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Transcriptomic response of *Enterococcus faecalis* to iron excess

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Abstract Iron is an essential nutrient for sustaining bacterial growth; however, little is known about the molecular mechanisms that govern gene expression during the homeostatic response to iron availability. In this study we analyzed the global transcriptional response of *Enterococcus faecalis* to a non-toxic iron excess in order to identify the set of genes that respond to an increment of intracellular iron. Our results showed an up-regulation of transcriptional regulators of the Fur family (PerR and ZurR), the cation efflux family (CzcD) and ferredoxin, while proton-dependent Mn/Fe (MntH) transporters and the universal stress protein (UspA) were down-regulated. This indicated that *E. faecalis* was able to activate a transcriptional response while growing in the presence of an excess of non-toxic iron, assuring the maintenance of iron homeostasis. Gene expression analysis of *E. faecalis* treated with H_2O_2 indicated that a fraction of the transcriptional changes induced by iron appears to be mediated by oxidative stress. A

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comparison of our transcriptomic data with a recently reported set of differentially expressed genes in *E. faecalis* grown in blood, revealed an important fraction of common genes. In particular, genes associated to oxidative stress were up-regulated in both conditions, while genes encoding the iron uptake system (*feo* and *ycl* operons) were up-regulated when cells were grown in blood. This suggested that blood cultures mimic an iron deficit, and was corroborated by measuring *feo* and *ycl* expression in *E. faecalis* treated with the iron chelating agent 2,2-dipyridil. In summary, our group identified an adaptive transcriptional mechanism in response to metal ion stress in *E. faecalis*, providing a foundation for future in-depth functional studies of the iron-activated regulatory network.

Keywords *Enteroccocus faecalis* · Iron transporters · Global gene expression · qPCR · Iron content · Oxidative stress

Introduction

Iron is an essential nutrient for bacterial growth. A large number of enzymes that participate in respiration, nitrogen fixation, central metabolism, redox stress resistance and amino acid synthesis require one or more atoms of iron for structure stability and function (Beinert et al. 1997; Py and Barras 2010). However, iron is not readily available for bacteria—particularly under aerobic conditions-, because it is present in the insoluble Fe^{3+} form. Thus, bacteria have evolved various mechanisms in order to acquire iron from diverse sources (Cornelis and Andrews 2010). In bacteria, iron uptake is a tightly regulated process due to the need of preventing its accumulation at toxic cell levels. Iron is responsible for the generation of reactive hydroxyls through the Fenton reaction, which are likely to promote DNA, protein and lipid damage (Imlay 2003). Therefore, intracellular levels of iron must be controlled in order to avoid toxicity while providing sufficient amounts for iron-dependent proteins (Beinert et al. 1997; Cornelis and Andrews 2010).

Bacteria respond to environmental changes of iron availability by shutting down or activating the transcription of key genes encoding proteins that directly control iron metabolism (Andrews et al. 2003; Salvail and Masse 2012). In general, the regulatory circuit operated by iron deficit in bacteria involves Fur family transcriptional regulators that are able to sense changes in the availability of iron and other metals, as well as regulate the expression of relevant target genes that encode proteins involved in iron uptake, storage and efflux (Hantke 2001; Semsey et al. 2006; Lee and Helmann 2007; Cornelis et al. 2011). Together with affecting iron metabolism, iron deficit has an impact on different cellular processes such as pathogenicity and response to oxidative stress (McHugh et al. 2003; Bronstein et al. 2008).

Enterococcus faecalis is a facultative aerobic Gram-positive bacterium that belongs to the Firmicutes Phylum and *Lactobacillales* order. This bacterium, a common member of the human gastrointestinal tract microbiota and protagonist in several study fields due to its relevance in the food industry (Foulquie Moreno et al. 2006), is the causative agent of nosocomial infections and is also responsible of urinary tract and soft tissue infections, bacteremia, and endocarditis (Shankar et al. 2002; Fisher and Phillips 2009). Over the past three decades, *E. faecalis* has emerged as an important nosocomial pathogen (Murray 1990; Giridhara Upadhyaya et al. 2009). Efforts have been made to explore its genomic diversity by sequencing several *E. faecalis* strains; among them, the genomes of *E. faecalis* V583 (Paulsen et al. 2003), OG1RF (Bourgogne et al. 2008) and strains from antibiotic-resistant clinical isolates, as well as environmental and animal samples (Domann et al. 2007; Palmer et al. 2010). As a complementary "omic" approach, genome-scale gene expression analyses have led to the identification of differentially expressed genes in *E. faecalis* grown in manganese, copper and zinc supplemented media (Reyes-Jara et al. 2010; Coelho Abrantes et al. 2011) and under conditions that mimic the in vivo host environment during infection (Solheim et al. 2007; Vebo et al. 2009; Lenz et al. 2010).

