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Interplay between copper and zinc homeostasis through the transcriptional regulator Zur in *Enterococcus faecalis*†

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By integrating the microarray expression data and a global *E. faecalis* transcriptional network we identified a sub-network activated by zinc and copper. Our analyses indicated that the transcriptional response of the bacterium to copper and zinc exposure involved the activation of two modules, module I that contains genes implicated in zinc homeostasis, including the Zur transcriptional repressor, and module II containing a set of genes associated with general stress response and basal metabolism. Bacterial exposure to zinc and copper led to the repression of the zinc uptake systems of module I. Upon deletion of Zur, exposure to different zinc and copper conditions induced complementary homeostatic mechanisms (ATPase efflux proteins) to control the intracellular concentrations of zinc. The transcriptional activation of zinc homeostasis genes by zinc and copper reveals a functional interplay between these two metals, in which exposure to copper also impacts on the zinc homeostasis. Finally, we present a new zinc homeostasis model in *E. faecalis*, positioning this bacterium as one of the most complete systems biology model in metals described to date.

Introduction

Zinc (Zn) is one of the most abundant transitional metals in the cell; it is a strong Lewis acid with no redox activity under physiological conditions and is considerably less toxic than redox active metals.[1] Zn functions include structural and catalytic roles in a large number of proteins such as RNA polymerase, superoxide dismutase, and Zn finger proteins.[2] However, Zn at high concentrations can act as a potent disruptor of the respiratory electron transport systems interrupting essential metabolic and cellular pathways.[3]

In bacteria, efforts have been made to identify mechanisms of resistance and pathogenesis related to Zn.[4] In this area, the transcription factor Zur has been described as one of the main components involved in the control of the expression of Zn homeostatic genes. This protein belongs to the Fur family (ferric uptake regulators),[5] operating as a Zn-dependent transcriptional repressor, which regulates high-affinity Zn uptake systems under conditions of Zn starvation in different types of bacteria.[6] While research of the Zur protein has led to important advances in Zn homeostasis, how fluctuations in bio-availability of this micronutrient are correlated with the response to other micronutrients, such as copper (Cu) and iron, or particularly the relationship of Zur with other metals are far from being understood.

Recently we reported the first global transcriptional regulatory network in the pathogen *Enterococcus faecalis*,[7] providing a new model and relevant data for understanding how a microorganism modulates gene transcription in the presence of different stimuli (mainly metals). In addition, genome-scale gene expression approaches led to the identification in *E. faecalis* of a set of genes differentially expressed by an excess of Zn,[8] some of them were also activated by Cu exposure. This result suggests the presence of transcriptional regulators that are able to respond to both Zn and Cu and reveals a putative interplay between these two metals at the transcriptional level.

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By integrating both sets of information, the microarray data and the transcriptional regulatory network, in the present work, we identified a putative transcriptional mechanism activated by Zn fluctuations that also responds to Cu exposure. The resulting Zn–Cu activated sub-network led to the identification of the transcription factor Zur as the principal regulatory protein able to respond to Zn and Cu stimuli in order to coordinate the expression of Zn homeostasis genes. This information provides evidence about the capacity of Cu to induce and affect the Zn homeostatic regulatory process, describing a regulatory interplay between these two metals through the transcription factor Zur in *E. faecalis*.

Results & discussion

Transcriptional regulatory sub-networks activated by Zn and Cu

The study of transcriptional regulatory networks permits the identification of proteins (transcriptional regulators) that directly or indirectly modulate gene transcription in response to different stimuli.[9,10] The functioning of the network integrates the operation of specific modules whose activation directly impacts the bacterial response to the stimuli.[7]

In this context, *E. faecalis* has become one of the most complete metal-metabolism models available today.[7,8,11–14] As mentioned, we built a global transcriptional regulatory network in this bacterium that allowed us to integrate and describe different global gene expression data. By using the same strategy employed in the identification of sub-networks activated by Cu fluctuations,[7] the microarray expression data for *E. faecalis* exposed to 4 mM $ZnSO_4$[8] (NCBI-GEO database GSE30947) were combined with the global network model in order to predict the transcriptional regulatory sub-network activated by Zn in *E. faecalis* (Fig. 1).

The sub-network is composed of a total of 33 operons connected by eight putative transcriptional factor families. Topological analysis indicated that the in-degree coefficient followed the classical power law distribution, with γ equal to 2.72 (similar to the global network 3.17).[6,7] In terms of connectivity patterns, the network contains auto-regulatory systems (n = 4), chain regulation (n = 5), single input motifs (n = 5) and feed-forward loops (n = 3). Most of these patterns were generated by the global transcription factors ArgR (arginine metabolism)[15] and LysR (general metabolism),[16] which also connect the largest number of operons within the network. The structure shown by the sub-network activated by Zn has the same classical topological features described in other bacterial network models,[17,18] suggesting that this is an effective and reliable model to understand the transcriptional mechanism activated by Zn in *E. faecalis*.

The topology analysis and coverage percentage (11% in relation to the global network) have similar features and values to those obtained previously for the *E. faecalis* network activated by a high concentration of Cu (0.5 mM $CuSO_4$).[7] The results indicate that Zn can be classified as a complex stimulus, since an elevated number of operons encoding proteins involved in different metabolic processes are transcriptionally responding to the same metal treatment.

