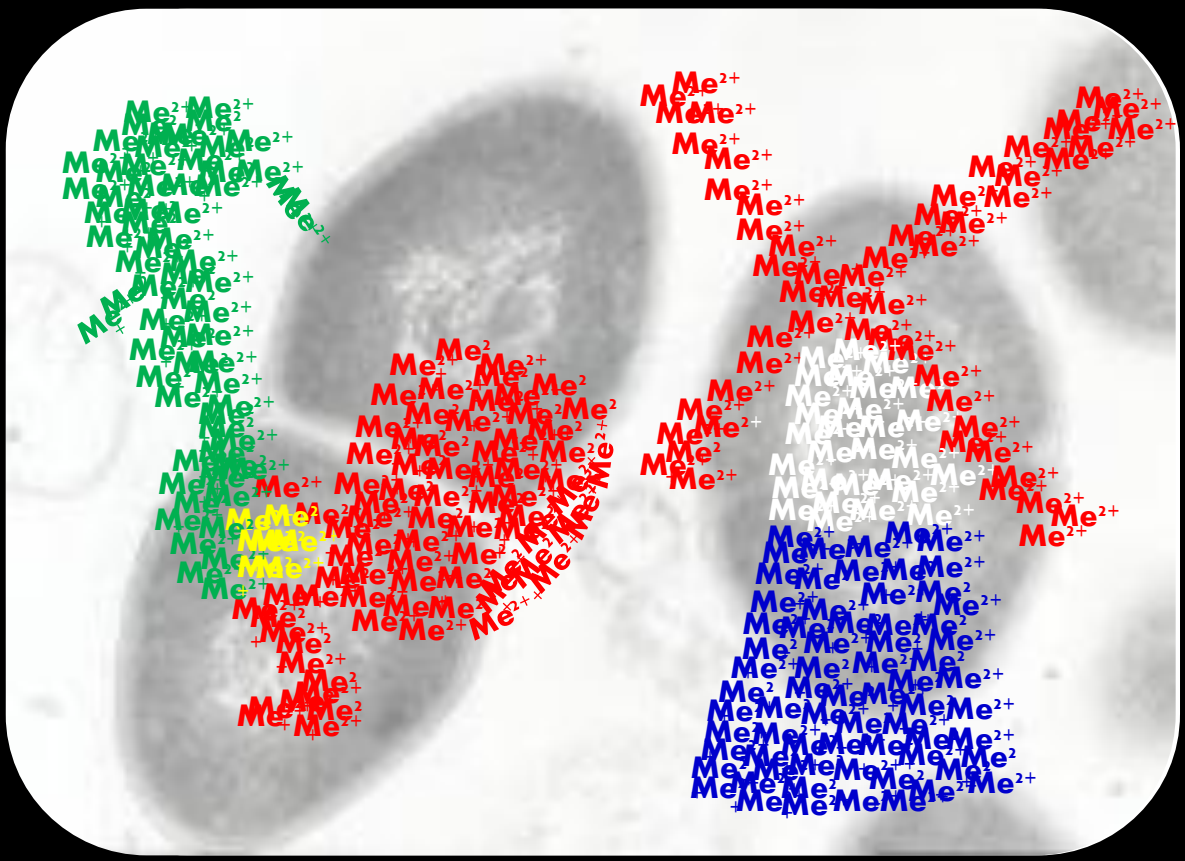
Precious transition metals: The importance of Zn^{2+}, Mn^{2+} and Cu^{2+} in the human pathogen *Enterococcus faecalis*

Marta Coelho Abrantes



Dissertation presented to obtain the Ph.D degree in Biology

Instituto de Tecnologia Química e Biológica | Universidade Nova de Lisboa

Oeiras,
July, 2012

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Marta Maria Coelho dos Santos Abrantes

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“It is not the strongest of the species that survives, nor the most intelligent, but the one most responsive to change”

Charles Darwin

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Welcome to what is generally the most read part of a thesis!

