* operon, we compared the transcriptome of *E. faecalis* grown in basal medium to the transcriptome of cells grown in medium supplemented with 0.5 mM $CuSO_4$ for 3 h. RNA was isolated from two independent exponential phase cultures of *E. faecalis* and hybridized to an anti-sense oligonucleotide array in which 11 probes per gene were utilized to estimate the transcriptional level of each gene in each replicate. Genes with a fold-change ≥4 were considered significantly different if there was a probability of differential expression of $P < 0.05$ based on Student's *t*-test statistics. The results indicated that 287 genes were differentially expressed between Cu-supplemented and basal medium cultures, representing 9% of the *E. faecalis* genome. Among them, 145 genes were up-regulated including the three members of *cop* operon and 142 were down regulated in response to Cu (supplementary Table 1). A similar proportion of transcriptional

changes were observed for *E. coli* exposed to Cu (0.75 and 2 mM $CuS0_4$) (Kershaw et al. 2005).

When we integrated these results with the gene annotation data, except for the *cop* operon genes, we found no other genes that showed obvious association to Cu metabolism. A large number of genes (n = 80) corresponded to hypothetical proteins and 42 of them have no COG assigned, the other fraction of genes mainly encode proteins involved in energy production, iron and amino acid metabolism and transcriptional gene regulation (supplementary Table 3). Regarding iron metabolism, three genes encoding subunits of iron compound ABC transporters family (EF1639, EF1640 and EF1641) were down-regulated in *E. faecalis* exposed to Cu, while two genes (EF0098 and EF0099) which encode subunits of iron-dependent L-serine-dehydratase were up-regulated. In this regard, it has been described in *E. coli* that Cu inactivates the catalytic clusters of L-serine dehydratase by displacement of iron atoms (Macomber and Imlay 2009). Additionally, expression changes of genes involved in iron metabolism have been observed in transcriptomic studies of *E. coli* and *P. aeruginosa* exposed to Cu excess (Kershaw et al. 2005; Teitzel et al. 2006) or under Cu starvation (Frangipani et al. 2008), suggesting the existence of common steps in the control of Cu and Fe homeostasis. In this context, it has been shown that both nutrients are intimately related at metabolic and transcriptional level in both human cells (Arredondo and Núñez 2005) and yeast (de Freitas et al. 2003).

Next, we assessed whether the absence of CopY repressor affected the transcriptional response of *E. faecalis* to Cu exposure. Microarray analysis revealed that after Cu exposure, 154 and 148 genes were up and down-regulated, respectively, in the Δ*copY* strain (Fig. 2 and supplementary Table 1). We detected an extensive overlap in the transcriptional response of WT and mutant strains, which is illustrated in the Venn diagram of Fig. 2. Most of the genes that displayed a significant change in their expression levels in WT strain (135 of the 145 up-regulated genes and 115 of the 142 down-regulated genes) were also differentially expressed in the *E. faecalis* Δ*copY* strain after Cu exposure (Fig. 2). Thus, these results indicated that only a small fraction of the transcriptional changes induced by Cu were dependent on CopY repressor activity. Data analysis showed that only ten genes were exclusively

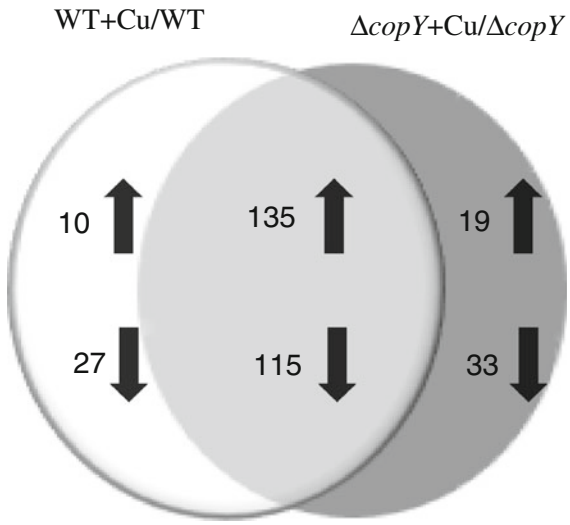

Fig. 2 Venn diagram of common and unique sets of genes differentially expressed in WT and Δ*copY* strains exposed to Cu The diagram indicates the number of genes that were up-regulated (*upward arrow*) or down-regulated (*downward arrow*) after Cu exposure

up-regulated by Cu in WT cells and, as expected, three of them corresponded to *cop* genes. Moreover, only *cop* operon genes were differentially expressed in microarray experiments designed to compare changes in gene expression levels between WT and Δ*copY* strains growing in basal culture medium (data not shown).