Despite the importance of iron availability for bacterial growth (Cornelis et al. 2011) and virulence, little is known regarding the molecular mechanisms that govern the expression of genes involved in the iron-excess homeostatic response. In this work, we evaluated the transcriptomic response of *E. faecalis* to iron excess in order to understand the bacterial cellular responses to metal ion stress, and to identify new putative components that respond to these conditions.

We found that the cellular content of iron was strongly associated to the differential expression of genes involved in an array of biological functions that include metal ion homeostasis, oxidative stress responses, membrane stress responses and transcriptional regulation in *E. faecalis*. Moreover, we compared our results on gene expression changes in *E. faecalis* grown in an iron-supplemented media with published data from blood studies (Vebo et al. 2009). This revealed common and unique sets of genes that characterize the transcriptional response of *E. faecalis* to an iron excess, as well as its response to environmental conditions while gaining access to the bloodstream and establishing a persistent infection. Overall, this study provides information regarding the adaptive transcriptional mechanisms of *E. faecalis* in response to iron availability fluctuations, as well as a background for the identification of the gene regulatory network activated by iron.

Materials and methods

Bacterial strains and growth conditions

Enterococcus faecalis strain OG1RF was grown in N medium (Peptone 1%, yeast extract 0.5%, Na_2HPO_4 1%, glucose 1%) (Odermatt and Solioz 1995) containing 2.8 μM iron as basal concentration. Solid media was prepared by adding technical agar at a final concentration of 1.0% w/v. The effect of iron on the growth of *E. faecalis* was determined by the addition of N media with different concentrations of $FeCl_3 \cdot 6H_2O$ in equimolar proportions with nitrilotriacetic acid ($FeCl_3$-NTA). To create iron limiting conditions, cells were grown during 6 h in presence of the chelating agent 2,2-dipyridil (2,2-DPD). Oxidative stress was generated by adding 2.4 mM hydrogen peroxide (H_2O_2) for 45 min during exponential growth phase.

In order to obtain the growth curves; an overnight-grown culture of *E. faecalis* was used to adjust the bacterial suspension to 0.05 at OD_{600nm} in N medium. *E. faecalis* was grown at 37°C with shaking at 140 rpm. Bacterial growth was monitored every hour during 8 h and at 24 h by two methods: measuring OD_{600nm} and determining the number of colony forming units (CFUs) by plate-counting in N agar-medium. All growth curve experiments were carried out in triplicate.

Minimal inhibitory concentration of iron (MIC-Fe)

The MIC-Fe was determined by measuring the effect of iron on *E. faecalis* OG1RF growth in liquid medium. N medium was supplemented with 0, 10, 20, 30, 40, 50 and 60 mM of $FeCl_3$-NTA. All experiments were repeated at least six times. The MIC-Fe was defined as the lowest concentration at which no growth was observed following an overnight incubation at 37°C.

Measurement of the intracellular iron content

To determine intracellular iron content, cells exposed to media supplemented with different $FeCl_3$-NTA concentrations were harvested by centrifugation, sequentially washed with sterile NaCl 0.85%, EDTA 1 mM pH 7 and NaCl 0.85%, and resuspended in 1 mL NaCl 0.85%. Cells were disrupted by sonication at 4°C and cell debris was removed by centrifugation at 28,600×*g* for 30 min at 4°C. Supernatant protein concentration was measured by the Folin-Lowry assay. One hundred μL of culture supernatant was treated with concentrated nitric acid (1:2) and incubated for 24 h at 65°C. Iron content was measured by AAS as described in (Gonzalez et al. 1999) with a spectrometer with graphite oven SIMMA 6100 (Perkin Elmer Massachussets, USA). Calibration curves were built with serial dilutions of iron standards (JT Baker, New Jersey, USA). The iron contents were expressed in mmoles of Fe per milligram of protein.

Measurement of glutathione content

Total glutathione content was measured by using the protocol described by Griffith (1980). This enzymatic method required a 200 μL aliquot of the supernatant obtained after sonication (see details in: measurement of iron contents) that was deproteinized with 5-sulfosalicylic. After centrifugation (5 min at 10,000 rpm, 4°C), 50 μL of supernatant were incubated with NADPH, DTNB (5,5′-dithio-bis[2-nitrobenzoic acid]) and glutathione reductase (GSSG reductase) during 30 min. The rate of TNB (5-thio-2-nitrobenzoic acid) formation was followed at 412 nm. This methodology allowed to determine total GSH (GSH + GSSG, in

GSH equivalents) in *E. faecalis* samples obtained after iron exposure.