This was the most difficult part to write, as it takes me “on a trip down Memory Lane”…

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Abstract

Enterococcus faecalis is a commensal bacterium able to colonize different sites in the human host, such as the gastrointestinal tract, the genito-urinary tract and the oral cavity. It can also be found in numerous other environments, including soil, sand, water, food products and plants. These bacteria show a dual behavior: they can behave quite harmlessly as commensals, but are able to become opportunistic pathogens and cause serious infections, such as urinary tract infections and endocarditis, in hospital settings. The question as to how these bacteria are able to change from commensalism to pathogenicity has directed many recent studies to focus on the environmental host conditions that may trigger this transition as well as on the underlying molecular mechanisms.

Metals are very important elements in the host environment, as they are key components of many proteins and are involved in numerous cell processes in both the host and the invading pathogen. The maintenance of metal homeostasis is fundamental to both to ensure that metabolism and cell functions are functioning properly. Variations in this homeostasis must be tightly regulated. In several Gram positive pathogens, metal homeostasis and regulation has been linked to their pathogenicity. The lack of knowledge on this subject in *E. faecalis* motivated the work presented in this thesis.

Our studies initially addressed the genome-wide transcriptional responses of *E. faecalis* V583, a multi-resistant strain isolated from a bloodstream infection, to zinc, manganese and copper ions (Chapter 2). A set of differentially expressed genes common to the three metal transcriptional responses and related to manganese transport were further

studied (Chapter 3). Some of the highest up-regulated genes in response to zinc excess were examined in Chapter 4, focusing on their role in the ability of *E. faecalis* to cause infections.

The transcriptomic experiments provided new information on the responses of *E. faecalis* to zinc, manganese and copper excess and revealed that the most relevant mechanisms involved were transport systems (Chapter 2). Our findings provided the first evidence for the correct annotation of many *E. faecalis* genes, as most of those transporters were annotated as metal transporters. Furthermore, genes encoding other types of transporters and proteins involved in energy, amino acid metabolism and cellular processes were also shown to be relevant for metal homeostasis in *E. faecalis*. Several genes related to the cell wall appeared to be particularly important in the response to excess copper. Although the *cop* operon for copper transport and regulation is described as the main player in copper homeostasis in *Enterococcus hirae*, our results suggest that in *E. faecalis* other transport genes may also be involved.

In a subsequent study, we focused on manganese transport systems and their regulation (Chapter 3). The genes encoding these systems were identified and were shown to be regulated by EfaR, a DtxR family regulator. Furthermore, several transporter mutants were examined to evaluate the role of these proteins, and that of their regulator, on cell processes relevant for *E. faecalis* colonization and infection of the host. The studies revealed that EfaR is the key player for manganese homeostasis in *E. faecalis*. Moreover, EfaR was able to influence a number of cell processes important for colonization and infection, namely biofilm formation, oxidative stress and intramacrophage survival. Our observations provided a link between manganese homeostasis and *E. faecalis* pathogenicity.

In addition, analysis of the zinc transcriptome experiments prompted the study of genes found to be highly up-regulated under zinc excess. In Chapter 4 we focused on the gene encoding a cation P-type ATPase, denoted $ZntA_{Ef}$, and on *ef0759*, encoding a putative SapB protein. Mutations were made in these genes in order to elucidate their role in *E. faecalis* defense against host attack. The zinc-responsive ATPase $ZntA_{Ef}$ was shown to play a role in the response to zinc overload, lysozyme treatment, oxidative stress, all used by the host to defend itself against bacteria and contributing to intramacrophage survival. EF0759 also influenced *E. faecalis* survival inside macrophages, although likely by a different mechanism, as the corresponding mutant was not more susceptible to the other three host weapons mentioned. As saliva contains lysozyme and a relatively high concentration of zinc, $ZntA_{Ef}$ may be relevant for the success of *E. faecalis* as an orthodontia pathogen. This constitutes the first link between zinc homeostasis and pathogenicity in this organism.

The work here presented reveals the importance of transition metals and the mechanisms and regulation of their transport in processes involved in *E. faecalis* colonization and infection of the host. Thus, it establishes a novel link between metal homeostasis and pathogenicity of *E. faecalis* and provides new leads for future strategies to fight this important pathogen.