In Fig. 1 we also describe the response of the Zn sub-network components to the exposure of Cu (0.5 mM $CuSO_4$). As a first step, we assessed how specific is the response to Zn and Cu. The components of the Zn sub-network can be classified into: (i) down- or up-regulated specifically in response to Zn (17 operons)

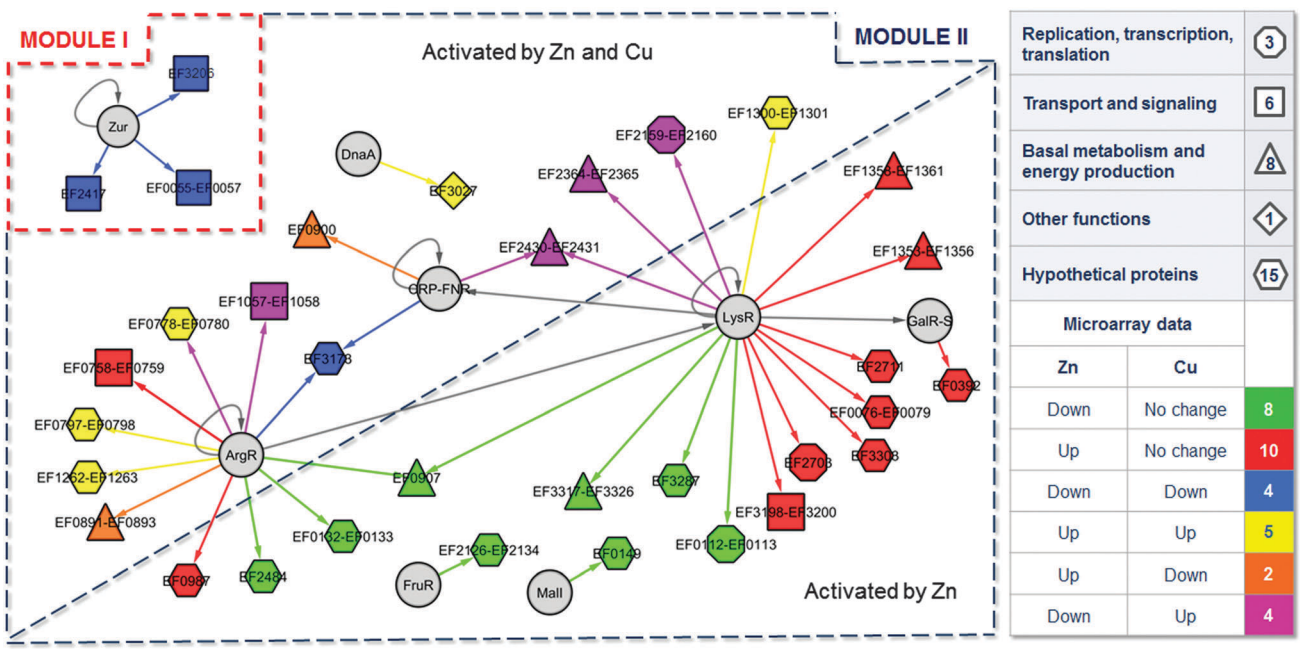

Fig. 1 Transcriptional regulatory network activated by Zn and Cu. The graph shows the transcriptional regulators affected by Zn and activated by Cu exposure from microarray data. The graph contains 40 nodes (99 genes in total) connected by 43 edges (putative binding sites). Grey circles denote transcriptional factor families. Node shape represents COG class classification. Numbers indicate the total elements in each group.

or (ii) modulated in response to Zn and Cu (15 operons). The reported sub-network activated by Cu showed that[7] more than 80% of the active operons are induced only by Cu and not by other metals. In contrast, a high number of operons activated by Zn are also induced by Cu (more than 50%), a result that is consistent with the higher requirement of Zn in different metabolic processes compared to Cu.

The second analysis sought to describe the specific metabolic processes represented in the sub-network.[7,19] In this context, we distinguished two modules: module I was isolated from the rest of the network and it was down-regulated by both metals. This module corresponds to the Zur regulon composed only of genes with functions in Zn homeostasis, the transcription factor Zur and Zn uptake transporters AdcABC and AdcA-II. Module II contains components involved in energy generation, synthesis of basic molecules and cellular damage; they are regulated by the global transcription factors LysR, ArgR and CRP-FNR. This module can also be divided according to the specificity in the response to Zn: (a) down- or up-regulated specifically in response to Zn or (b) modulated in response to both metals. Module II was the most represented in the network, connecting more than 90% of the operons and thus, describing complex regulatory events, as reflected in the up- and down-regulation of genes contained in it. In particular, the transcription factor DnaA (replication-initiator)[20] is up-regulated by both treatments. The induction of this gene not only denotes an active control of the transcriptional activity, but also determines multiple regulatory systems, which include the time of initiation of the replication phase and cell growth.[21,22] Interestingly, the isolated module I (Zur regulon) seems to independently control Zn homeostasis in *E. faecalis*, nevertheless this module was able to respond to both, Zn and Cu, revealing a particular transcriptional behavior, which may significantly impact on Zn homeostasis in the bacterium. To address this hypothesis our next steps were to further characterize the transcriptional regulator Zur and examine the transcriptional response of Zur regulon (*zur*, *adcABC* and *adcA-II* operons) under different Zn and Cu treatments.