RNA extraction and cDNA synthesis

Total RNA from untreated cells and cells exposed to 0.5 mM of $FeCl_3$-NTA was extracted as described by Reyes-Jara et al. (Reyes-Jara et al. 2010). Briefly, an *E. faecalis* OG1RF cell suspension was prepared to 0.05 at OD_{600nm} from an overnight culture in N medium supplemented with iron or the iron chelator 2,2-DPD, and incubated during 6 h at 37°C and 150 rpm. The cells were harvested by centrifugation and washed as described previously. The RNA was extracted using a Qiagen RNeasy mini kit (Qiagen) according to the instructions detailed by the manufacturer. Residual contaminating DNA was removed by RNase-free DNase (Qiagen) treatment according to the protocol detailed by the manufacturer. Extracted RNA integrity was assessed by gel electrophoresis. cDNA was synthesized by using two micrograms of total RNA, which were reverse transcribed using random primers (Invitrogen) with Moloney Murine Leukemia Virus Reverse (Promega, USA) according to the instructions detailed by the manufacturer.

Microarray experiments

Overall gene expression was assessed by using a microarray chip described in (Reyes-Jara et al. 2010). Four independent hybridizations (two biological replicates per treatment, paired with their respective controls) were carried out by the manufacturer (Nimblegen) in equal conditions in a single flask, thus reducing variability between hybridizations (pre-hybridization, hybridization and washing steps). Slide scanning was carried out by Nimblegen. Fold-change values were calculated from gene expression values between the iron treated and control samples. The GEO accession number GSE34432 contains access to all the microarray data generated in this paper. Student *t*-test was used to identify significant changes of gene expression levels between the average value of each gene and its corresponding reference ($P < 0.05$, DNASTAR Software Array Star 3.0). Data and significance analyses of blood microarrays of *E. faecalis* were obtained from Vebo et al. (2009), where the authors exposed *E. faecalis* to 100% v/v of defibrinated horse blood during 30 min at 37°C and used the bacterium growing in 2xYT medium as a reference.

Quantitative PCR and primer design

Quantitative PCR (qPCR) was performed using the real-time PCR system, LightCycler™ Roche. PCR primers were designed with Primer Premiere 5.0 Software using the *E. faecalis* V583 genome sequences listed in Supplementary Table S1. Each reaction was conducted in triplicate using two independent RNA samples. Fluorescence was analyzed using LightCycler™ Analysis Software. The number of threshold cycles per reaction was determined by using the Second Derivate Maximum algorithm and a manual baseline adjustment. Amplification efficiencies were calculated using LinRegPCR Software. The relative expression level of each gene of interest was calculated using the $2^{-\Delta\Delta}$Ct method (Livak and Schmittgen 2001), using the *gdh* gene (EF 1004) as a reference (Ruiz-Garbajosa et al. 2006). Data was expressed as fold-change between treated and untreated cells. Significant differences in fold-change values were assessed by the REST 2008 algorithm and ANOVA test.

Results

Effects of iron exposure on *E. faecalis* growth and intracellular iron content

In order to evaluate the global transcriptional response of *E. faecalis* towards an iron excess, we established an extracellular concentration of iron that did not affect its doubling time, and allowed to measure changes in the intracellular iron content (Fig. 1a). Results indicated that after 6 h of exposure to 0.25, 0.5 and 1.0 mM $FeCl_3$-NTA, *E. faecalis* growth was equivalent to that observed in cultures without iron supplementation (Fig. 1a). Additionally, we detected growth inhibition at 40 mM $FeCl_3$-NTA while testing the tolerance of *E. faecalis* to iron using a MIC-Fe assay. AAS was used to measure intracellular iron content after 6 h of exposure to $FeCl_3$-NTA. Cells increased their iron content in 3-, 6- and 7- fold after an exposure to 0.25, 0.5 and 1.0 mM of Fe respectively, compared to control cells grown in the absence of FeCl3-NTA (Fig. 1b). Based on these results, we