Resumo

Enterococcus faecalis é uma bactéria comensal que coloniza naturalmente mamíferos e insectos. No hospedeiro humano é capaz de colonizar diferentes locais, tais como o tracto gastrointestinal, o tracto génito-urinário e a cavidade oral; também pode estar presente noutros ambientes, nomeadamente solo, areia, água, produtos alimentares e plantas. Estas bactérias têm um comportamento dualístico: como comensais, podem ser inofensivas, mas também se podem tornar patogéneos oportunistas e causar infecções graves em ambiente hospitalar, tais como infecções urinárias e endocardites. A questão de como os enterococcus são capazes de passar de comensais a patogénicos tem levado muitos estudos recentes a focarem-se nas condições ambientais do hospedeiro que possam potenciar esta transição e também nos mecanismos moleculares subjacentes.

Os metais são elementos importantes nos ambientes do hospedeiro pois são componentes fundamentais de muitas proteínas e estão envolvidos em inúmeros processos celulares tanto no hospedeiro como nas bactérias. A homeostase da concentração de metais é fulcral em ambos para garantir que o metabolismo e as funções celulares decorrem de forma adequada. As variações nesta homeostase têm de ser reguladas de forma rigorosa. Em vários patogéneos Gram positivos, a homeostase e a regulação dos metais foram correlacionadas com a patogenicidade dessas bactérias. A falta de conhecimento sobre esta temática relativamente à bactéria *E. faecalis* motivou o trabalho apresentado nesta tese.

Inicialmente, os nossos estudos foram dirigidos para o perfil transcriptómico, na presença de excesso de iões de zinco, manganês ou

cobre, da estirpe *E. faecalis* V583, uma estirpe multi-resistente isolada de uma infecção sanguínea (Capítulo 2). Um conjunto de genes que foram diferencialmente expressos em comum aos três metais e relacionados com o transporte de manganês foram objectos do estudo apresentado no Capítulo 3. Alguns dos genes que foram mais sobrexpressos na presença de excesso de zinco foram estudados no Capítulo 4, focando o papel destes genes na capacidade de *E. faecalis* causar infecções.

As experiências de transcriptómica forneceram novas informações evidenciando que os transportadores são os mecanismos mais relevantes na resposta ao excesso de zinco, manganês e cobre. Os nossos resultados concederam a primeira prova empírica da correcta anotação de vários genes de *E. faecalis*, pois a maioria dos sistemas de transporte revelados nestas experiências (Capítulo 2) estavam anotados como associados ao transporte de metais. Adicionalmente, genes que codificam outros tipos de transportadores e proteínas envolvidas no metabolismo em geral e em processos celulares mostraram também ter um papel relevante na homeostase de metais em *E. faecalis*. Vários genes relacionados com a parede celular parecem ter um papel particularmente importante na resposta ao excesso de cobre. Embora o operão *cop*, responsável pelo transporte e regulação do cobre, esteja descrito como tendo o papel principal na homeostase deste metal em *Enterococcus hirae*, os nossos resultados sugerem que em *E. faecalis* há outros genes que codificam transportadores que também podem estar envolvidos na homeostase do cobre.

Os estudos seguintes focaram-se nos sistemas de transporte de manganês e na sua regulação (Capítulo 3). Os genes que codificam estes sistemas, identificados no Capítulo 2, revelaram ser regulados pela proteína EfaR, pertencente à família de reguladores DtxR. Adicionalmente, várias estirpes mutantes nestes sistemas de transporte foram testadas no

sentido de avaliar o papel dos genes inactivados e do seu regulador, em processos celulares relevantes para a colonização e infecção do hospedeiro. Os estudos mostraram que o regulador EfaR tem uma função crucial na homeostase do manganês em *E. faecalis*. Os mesmos estudos revelaram também que EfaR é capaz de influenciar um número de processos celulares importantes para a colonização e infecção, nomeadamente a formação de biofilmes, a resistência ao stress oxidativo e a sobrevivência no interior de macrófagos. As nossas observações demonstram a existência de uma ligação entre a homeostase do manganês e a patogenicidade em *E. faecalis*.