Bioinformatic characterization of Zur regulon

Members of the ferric uptake regulator family (Fur) are some of the main transcriptional factors capable of sensing changes in the availability of metals in bacteria to regulate the expression of genes encoding proteins with relevant roles in metal homeostasis.[23] Although the *E. faecalis* genome annotation denotes that the EF2417 gene codes for Zur, there are high levels of sequence similarity between this protein with other Fur family members encoded in this bacterium genome.[13] Therefore, we perform several bioinformatics analyses to identify the specific features of Zur, known to be present in this regulator, but are absent in other members of the Fur family (Fig. 2).

The protein global alignment showed high sequence conservation (more than 60% similarity) among Zur archetypes present in other bacteria, including the three Zn binding site motifs described as important for structural stability and DNA-binding activity.[24,25] The *in silico* tridimensional structure modeling of *E. faecalis* Zur showed a coherent putative tertiary protein folding with the Zur crystal from *Streptomyces coelicolor* (over 50% of structure homology).[25] Finally, the conserved position of the three classical Zn coordination motif (absent in other Fur family members like Fur and Per) strongly suggests

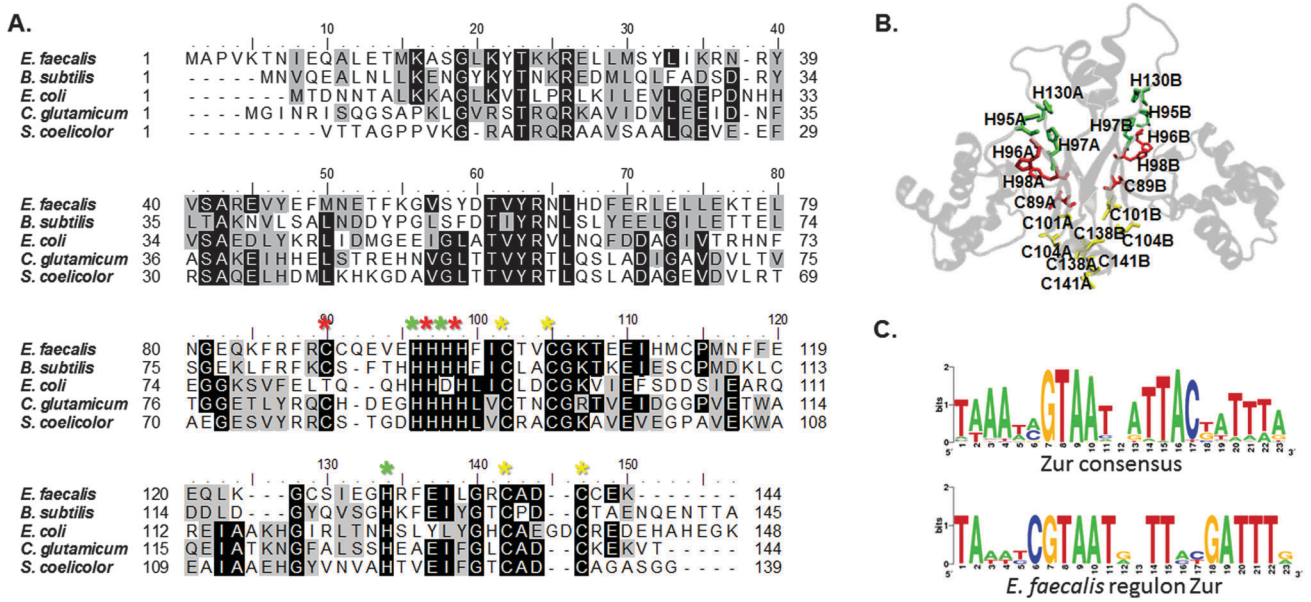

Fig. 2 Bioinformatic characterization of the Zur protein. (A) Sequence alignments among *E. faecalis* Zur and Zur archetypes from different bacterial species. Stars indicate conserved residues involved in Zn binding, black: identical residues, and grey: similar residues. (B) Molecular 3D projection of the Zur protein dimer. The model was generated using automatic sequence comparison (template PDB id: *S. coelicolor* 3MWM). Secondary structure in grey. (C) Sequence matrix logo of different Zur DNA binding sites (consensus) and the 3 putative binding sites found in the promoters of EF0055-57, EF3206 and EF2417 genes.

that EF2417 codes for the transcription factor Zur in *E. faecalis* (Fig. 2).

We then analyzed whether Zur regulates its expression and the expression of the two *adc* operons, EF0055-57(*adcABC*) and EF3206 (*adcA-II*), by searching putative Zur binding motifs within the promoters of the genes that conform the predicted Zur regulon. Using a conserved 23-bp palindrome (n**AAA**nn**GTAA** nnn**TTAC**nn**TTT**n)[23] of the Zur DNA binding-site, the following sequences were identified: T**AAA**CC**GTAA**TAT**TTAC**GA**TTT**G, T**AAA**TC**GTAA**TGG**TTAC**GA**TTT**G and TATTTC**GTAA**TGA**TT**CTG A**TTT**A within the promoters of EF0055-57, EF3206 and EF2417 (*zur*) operons, suggesting a transcriptional auto-regulatory feedback loop. Thus, Zur seems to control the expression of genes *adcABC* and *adcA-II*, which encode for Adc transporters; ABC systems required Zn uptake in several bacterial species.[26] Importantly, similar Zn binding motifs and high sequence identity were predicted among *E. faecalis* Adc systems and other previously characterized Adc transporters (Fig. S1, ESI†), supporting a similar role in Zn homeostasis.