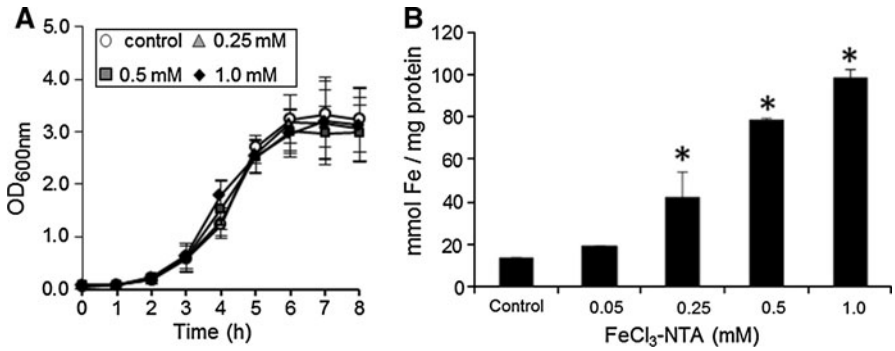

Fig. 1 Effects of iron in *E. faecalis* OG1RF. **a** Growth of *E. faecalis* with increasing concentrations of $FeCl_3$-NTA in the medium. **b** Iron contents of cells exposed to different concentrations of $FeCl_3$-NTA for 6 h. Each *bar* represents the average results from three independent experiments. *Error bars* indicate standard deviation (SD) values. *Asterisks* indicate significant differences with respect to the control (*t*-student test, $P < 0.05$)

selected 0.5 mM iron for our microarray experiments, since it was the lowest extracellular concentration that induced the highest increase in intracellular iron concentration Thus, the effect of iron excess on bacterial gene expression was evaluated after 6 h of exposure to 0.5 mM $FeCl_3$-NTA.

Global transcriptional response of *E. faecalis* to iron excess

To determine which *E. faecalis* genes are involved in the response to iron excess, we used cDNA arrays to characterize the transcriptional response of *E. faecalis* exposed to 6 h of 0.5 mM iron. All genes that changed their expression level at a *P* value lower than 0.05 (cutoff value) were included. Under these conditions, a set of 475 genes (nearly 15% of the *E. faecalis* V583 genome) showed a significant differential expression after iron exposure. Of these genes, 249 were up-regulated and 226 were down-regulated in response to iron (Supplementary Table S2).The differentially expressed genes were categorized based on the Clusters of Orthologous Groups (COG) category. We then compared the number of up or down-regulated genes within each category against the total number of predicted genes from the *E. faecalis* V583 genome (Supplementary Fig. S1). Results indicated that the major groups of differentially up-regulated genes were those involved in post-translational modification (>20%), ribosomal structure translation and biogenesis (>15%), showing a significant enrichment ($P < 0.05$) compared to their expected proportion in the bacterial genome.

Iron-regulated expression of genes encoding proteins involved in iron metabolism and redox stress response

While analyzing the annotated *E. faecalis* V583 genome (Paulsen et al. 2003), we identified 19 genes encoding proteins involved in iron homeostasis, including three members of the Fur family of transcriptional regulators (Table 1). We selected all 19 genes to validate our microarray results by using qPCR assays with the same RNA samples used in the previous array hybridizations. Significant changes in relative transcript abundance were detected for six of the 19 genes that also showed a good correlation in up- and down-regulation between the qPCR and the microarray results. The remaining genes did not show significant expression changes when measured by qPCR assays and were not differentially expressed in the microarray data. However, two orthologous ferric hydroxamate uptake (*fhu*) genes (*fhuB* and *fhuC*) appeared to be down-regulated in the microarray analysis; nevertheless, these changes were detected in only one of the biological replicates (Table 1).

Among the genes that showed a significant increase in their relative expression levels, we found genes encoding proteins of the cation efflux family (*czcD*, EF0859), a ferredoxin protein (EF1543) and two transcriptional regulators of the Fur family (PerR and ZurR) (see Table 1). Additionally, EF1057 and EF1058 showed a significant decrease in their expression. These two genes form a predicted operon (operondb.cbcb.umd.edu) that encodes a transporter and a universal stress protein, respectively.

Table 1 qPCR validation of microarray data

Locus gene	Gene	Protein	qPCR fold change[a]	Microarray change[b] (bio1)[c]	Microarray change[b] (bio2)[c]
Iron metabolism					
EF0188	*fhuD*	Dicitrate iron ABC transporter, substrate-binding protein	0.92 ± 0.3	–	–
EF0191	*fhuC*	Ferrichrome ABC transporter, ATP-binding protein	1.2 ± 0.3	Down	–
EF0192	*fhuB*	Ferrichrome ABC transporter, permease protein	–	Down	–
EF0193	*fhuG*	Ferrichrome ABC transporter, permease protein	0.78 ± 0.6	–	–
EF0475	*feoA*	Ferrous iron transport protein A	−0.07 ± 0.2	–	–
EF0476	*feoB*	Ferrous iron transport protein B	−0.01 ± 0.2	Down	Down
EF0859	*czcD*	Cation efflux family protein	5.4 ± 1.0*	Up	Up
EF1057	*mntH*	Mn2 +/Fe2 + transporter, NRAMP family	−49.7 ± 5.1*	Down	Down
EF1058	*uspA*	Universal stress protein family	−74.0 ± 13.3*	Down	Down
EF1543	*fer*	Ferredoxin	4.1 ± 1.8*	–	Up
EF1639	*hmuV*	Heme iron ABC transporter, ATP-binding protein	1.4 ± 1.1	–	–
EF1640	*hmuU*	Heme iron ABC transporter, permease protein	1.0 ± 0.9	–	–
EF1641	*hmuT*	Heme iron ABC transporter, iron compound-binding protein	1.6 ± 0.7	Up	–
EF3082	*ycIQ*	Hidroxamete-iron ABC transporter, substrate-binding protein	0.32 ± 0.2	–	–
EF3083	*ycIP*	Hidroxamate iron ABC transporter, ATP-binding protein	−1.1 ± 1.4	Down	–
EF3085	*ycIN*	Hidroxamete-iron ABC transporter, permease protein	0.11 ± 0.1	Down	–
Transcription factors					
EF1525	*fur*	Transcriptional regulator, Fur family	1.1 ± 1.1	–	–
EF1585	*perR*	Transcriptional regulator, Fur family	7.4 ± 1.6*	Up	–
EF2417	*zurR*	Transcriptional regulator, Fur family	3.8 ± 2.5*	Up	–