A análise dos resultados das experiências de transcriptómica com zinco impulsionou o estudo, apresentado no capítulo 4, de genes consideravelmente sobrexpressos na presença de excesso de zinco, nomeadamente um gene que codifica uma ATPase de tipo P, denominada $ZntA_{Ef}$ e o gene *ef0759*, codificando uma proteína SapB putativa. Foram construídas estirpes com mutações nestes genes, com o intuito de esclarecer as suas funções na resposta de *E. faecalis* às defesas do hospedeiro. A ATPase $ZntA_{Ef}$, que responde à presença de zinco, mostrou ter um papel relevante na resistência ao excesso de zinco, à lisozima e ao stress oxidativo. Estes três elementos de defesa do hospedeiro são particularmente importantes nos macrófagos, onde o transportador $ZntA_{Ef}$ demonstrou ser crucial para a sobrevivência de *E. faecalis*. A proteína EF0759 demonstrou ter também um papel activo na sobrevivência de *E. faecalis* dentro de macrófagos. No entanto, o mecanismo pelo qual contribui para este fenótipo deverá ser diferente do mecanismo da proteína $ZntA_{Ef}$, já que o respectivo mutante não se revelou mais susceptível às três referidas defesas do hospedeiro, comparativamente com a estirpe selvagem. Dado que a saliva contém lisozima e uma concentração relativamente elevada de zinco, a proteína $ZntA_{Ef}$ pode ser relevante no

sucesso de *E. faecalis* como patogéneo periodontal. Este trabalho constitui a primeira ligação entre a homeostase do zinco e a patogenicidade deste microrganismo.

O trabalho apresentado nesta tese revela a importância dos metais de transição e dos mecanismos de regulação dos seus transportadores, e permite o reconhecimento da ligação entre homeostase de metais e a patogenicidade de *E. faecalis*, sendo fornecidas novas pistas para futuras estratégias de combate a este importante patogéneo.

Nota: Este texto não foi escrito em conformidade com o Novo Acordo Ortográfico.

Abbreviations

Δ	deletion
ABC	ATP-binding cassette
ADI	arginine deiminase
ATP	Adenosine-5'-triphosphate
BHI	Brain Heart Infusion
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
CDF	cation diffusion facilitators
cDNA	complementary DNA
CFU	colony forming unit
chelGM17	chelated GM17
cm	chloramphenicol
DISCLOSE	DISsection of Clusters Obtained by Series of transcriptome data
DNA	Deoxyribonucleic acid
ebm	EfaR binding motif
ECM	ExtraCellular Matrix
Ehk	Enterococcal histidine kinase
Err	Enterococcal response regulator
ery	erythromycin
GEO	Gene Expression Omnibus
GM17	M17 medium with 0.5% glucose
GRAS	Generally Recognized As Safe
GSH	Glutathione
kan	kanamycin

LAB	Lactic Acid Bacteria
LB	Luria-Bertani medium
LTA	Lipotheichoic acids
MCO	multi-copper oxidase
MCS	multiple cloning site
moi	multiplicity of infection
MOODS	MOtif Ocurrence Detection Suite
NCBI	National Center for Biotechnology Information
NRAMP	Natural Resistance-Associated Macrophage Protein
OD	Optical Density
PAI	Pathogenicity Island
PCR	Polymerase Chain Reaction
PTS	Phosphotransferase system
^{r}	resistance
RNA	Ribonucleic acid
RND	resistance nodulation family
ROS	Reactive Oxygen Species
rpm	rotations per min
SDS	Sodium Dodecyl Sulfate
SI	Survival Index
sqRT-PCR	semi-quantitative Reverse Transcriptase PCR
TCS	Two Component System
tet	tetracycline
v/v	volume/volume
YT	Yeast Extract with Tryptone
zim	zinc motif

Table of Contents

Acknowledgements VII

Abstract XI

Resumo XV

Abbreviations XIX

Table of Contents XXI

Chapter 1

General Introduction 1

Chapter 2

Impact of manganese, copper and zinc ions on the transcriptome of the nosocomial pathogen *Enterococcus faecalis* V583 53