Previous work in *Escherichia coli* showed that Zur repressed the expression of the uptake systems ZnuABC and ZinT, which are activated in response to Zn abundance.[27] On the other hand, AdcABC and AdcA-II components were previously reported in other species as transcriptional targets of the regulator AdcR.[28] After BLASTP search using as templates different AdcR, ZinT and ZnuABC sequences described in other bacteria (Table S1, ESI†), putative homologs of all of these proteins were not found in the *E. faecalis* genome, ruling out the possibility that these components were transcriptionally controlled by Zur in *E. faecalis* or participate in Zn homeostasis.

These results not only support the initial transcriptional prediction used in the construction of the global network, but also predict the presence in *E. faecalis* of an unreported Zur regulon, in which the Zn uptake system AdcABC and AdcA-II components are transcriptionally controlled by this regulator.

To study the capacity of module I to respond to Zn and Cu, the next step was to quantify the relative gene expression changes in its components under different scenarios of Zn bioavailability and Cu exposure.

Zn and Cu exposure affects Zn homeostasis and expression of Zur regulon

Currently, no data have been published characterizing the Zn homeostasis response in *E. faecalis*, thus we first sought to evaluate the physiological response of this bacterium towards changes in Zn bioavailability and Cu exposure. *E. faecalis* growth was affected by concentrations higher than 5 μM TPEN (a membrane-permeable Zn chelator) and 0.5 mM Cu. However the cell viability was unaffected by Zn concentrations up to 3 mM (Fig. S2, ESI†). Regarding the internal metal concentrations, cells doubled their Zn content after three hours of exposure to 0.5 mM Zn and decreased their Zn content by 40% after exposure to 5 μM TPEN compared to control cells without treatment (Fig. 3C). These results showed that a deficit in Zn reduced the intracellular metal concentration and impacted on the cellular growth (Fig. S2B, ESI†), an observation that confirms previous reports on other pathogenic bacteria.[27,29–31] In contrast, the increment in the Zn content did not affect the bacterial growth (Fig. S2A, ESI†), suggesting that the bacterium was able to manage a two-fold increase in the intracellular Zn content, a phenotype also reported in other organisms.[32] Regarding copper treatment, significant differences were observed after three hours of Cu supplementation (cell viability was affected in *E. faecalis* treated with >1 mM $CuSO_4$, Fig. S2C, ESI†). The detrimental effect of a high extracellular Cu concentration can be explained by a possible toxic effect (free-radical stress) induced by the metal at high concentrations.[11] We have reported earlier that WT cells exposed to 0.5 mM $CuSO_4$ increase their cellular metal content by 8-fold, when compared to cells grown without Cu.[11] In this work, we showed that cell exposure to 0.5 mM Cu results in low but a

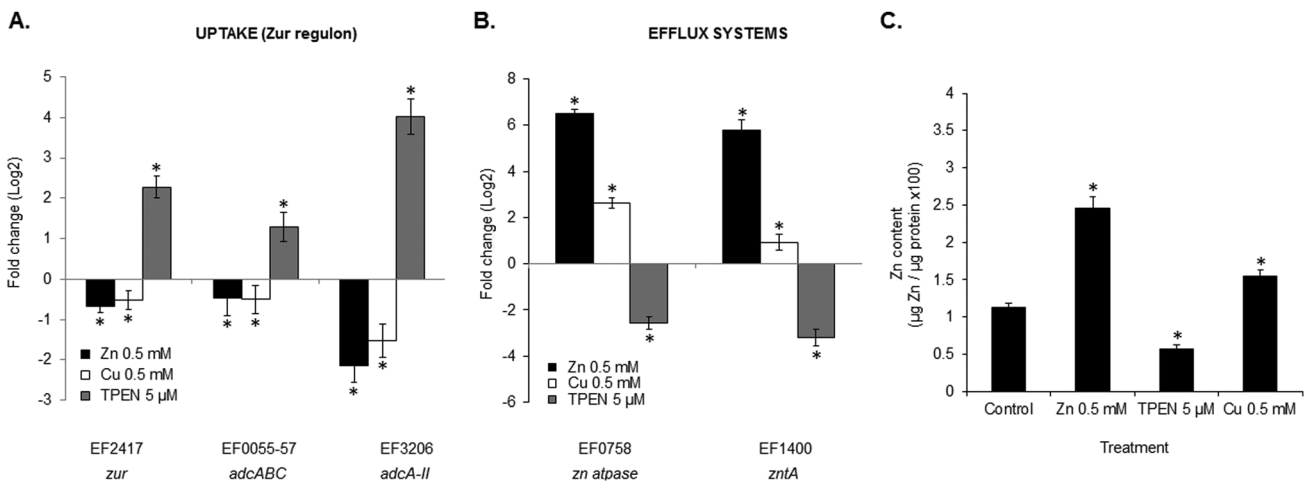

Fig. 3 Relative abundance of transcripts from Zn uptake and efflux genes in the WT strain exposed to Zn, TPEN and Cu. (A) Uptake system (Zur regulon), (B) efflux system. Transcript abundances were quantified by qPCR and expressed as the fold change (log_2) between treated and control cDNA samples. (C) Intracellular metal content was determined in WT cells. The internal Zn concentration was quantified after 3 h of exposure. Asterisks = significant differences (REST test, $p < 0.05$).

significant increase in the Zn content by 0.35-fold (Fig. 3C), without consequences on cell growth (Fig. S2C, ESI†). Therefore, the increment of Zn seems to be a secondary effect of the intracellular Cu increase, which probably affects the normal efflux rate of Zn, an aspect that requires further analysis. Taken together, these results allowed us to characterize the Zn homeostatic response of *E. faecalis* against fluctuation of Zn and Cu, and suggest the possibility that different intracellular Zn concentrations can differentially activate Zn homeostatic genes, mainly the Zur target genes involved in the uptake of the metal.