[a] qPCR data was expressed as fold-change for each gene expression level between treated and untreated cells. The values correspond to the mean and SD of triplicate determinations from two independent RNA samples. Asterisks indicate significant differences with respect to the control (*t*-student test, $P < 0.05$)

[b] Data was expressed as up- or down-regulated according to microarray data analysis (Details in Supplementary Table S2)

[c] Two biological replicates were independently hybridized onto microarrays and analyzed

Considering that iron excess is also responsible for the generation of a redox stress response (Cornelis et al. 2011), we evaluated if a supplement of 0.5 mM $FeCl_3$-NTA was enough to generate transcriptional changes in genes that encode proteins involved in stress protection. In order to measure oxidative stress generated by iron excess, we determined glutathione levels in *E. faecalis*. Our results indicated that after 6 h of iron exposure, cultures showed a significant decrease in the amount of total glutathione (Fig. 2a), which was associated with an increase in the relative abundance of transcripts encoding superoxide dismutase (*sodA*), catalase (*katA*), thioredoxin (*trx*), hydroperoxide resistance protein (*ohrA* and *ohrB*) and peptide methionine S-sulfoxide reductase (*msrA*) (Fig. 2b). Most of these transcriptional changes were also observed when cells were exposed to H_2O_2 (Fig. 2c), suggesting that in our experimental conditions the transcriptional response of *E. faecalis* to iron excess includes a general oxidative stress response.

Comparison of gene expression changes in *E. faecalis* exposed to iron and blood

Withholding iron from infecting bacteria is essential in host defense mechanisms, as well as virulence of many pathogens can be increased by injecting iron compounds into animal hosts (Ratledge and Dover 2000).

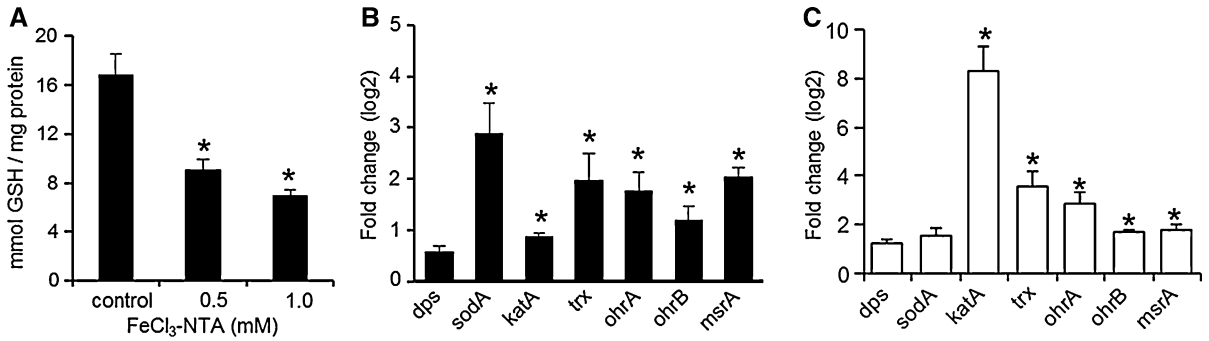

Fig. 2 Oxidative stress response of *E. faecalis* OG1RF. Total glutathione content (GSH) was determined in cells exposed to 0, 0.5 or 1.0 mM of $FeCl_3$-NTA for 6 h (**a**). qPCR analysis of the expression of genes *dps*: DNA-binding proteins from starved cells (EF3233); *sodA*: superoxide dismutase, Mn (EF0463); *katA*: catalase/peroxidase (EF1597); *trx*: thioredoxin (EF1405); *ohrA*: OsmC/Ohr family protein (EF0453); *ohrB*: OsmC/Ohr family protein (EF3201); *msrA*: peptide methionine sulfoxide reductase (EF1681) in *E. faecalis* treated with 0.5 mM iron ($FeCl_3$-NTA) for 6 h (**b**) or with 2.4 mM H_2O_2 for 45 min (**c**). *Data* are expressed as fold-change for each gene expression level between treated and untreated cells. The values correspond to the mean and SD of triplicate values of two independent RNA samples. *Asterisks* indicate significant differences with respect to the control (*t*-student test, $P < 0.05$)