Chapter 3

EfaR is a major regulator of *Enterococcus faecalis* manganese transporters and influences processes involved in host colonization and infection 109

Chapter 4

$ZntA_{Ef}$ mediates *Enterococcus faecalis* defense against zinc overload, lysozyme and oxidative stress 143

Chapter 5

General Discussion 175

Chapter 1

Introduction

Table of Contents

Enterococcus 5
Transition from commensalism to pathogenicity 7
Virulence 8
Environmental stresses 10
Metals 12
Metal sensor proteins 15
Metal transport 17
Uptake systems 17
Efflux systems 17
Zinc 18
Zinc regulation 20
Manganese 21
Manganese regulation 24
Copper 26
Copper regulation 28
Metal regulation in enterococci 29
Scope of this thesis 31
References 33

Enterococcus

Enterococci are commensal inhabitants of the gastrointestinal tract of humans and other animals and can colonize the genitourinary tract and the oral cavity [1]. They are also capable of surviving in the most diverse environmental niches such as soil, sand, water, food products and plants [2]. These bacteria are differentiated by their capacity to grow between 10°C and 45°C, in 6.5% NaCl and at pH 9.6, to survive upon heating at 60°C for 30 min, to hydrolyze esculine into esculitine [3] and to react with the Lancefield group D antisera [1]. Enterococci produce L(+)-lactic acid homofermentatively from glucose. They belong to the large group of the lactic acid bacteria (LAB). Contrary to other LAB, enterococci are not considered “Generally Recognized As Safe” (GRAS) and their detection in water is regarded as an indicator of fecal contamination [4].

Presently forty-one species of *Enterococcus* are recognized (http://old.dsmz.de/microorganisms/bacterial_nomenclature_info.php?genus=Enterococcus); *Enterococcus faecalis* and *Enterococcus faecium* are the two most common species found in the human microbiota [3].

Enterococci are important in several areas of our daily lives. In the food industry, they have a beneficial role due to their ability to degrade casein and stimulate the growth of certain LAB, which is exploited for the ripening of cheeses such as cheddar and mozzarella [5]; their capacity to grow under harsh conditions makes them suited for fermentation of other products as well, such as sausages [6-8]. The putative negative aspect of enterococci in food is their ability to produce biogenic amines in cheese and fermented sausages [9, 10]. Enterococci have also been successfully used as probiotics in the promotion of a positive gut environment [11-13].

Although harmless in healthy individuals, enterococci have been emerging as important nosocomial pathogens causing wound-, bloodstream- and urinary tract infections and endocarditis mainly in hospitalized patients with severe underlying diseases or with an impaired immune system under prolonged antibiotic treatments. Enterococci rank second as a cause of urinary tract infections in both the United States and Europe [14, 15] and are the third leading cause of endocarditis after streptococci and *Staphylococcus aureus*, being responsible for 5 to 20% of all cases of endocarditis (predominantly *E. faecalis*) [16]. *E. faecalis* and *E. faecium* are associated with approximately 60% and 40%, respectively, of hospital-acquired infections caused by enterococci [17, 18]. The strong association of enterococci with infections in hospital settings and the difficulty in devising a successful medical treatment have been attributed to their inherent capacity to withstand environmental stresses and their innate and acquired resistance to many commonly used antibiotics [1, 19, 20] making them well equipped to survive and colonize hospital environments [21, 22]. Also, the pathogenicity of enterococci is potentially increased by their highly efficient ability to transfer genetic material [23]. Vancomycin has been used as the drug of last resort in the treatment of Gram positive bacterial infections, including those caused by enterococci. Resistance to vancomycin poses a serious problem in the treatment of enterococcal infections [19], and favors propagation and persistence of enterococci in hospitals; furthermore, there is an increased risk of horizontal transfer of this resistance determinant to other medically relevant vancomycin-susceptible species [19, 24, 25], as has already been observed for *S. aureus* [26]. Vancomycin resistance genes have been found in both clinical and dairy enterococcal isolates, which facilitates the propagation of these genes and potentiates the proportion of this health problem [27].