Our next step was determining changes in mRNA abundance of the genes that form the predicted Zur regulon under different conditions of Zn exposure (Fig. 3A). The extracellular concentrations of Cu (0.5 mM), Zn (0.5 mM) and TPEN (5 μM) were selected considering that they elicited a significant change in the intracellular Zn content without affecting the bacterial growth. The results of using real time PCR (qPCR) showed that relative expression levels of the uptake system (*adcABC* and *adcA-II* encoded by EF0055-EF0057 and EF3206 operons, respectively) decreased under Zn and Cu treatment. In contrast, TPEN exposure increased the RNA abundance of these components. Therefore, changes in the transcriptional profiles of the Zn uptake systems were directly correlated with the increase or decrease in the internal Zn concentration induced by the treatment (Fig. 3C). In our experimental conditions, the Zur–Zn complex can repress the Zn uptake system as a mechanism of defense avoiding the overload of Zn in cells exposed to the metals, a function previously proposed for *Bacillus subtilis* Zur regulon.[33] These results support the idea that under Cu treatment *E. faecalis* was able to coordinate a transcriptional response that mimicked the response to increased intracellular concentration of Zn. As expected, TPEN in turn decreases the internal concentration of the metal (Fig. 3C). In Zn deficiency, the three target genes of Zur (entire module I) were induced, probably by a de-repression mechanism dependence of Zur, as previously described in other bacteria.[25,33]

However, we cannot disregard that additional proteins play a role in controlling the intracellular concentration of Zn. To examine this possibility, we performed a BLASTP bioinformatic strategy to identify, in the *E. faecalis* genome, genes encoding additional Zn homeostasis proteins previously described in other bacteria (Table S1, ESI†). We found two homologs of ZntA ATPase type P (EF0758 and EF1400) and one homolog of Fief (CDF, EF0859) proteins; all of them showed in their primary protein structure, characteristic functional amino acid motifs described in other Zn efflux pump proteins directly involved in the metal transport[34,35] (Fig. S1, ESI†).

Using the strategy described above, we did not identify putative Zur binding motifs in the promoter of these genes. Consistently, no previous reports indicate that these components are regulated by Zur in other bacteria, suggesting that they do not belong to the Zur regulon, however they still may be a part of the Zn homeostasis mechanism of *E. faecalis*.

Our results showed that during Zn and Cu exposure both putative ATPases significantly increased their transcriptional levels (Fig. 3B), supporting a function in the Zn efflux that is activated when the internal Zn concentration increases.[36] During deprivation of Zn (TPEN condition), this system responded as a compensatory mechanism reducing its transcript levels, possibly decreasing the efflux of Zn to avoid the loss of this metal.

The transcriptional activation of both *zntA* genes during Zn and Cu exposure indicates the presence of a second transcriptional factor capable of translating both stimuli and affecting the Zn homeostasis mechanism. In a recent work,[37] Abrantes *et al.* described a DNA-motif called zim present in the promoter region of EF1400 and EF0759 (SapB, contributed to the intramacrophage survival, which shares the same operon with EF0758). While, the zim motif showed an active response against different Zn treatments, the authors declared that it was impossible to identify the transcriptional factor able to recognize this sequence. In this context, knowing that in several bacterial species the regulator ZntR controls the expression of *zntA*,[38,39] we performed a BLASTP bioinformatics analysis, but we were unable to find a homolog of this transcriptional regulator in the *E. faecalis* genome, suggesting that this bacterium apparently controls Zn homeostasis throughout an unusual non-classical transcriptional regulator, corroborating the previous findings.[37] Promoter specific analyses are currently underway to identify a putative transcription factor for *zntA* genes to place this system into the network. Regarding Fief efflux pumps (Fig. S3, ESI†), the absence of transcriptional changes in the *fief* gene under the conditions tested here may be explained by assuming that CDF family proteins participate as a secondary efflux system during the extremely toxic Zn exposure concentration, when the cell viability is affected, as demonstrated in other bacteria.[34]

The transcriptional changes induced by the different experimental treatments (Zn, Cu and TPEN) are directly correlated with fluctuations in the internal Zn concentration. Moreover, the common phenotype observed under Cu and Zn exposure denotes a regulatory interplay between both metals. Previous reports on *Pseudomonas protegens* and *Corynebacterium glutamicum*[40,41] indicate that changes in Zn bioavailability induce the activation of a mechanism involved in Cu homeostasis, reinforcing the idea of a transcriptional interplay between the homeostasis of Zn and Cu and supporting the results observed in *E. faecalis*.

Into the sub-network activated by Zn and Cu, Zur seems to regulate the expression of components involved in the uptake of Zn (module I) in response to different intracellular Zn changes. In order to analyze the implication of the control of Zur over the Zn uptake systems, we removed Zur from module I, with the aim of understanding the importance of this regulator in Zn homeostasis in *E. faecalis*.