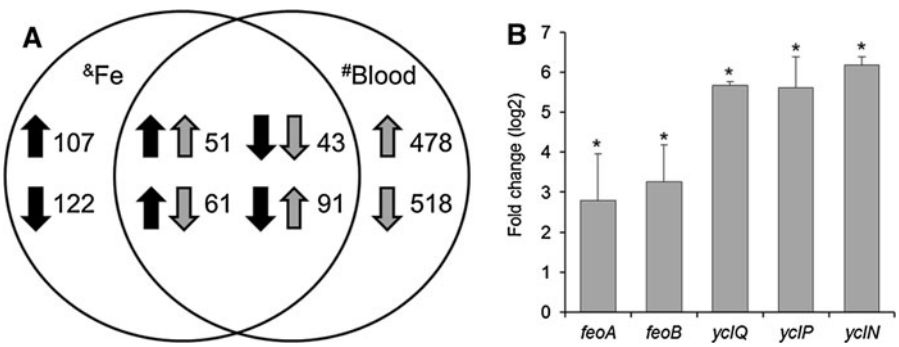

Fig. 3 Comparison of microarray gene expression data between *E. faecalis* exposed to iron and blood. **a** The numbers of common and unique differentially expressed genes are shown in the Venn diagram. *Black* or *grey arrows* indicate the microarray results (up- or down-regulation) for iron or blood, respectively. [&]The iron microarray data was generated in the present study. [#]The blood data and significance test analysis was directly obtained from Vebo et al. (2009). **b** qPCR analysis of the expression of the *feoAB* operon (EF0475 and EF0476) and *yclQPN* operon (EF3082, EF3083 and EF3085) in *E. faecalis* grown in media supplemented with 0.5 mM of 2,2-DPD (iron chelator) for 6 h. *Data* are expressed as fold-change for each gene expression level between treated and untreated cells. The values correspond to the mean and SD of triplicate values of two independent RNA samples. *Asterisks* indicate significant differences with respect to the control (*t*-student test, $P < 0.05$)

For this reason, it was of interest to compare the transcriptomic response of *E. faecalis* to iron against the transcriptional response of this bacterium cultured in blood. As abovementioned, *E. faecalis* is an agent of nosocomial infections, including bloodstream infections (Lyytikainen et al. 2002; Luzzaro et al. 2011). The transcriptomic analysis of *E. faecalis* V583 exposed to defibrinated horse blood has been recently reported (Vebo et al. 2009). The Venn diagram (Fig. 3a) shows overlapping of the two datasets. Results indicate that about 50% of the iron-responsive genes were also differentially expressed when *E. faecalis* was grown in blood. However, four times more genes were differentially expressed after blood exposure than after iron exposure, which might be explained by the complexity of the blood stimulus (Fig. 3a).

In this regard, the Mn^{2+}/Fe^{2+} transporter (EF1057) and the universal stress protein (EF1058) (involved in iron metabolism and stress response) were down-regulated in response to iron, but increased their expression when exposed to blood, while the cation efflux CzcD protein (EF0859) showed the inverse pattern (Table 2). In addition, the *feo* (EF0475,

EF0476) and *ycl* (EF3082, EF3083 and EF3085) operons, both up-regulated in blood cultured *E. faecalis*, were not affected by iron excess. Since both operons are involved in iron uptake, this observation suggested that blood might induce a transcriptional response that is equivalent to that of cells growing under limiting iron conditions. In order to evaluate this possibility, we used qPCR assays to measure changes in the expression levels of *feo* and *ycl* genes when *E. faecalis* was grown in a medium supplemented with 0.5 mM of the iron chelator 2,2-DPD. Our results indicated that after 6 h of exposure to 2,2-DPD, the intracellular iron content was reduced in 60% (data not shown), whereas both operons (*feo* and *ycl*) were in fact up-regulated (Fig. 3b).