In addition to transcriptional regulators *copY*, a hypothetical protein of COG class K (EF1752), the transcription elongation factor GreA (EF2914), and members of transcription factor Rrf2 (EF3175), Cro/CI (EF0873) and SorC/DeoR (EF1965) families, increased their expression levels at 15 or 45 min after Cu exposure. However, none of these transcriptional regulators showed the temporal gene expression pattern of *copY* repressor (EF0297), which reached its maximum expression level at the earliest time measured (15 min) (Fig. 3, *copY*). These results suggested that the transcriptional activation of these genes requires a further increment of Cu content, which is reached after a longer period of Cu exposure, as it has been proposed by us using *E. hirae* as a model (Pecou et al. 2006). Alternatively, these genes might be induced in response to a secondary signal such as the oxidative stress that is generated by Cu, as it has been shown in *E. coli* (Kershaw et al. 2005). In this

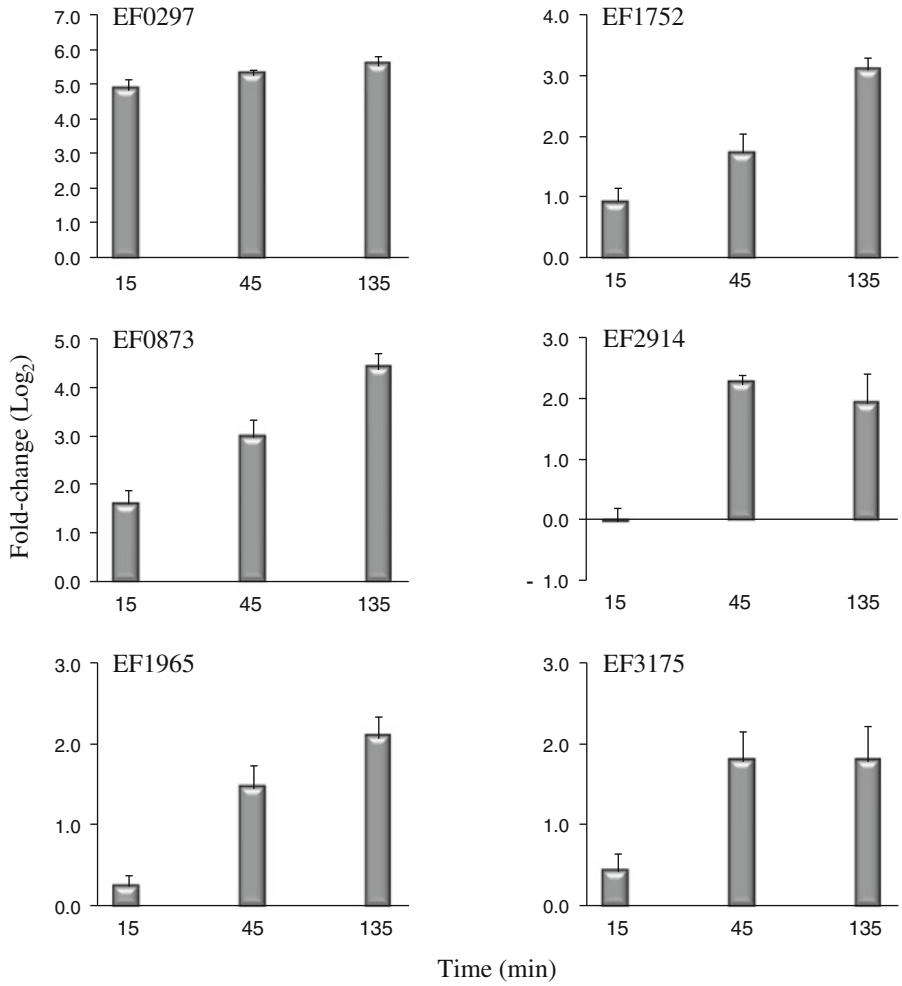

Fig. 3 Temporal expression pattern of Cu-induced genes encoding transcriptional regulator WT cells were grown in basal medium or in medium supplemented with 0.5 mM of $CuSO_4$ for 15, 45 and 135 min before harvesting. Transcripts of selected genes were quantified by real-time RT-PCR. Data were expressed as fold-change between Cu-treated and untreated cDNA samples. The values correspond to the mean and SD of four determinations. EF0297: CopY repressor; EF3175: rrf2 family protein; EF2914: transcription elongation factor GreA; EF0873: transcriptional regulator, Cro/CI family; EF1965: transcriptional regulator, SorC/DeoR family; EF1752: hypothetical protein

context, several transcription factors were postulated to sense changes in redox state (O'Halloran 1993), they are good candidates to mediate regulation of gene expression in response to Cu-induced oxidative stress. Therefore, our results provided a new group of transcriptional regulators that will help us to understand the effect of Cu exposure on global gene expression regulation.

In conclusion our results indicate that in the *E. faecalis*, the *cop* operon contains a functional conserved CopY repressor, which is required for cellular adaptation to Cu excess and seems to regulate only *cop* genes. Global gene expression analysis revealed new putative components of Cu homeostasis and the presence of several transcriptional regulators that might complement the CopY role in the regulatory network activated by Cu.

Acknowledgements This work was supported by grants Basal-CMM, Fondecyt 1071083 and 1090211 and Fondef D04I1257. AR-J was recipients of an 'A. Steckel' fellowship from INTA and MAL is a recipient of doctoral fellowship from Conicyt. The authors thank Arely González and Andres Aravena for COGs assignments and manual revision of V583 genome.

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