Effects of the absence of Zur transcriptional control on Zn uptake system expression and metal homeostasis

Analysis by qPCR of the *zur* null deletion mutant (Δ*zur*) indicated that the transcript abundance of *adcABC* and *adcA-II* operons increased in the Δ*zur* strain compared to the wild type strain (WT) growing in control media (Fig. 4A). This result is in line with the predicted role of Zur as a transcriptional

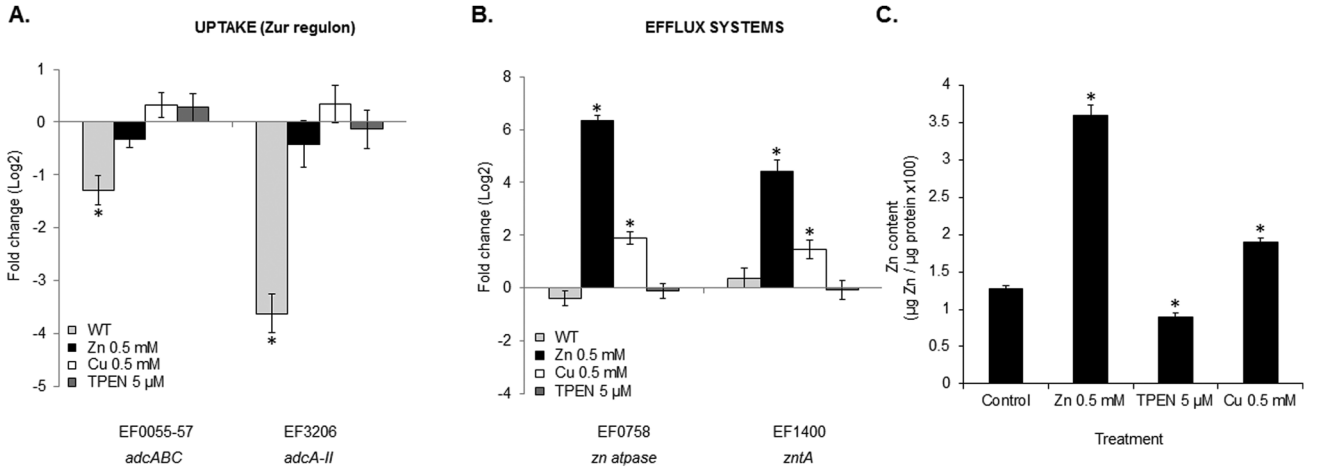

Fig. 4 Relative abundance of transcripts from Zn uptake and efflux genes in the Δ*zur* strain exposed to Zn, TPEN and Cu. (A) Uptake system, (B) efflux system. Light grey bars indicate the fold change (log_2) in transcript abundance between WT and Δ*zur* strains growing in the control media. Black, white and dark grey bars indicate fold changes in transcript abundance between the Δ*zur* strain exposed to 0.5 mM $ZnCl_2$, 5 µM TPEN or 0.5 mM $CuSO_4$ and the Δ*zur* strain growing in control media. (C) Intracellular metal contents measured in the Δ*zur* strain. Internal Zn concentrations were quantified after 3 h of exposure. Asterisks = significant differences (REST test, $p < 0.05$).

repressor of Zn uptake systems[42] and corroborates the *in silico* prediction of the *E. faecalis* network. The other experimental treatments (Zn, Cu and TPEN) did not induce transcriptional changes in the Zur regulon genes in the mutant strain, strongly suggesting that the control of the uptake system is regulated only by Zur, without the presence of a second transcriptional factor which is able to respond to Zn, Cu or TPEN at the concentrations used in this study. On the other hand, in the absence of metal treatment (control media), no differences were observed in the transcript abundance of *zntA* genes between Δ*zur* and the WT strain (Fig. 4B), supporting the fact that Zur was not regulating the Zn efflux components. The transcriptional induction observed at 0.5 mM Zn in the mutant is comparable to the change in abundance obtained in the WT exposed to the same concentration; a similar phenotype was detected during the exposure of 0.5 mM Cu in both strains. However, the decrease in transcript abundance observed in *zntA* genes when the WT bacterium is exposed to TPEN was not observed in the Δ*zur* strain. According to the hypothesis that changes in the internal Zn concentration are directly impacting on the transcriptional activation of the homeostasis systems, these differences in the expression of *zntA* genes between the Δ*zur* and the WT can be explained by differences in the internal Zn concentration in both strains under the same metal treatments. To address this assumption, Fig. 4C shows the Zn content in the WT and Δ*zur* strains over all the experimental conditions analyzed.

The absence of Zur did not generate a significant change in the internal Zn concentration when *E. faecalis* is growing in the control media, which is correlated with no changes in the expression of *zntA* genes. When the bacteria was exposed to 0.5 mM Zn, the intracellular metal content increased in both strains (Fig. 3C and 4C), however, in Δ*zur* the Zn content increased significantly more than in the WT strain (almost a 35% increase). This internal Zn increment in the mutant can be explained by the constant expression level of the uptake mechanism (module I) generated by the absence of the repressor Zur, which also explains the increase in the mRNA abundance of *adc* genes observed in Δ*zur* during the Cu and TPEN treatment compared with the WT strain under the same conditions.

In terms of the transcriptional activation of *zntA* genes, while in Δ*zur* occurs a significant increase in the internal Zn concentration during the exposure to this metal (Fig. 4C), this increment does not generate a difference in the mRNA abundance between the mutant and the WT (Fig. 3B and 4B). This result suggests that the internal Zn concentration achieved in the WT during the exposure to 0.5 mM Zn (more than 2 times compared with the control, Fig. 3C and 4C) already exceeds the threshold of maximum transcriptional induction of the efflux components, therefore any increment above this internal Zn concentration (as in the Δ*zur* strain) will not increase the mRNA abundance of *zntA* genes. As mentioned, during Cu exposure, there is a small increase in the concentration of Zn in the mutant strain in comparison to WT (18% increase), which can also be explained by the induction of *zntA* genes.