Discussion

In the last 10 years, genomic (Paulsen et al. 2003; Domann et al. 2007; Bourgogne et al. 2008; Palmer et al. 2010) and functional genomics studies (Solheim et al. 2007; Vebo et al. 2009; Reyes-Jara et al. 2010; Coelho Abrantes et al. 2011) have contributed to turn *E. faecalis* into an important biological model to explore the role of transcriptional regulation during adaptation to metal availability fluctuations, including copper (Reyes-Jara et al. 2010; Coelho Abrantes et al. 2011), zinc and manganese (Coelho Abrantes et al. 2011). In the present study, we exposed *E. faecalis* to an excess of extracellular iron in order to complement the "omic" information of this bacterium by characterizing its transcriptomic response to metal ion stress.

Growth of *E. faecalis* under an iron excess (0.5 and 1 mM of $FeCl_3$-NTA) showed an increase of intracellular iron content without changes in cell viability, suggesting that under these conditions the bacterium was capable of adjusting its iron requirement and able to control the potential associated effects of iron overload (Bronstein et al. 2008). Several proteins involved in iron metabolism (Table 1) were up-regulated in iron-exposed *E. faecalis*. Among them

Table 2 Comparative analysis of data from *E. faecalis* growing in iron (qPCR Table 1) and blood [microarray data from Vebo et al. (2009) (colour online)]

Locus gene	Gene name	Gene description	Iron qPCR	Blood micro-array
Iron metabolism				
EF0475	*feoA*	ferrous iron transport protein A		
EF0476	*feoB*	ferrous iron transport protein B		
EF0859	*czcD*	cation efflux family protein		
EF1057	*mntH*	Mn2+/Fe2+ transporter, NRAMP family		
EF1058	*uspA*	universal stress protein family		
EF1543	*fer*	ferredoxin		
EF3082	*yclQ*	hidroxamete-iron ABC transporter, substrate-binding protein		
EF3083	*yclP*	hidroxamate iron ABC transporter, ATP-binding protein		
EF3085	*yclN*	hidroxamete-iron ABC transporter, permease protein		
EF1585	*perR*	transcriptional regulator, Fur family		
EF2417	*zurR*	transcriptional regulator, Fur family		
Stress response				
EF0453	*ohrA*	OsmC/Ohr family protein		
EF0463	*sodA*	superoxide dismutase, Mn		
EF1405	*trx*	thioredoxin		
EF1597	*katA*	catalase/peroxidase		
EF1681	*msrA*	peptide methionine sulfoxide reductase		
EF3201	*ohrB*	OsmC/Ohr family protein		
EF3233	*dps*	Dps family protein		

Genes in red were up- regulated, in green were down-regulated and in grey were unchanged

were the cation efflux system protein (*czcD*) and ferredoxin (EF1543). *czcD* was also reported as overexpressed in response to manganese (Coelho Abrantes et al. 2011), suggesting that CzcD enables metal extrusion under metal excess conditions. Moreover, an ortholog CzcD protein has been characterized in *Streptococcus pneumoniae* (Kloosterman et al. 2007) and its role in zinc resistance was confirmed through a mutation of *czcD*, whereas the expression of *czcD* was shown to be activated by zinc, cobalt and nickel. Ferredoxin (EF1543) is an iron-sulfur protein involved in electron transport that exhibits diverse functions in bacteria (Andrews 1998; Sevrioukova 2005), for example, synthesis of unsaturated fatty acids (Chazarreta-Cifre et al. 2011). In *Campylobacter jejuni*, ferredoxin is also an iron-induced protein and is involved in aerotolerance (van Vliet et al. 2001). We demonstrated that EF1543 was up-regulated by iron in *E. faecalis,* and in a previous study we showed that EF1543 was also up-regulated in response to copper (Reyes-Jara et al. 2010). Given that both iron and copper are redox active metals, we cannot discard that in *E. faecalis* ferredoxin might be involved in electron transference, as previously suggested for different bacterial species (van Vliet et al. 2001; Sevrioukova 2005; Chazarreta-Cifre et al. 2011).

Two transcriptional factors of the Fur family (ZurR and PerR) (Faulkner and Helmann 2010; Dowd et al. 2012; Shin et al. 2011) increased their expression after iron exposure (Table 1). In addition, the *perR* transcripts (EF1585) enhanced their abundance in cells exposed to 2.4 mM of H_2O_2 for 45 min (data not shown), suggesting that this response is a consequence of the oxidative stress induced by an excess of intracellular iron. However, data obtained from a functional screening of genes with a potential role in *E. faecalis* virulence showed that the absence of *perR* or *zurR* did not affect the sensitivity of *E. faecalis* towards hydrogen peroxide (Rigottier-Gois et al. 2011). Interestingly, the same report, showed a significant decrease of *E. faecalis* virulence in *perR* and *zurR* mutants (Rigottier-Gois et al. 2011). This supports a relationship between PerR and ZurR and their target genes with *E. faecalis* virulence capacity when iron availability is increased.