Treatment with TPEN in the Δ*zur* strain did not induce the same decrease in the internal Zn concentration (from 1.26 ± 0.04 to 0.89 ± 0.05; Fig. 4C) as observed in the WT (from 1.12 ± 0.06 to 0.57 ± 0.04; Fig. 3C), probably due to the absence of Zur, since in the Δ*zur* strain the expression of uptake systems is up-regulated in both conditions: exposed and not exposed to TPEN (Fig. 3B). In this context, the cellular Zn content and the cell growth were determined in the WT and Δ*zur* exposed to different TPEN concentrations (Fig. 5). At 10 µM TPEN the mutant strain grew faster than WT cells (Fig. 5B), which was correlated with a higher intracellular Zn content (Fig. 5A), a phenotype directly related to the constant activation of module I, which is responsible for Zn uptake (Adc systems).

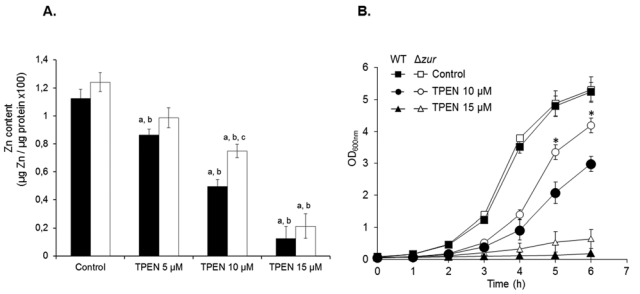

Fig. 5 Effect of TPEN supplementation on the Zn cellular content and growth in *E. faecalis* WT and Δ*zur*. (A) Cellular content of Zn measured after 3 h of metal exposure. Black bars indicate WT and, white bars Δ*zur* strains. a and b denote the significant difference between Zn contents of TPEN-treated cells and WT or Δ*zur* cells grown in control medium; c = significant differences in Zn contents of the Δ*zur* strain with respect to the WT strain. (B) Growth curves at an increasing concentration of TPEN. Asterisk = significant differences between WT and Δ*zur* strains at 10 μM TPEN. Error bars = standard deviation (SD) values. (Mann–Whitney test, $p < 0.05$).

Conclusion

In the last decade, our understanding about transcriptional regulatory networks has contributed to important advances in the systems biology field.[43] In this work we presented a Zn and Cu activation model that describes a specific and common transcriptional mechanism capable of responding to both metals. The activated network showed that response to Zn can be classified as a complex perturbation and can be divided into two specific modules that are comprised of genes with predicted functions in Zn homeostasis (module I, Zur regulon) and basal metabolism (module II). This response was similar to that observed previously when the bacterium was exposed to the same concentration of Cu.[7]

Unlike the *E. faecalis* Cu homeostasis systems (*cop* genes), which are strictly activated by Cu and no other metals,[11] module I encoding the Zn uptake system was able to respond to Zn and Cu fluctuations, suggesting the importance of Zn during Cu exposure. In terms of the specificity of response, *E. faecalis* exposed to iron and blood (iron-like deficient scenario) can also transcriptionally activate the Zur regulon (microarray data).[13,44] As one of the principal co-factors in the cell, Zn can be utilized by different metabolic processes, mostly related to the activation of transcriptional mechanisms and oxidative stress response,[45] two processes that are highly required during Cu and iron exposure.

Taking advantage of the *E. faecalis* gene regulatory network, our approach allowed us to identify the transcription factor Zur as one of the primary regulators activated by Zn and Cu. In previous work,[42,46] this protein had been described as a repressor of processes involved in Zn uptake during deprivation of this metal. Here, we contribute to its characterization, adding an important new capacity, namely its ability to response to Cu.

Through the construction of a Δ*zur* mutant not only the bioinformatics prediction of Zur regulon in *E. faecalis* was confirmed, but it helped us to study the impact of this regulator over Zn homeostasis and Cu response. However, more analysis is needed to make an accurate interpretation about the mechanism of how Cu can interfere with the DNA binding capacity of Zur.

Finally, we present a new Zn homeostasis model in *E. faecalis*, adding to the current knowledge in terms of Cu and iron homeostasis and positioning this bacterium as one of the most complete cellular metal models described. This bacterium may be an excellent alternative to understand how cells can adapt to the presence of transcriptional factors that are able to connect different stimuli. It is important to declare that the response of *E. faecalis* to different metal scenarios and the corresponding interpretations are limited to the concentrations used during the experiments. In this context, further experiments are underway to assess the effect of Cu addition in cells facing the metal deficiency condition produced by TPEN. They will improve our understanding of *E. faecalis* metal response and the participation of Zur regulon in this homeostatic process. Furthermore, our data also provide potential insights in terms of pathogenesis of *E. faecalis*. Zn is known to play an important role in bacterial infection;[47] the Zn homeostatic genes studied in this work could become a target for new drugs.

Materials & methods

Bioinformatics

Cu and Zn microarray data were collected directly from the NCBI-GEO database (accession numbers GSE20453 and GSE30947).[8,11] The global transcriptional regulatory network model of *E. faecalis* (EfaecalisGTN.gbk file) was obtained from Latorre *et al.*[7] Crossing information between the microarray data, transcriptional network model and graphic displays was performed using Cytoscape software.[48] Network topology analyses were performed using R software using the iGraph package.

Search and verification of Zn homeostasis components in *E. faecalis* was performed using BLASTP,[49] using sequences of proteins described in other bacterial species (Table S1, ESI†) and the entire NCBI *E. faecalis* V583 genome.[50] Global protein alignments were performed using ClustalW.[51] The efZur 3D molecular model was generated using SWISS-MODEL (PDB Id. 3MWM)[25] and displayed using VMD v1.8.6 software.[52,53] Binding site logos were made using the WebLogo application.[54]

Deletion of *zur*

E. faecalis OG1RF Δ*zur* strain was constructed using the PheS* system, resulting in a non-polar deletion mutant.[55] Briefly, fragments of *ca.* 900 bp located downstream and upstream of the zur target gene (NCBI id EF2417) were amplified by PCR using the primers shown (Table S2, ESI†). The resulting amplicon was first cloned in pGEM-T Easy (Promega) and then assembled in the pCJK47 vector. *E. coli* JM109 was used for cloning the first resultant vector and Ec1000 for the pCJK47 final construct. The final construct was transferred to *E. faecalis* CK111 by electroporation and finally to *E. faecalis* OG1RF by conjugation (single cross-over insertions). Gentamicin was added at 150 μg ml^{-1} for *E. faecalis* and 25 μg ml^{-1} for *E. coli* to select positive transformants. The loss of the plasmid was then selected using MM9YEG agar

medium supplemented with 10 mM p-Cl-Phe and 200 μg ml^{-1} X-gal. The possible mutants were first screened by PCR, then the junction area was sequenced, and the strain background was confirmed by pulsed field gel electrophoresis.

Bacterial strains and growth conditions

E. faecalis OG1RF WT and *E. faecalis* OG1RF Δ*zur* strains were grown in N media (Peptone 1%, yeast extract 0.5%, Na_2HPO_4 1%, glucose 1%)[56] containing 3.52 μM Zn as a baseline concentration. All experiments involving bacterial growth were performed by pre-culturing WT and Δ*zur* strains overnight in N medium at 37 °C and 140 rpm. The next day, the culture was refreshed by diluting it 1/10 in N-media and letting it grow under the same conditions for 2 more hours, then inoculating 50 ml of N medium, adjusting the initial concentration to an OD_{600nm} of 0.05 and growing it at 37 °C and 140 rpm, at this point: (i) Zn excess conditions were achieved by the addition of 0.5, 1 or 3 mM $ZnCl_2$ (Sigma) to the N medium and (ii) for Zn limiting conditions, cells were grown in the presence of 5, 10 or 15 μM chelating agent *N*,*N*,*N*′,*N*-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN). Treatment with Cu (0.5 mM, 1 mM or 3 mM $CuSO_4$) was realized as has been previously reported.[11] For the growth curves the OD_{600nm} was registered every hour for six hours.

Measurement of Zn content

To determine the Zn content, the culture was initiated with N medium broth supplemented with 0.5 mM $ZnCl_2$, 15 μM TPEN or 0.5 mM $CuSO_4$, including an untreated control (N media). After 3 h of incubation, at the mid-log phase, OD_{600nm} = 1.0, 6 ml of culture was taken. The cells were collected by centrifugation and washed sequentially with phosphate buffered saline (PBS) (136 mM NaCl; 2.7 mM KCl; 8.1 mM Na_2HPO_4; 1.5 mM KH_2PO_4; pH 7.4), 0.15 M NaCl, 1 mM EDTA and finally PBS. The cells were suspended in 1 ml of PBS and were disrupted by sonication; supernatants and cell debris were separated by ultracentrifugation at 14 000 rpm for 30 min. 100 μl of the culture supernatant was treated with concentrated nitric acid (1 : 2.5) and incubated for 24 h at 65 °C. The Zn content was determined in triplicate by atomic absorption spectrometry (AAS) as previously described.[11] Protein concentration of the supernatants was measured by the Bradford assay[57] to be used for normalization.

Quantitative PCR

Total RNA extraction and cDNA synthesis were performed as previously described.[11] *E. faecalis* WT and Δ*zur* strains, untreated (N media only) or exposed to 0.5 mM $ZnCl_2$, 15 μM TPEN or 0.5 mM $CuSO_4$, were grown for 3 hours, upon reaching the mid-log phase (OD_{600} = 1), 6 ml of culture was collected by centrifugation and washed with PBS three times for subsequent RNA extraction. The quantitative PCR and data analysis (qPCR) was performed using the real-time PCR system, LightCycler™ Roche. PCR primers were designed using the software Primer3Plus[58] using the *E. faecalis* V583 genome sequence as a template[50] (Table S2, ESI†). 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,[59] using *gdh* (EF1004) as a reference.[60] The results were expressed as the fold change (log_2) between treated and untreated cultures.

Statistical analyses

Data are expressed as mean value ± SE of at least three independent experiments. Statistical comparisons between different groups were conducted using the Mann–Whitney test. For qPCR assays, significant differences in fold-change values were assessed by the REST 2009 algorithm.[61] Differences of $p < 0.05$ were considered statistically significant.

Conflicts of interest

All the authors of this work declare that they have no conflict of interest.

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