In contrast, EF1057 and EF1058, were significantly down-regulated in cells exposed to iron (Table 1), appearing to form a transcriptional unit. Both genes decreased their abundance when *E. faecalis* was exposed to a high or moderate extracellular concentration of copper (Reyes-Jara et al. 2010; Coelho Abrantes et al. 2011). EF1057 is a bacterial orthologous of a proton-dependent transporter Mn^{2+}/Fe^{2+} (MntH), also known as a member of NRAMP family. It has been demonstrated that its expression increases the sensitivity of *E. coli* to Cd, Co, and Fe (Richer et al. 2003). Thus, the decrease of *mntH* transcript levels in *E. faecalis* might decrease iron uptake capacity, which in return results in a reduction of iron cellular content in response to an increase of iron availability. The universal stress protein (EF1058) belongs to an highly conserved orthologous group of proteins named the UspA superfamily (Nachin et al. 2005). The *usp* genes were first reported in *E. coli*, where changes in *uspA* expression have been observed in response to a variety of different stress and nutritional conditions that promote or inhibit cell growth (Nachin et al. 2005). The role of this protein during iron exposure in *E. faecalis* requires further analysis.

In regards to genes involved in redox stress response, our analysis indicated that the transcriptional response of *E. faecalis* to iron excess involved genes encoding proteins with antioxidant activities such as superoxide dismutase (EF0463), catalase (EF1597), thioredoxin (EF1405), two members of organic hydroperoxide resistance proteins (EF0453 and EF3201), and methionine sulfoxide reductases (EF1681), which catalyzes the reduction of methionine sulfoxide residues in proteins. The activity of these proteins has been previously described in *E. faecalis* (Rince et al. 2001; Frankenberg et al. 2002; Verneuil et al. 2006; Zhao et al. 2010). Thus, up-regulation of stress genes in *E. faecalis* exposed to iron excess is consistent with our observation that its growth in 0.5 mM $FeCl_3$-NTA induces a stress condition, which was evidenced by changes in GSH contents (Fig. 2a). All these genes, with the exception of catalase, increased their abundance in *E. faecalis* exposed to copper excess (Reyes-Jara et al. 2010) and blood (Vebo et al. 2009), suggesting that oxidative stress resistance is crucial for *E. faecalis* survival in the host environment. Consistently, *E. faecalis* strains with mutations in the catalase gene showed no significant differences in H_2O_2 sensitivity when compared to wild type cells (Rigottier-Gois et al. 2011). In contrast, cells that failed to express EF1681 (Zhao et al. 2010) or EF0463 (Bizzini et al. 2009) were more sensitive than wild type cells to H_2O_2 exposure. These

results suggest that a fraction of the common transcriptional response to iron and blood is induced by stress signals (Table 2). It is possible that the stress response of blood exposed bacteria might be due to a secondary effect of iron deficiency, such as the impaired function of one or more metal dependent enzymes, which in turn might lead to the activation of stress responses. In fact, essential metals might induce cellular stress by deficit or excess (Hobman et al. 2007).

On the other hand, the analysis of genes associated to iron metabolism indicated that iron excess and blood induced divergent transcriptional changes in two predicted operons (Table 2), which include the genes *feo* and *ycl* that are involved in iron uptake in Enterobacterial species and *Bacillus subtilis* respectively (Cartron et al. 2006; Zawadzka et al. 2009). Both the predicted function of genes *feo* and *ycl* and their up-regulation in response to blood suggested us that blood-exposed *E. faecalis* might be sensing a deficit of iron. In order to explore this possibility, we grew *E. faecalis* in the presence of the iron chelating agent 2,2-DPD (Fig. 3b), and showed that iron deficit was able to induce the expression of *feo* and *ycl.* These results are consistent with previous reports that describe up-regulation of a system related to iron uptake (Baichoo et al. 2002; Klitgaard et al. 2010) and reveal some of the mechanisms employed by *E. faecalis* to face changes in iron availability.

In summary, *E. faecalis* growing in the presence of iron excess was capable of activating a transcriptional response to ensure iron homeostasis maintenance. Genes encoding proteins involved in iron transport changed their expression levels in order to enhance iron extrusion or reduce metal uptake. As expected, the oxidative stress generated by iron triggers the expression of genes encoding proteins involved in stress protection. Around 50% of the genes differentially expressed in response to iron also changed their expression levels in blood-exposed *E. faecalis*. Metal transporter-encoding genes showed an inverse gene expression pattern for iron and blood, whereas genes encoding proteins involved in stress protection were up-regulated during both stimuli.

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