E. faecalis V583 was the first reported vancomycin resistant clinical isolate in the United States. It was obtained from a patient suffering from a persistent bloodstream infection [28]. V583 is part of the high-risk clonal complex 2, which comprises mostly isolates derived from hospital infections worldwide [29, 30], and was the first *E. faecalis* strain to have its genome sequence published [30]. Since then, 29 other enterococcal genomes were sequenced [31, 32]. Comparative genome hybridization-based studies have revealed considerable variation in the genomic content of different *E. faecalis* and *E. faecium* strains. This variation is mainly a result of the presence or absence of mobile genetic elements such as phages and conjugative transposable elements and differences within the *E. faecalis* and *E. faecium* pathogenicity islands [33, 34].

The mechanisms by which inoffensive commensal enterococci may become major hospital-acquired pathogens are still not understood. The identification of traits related to the pathogenicity of these bacteria will contribute to the understanding of the dual nature of this organism.

Transition from commensalism to pathogenicity

The invasion of the bloodstream or other internal areas of the host is a critical step in the transition of enterococci from commensals to pathogens. During the process of tissue invasion, enterococci encounter an environment vastly different from that at sites of colonization, where there is limited nutrient availability, high redox potentials and host defenses are present. Infecting enterococci likely express genes favoring growth under these different environmental conditions [20].

Virulence

E. faecalis possess several virulence factors that help establishing infection and persisting in the presence of host immune responses [35]. Several of these virulence factors have been characterized and are presented in Table 1 (reviewed in [24, 36, 37]).

Table 1: *E. faecalis* genes encoding virulence factors and their putative roles.

Gene	Virulence factor	Putative role	Reference
agg	Aggregation substance	Adhesion	[38]
efaA	Endocarditis specific antigen	Adhesion and infection	[39]
ace	Adhesin to collagen	Adhesion to ECM	[40]
esp	Enterococcal surface protein	Adhesion and infection	[41]
cylA-M	Cytolysin	Tissue damage	[42]
gelE	Gelatinase	Tissue damage	[43]
epa	Enterococcal polysaccharide antigen	Not determined	[44]
cpsA-K	Capsular polysaccharide	Resistance to host defense	[45]
sprE	Serine protease	Tissue damage	[46]
hypR	Hydrogen peroxide regulator	Resistance to host defense	[47]
gls24	Glucose starvation protein	Persistence in the host	[48]
cylR1-R2	Two-component system	cylA-M regulation	[42]
fsrA-C	Agr-like regulatory system	*gelE* and *sprE* regulation	[46]
etaRS	OmpR-like two-component system	Not determined	[49]
perR	Peroxide regulator	Not determined	[50]

ECM – Extracellular Matrix

Even though several factors may contribute to virulence of enterococci, a widespread distribution of putative virulence determinants in enterococcal isolates independent of their origin has been reported [51-53] and to date, no single virulence factor has been found to be ubiquitous in clinical isolates or has been demonstrated to be essential for enterococcal infections [54]. Furthermore, the importance of these virulence factors does not always seem to be supported by the findings of clinical studies [21]. In addition, though the detection of virulence genes may point to a virulence potential in food strains, foodborne enterococcal infections have never been reported. Nonetheless, food isolates may contribute to the spread of virulence genes by horizontal transfers rather than being a direct cause of infection [36].

The ability to cause infection can also be associated with a strain's competence to form biofilms [55], which would possibilitate the survival in less favorable conditions such as antibiotic-rich environments and promote persistance on medical devices. The capacity of enterococci to bind to various medical devices such as ureteral stents [56], intravascular catheters, biliary stents and silicone gastrostomy devices has been associated with their ability to produce biofilms [57, 58]. Hence, this ability enhances the capacity of enterococci to cause infections and contributes to the emergence of enterococci in hospitals [20, 59].

For enterococci to become pathogens, they had to develop mechanisms of adaptation that clearly include more than production of biofilms or expression of virulence factors and enabled them to cope with hostile host and non-host environments [54, 60].

Environmental stresses

In order to survive and colonize the human gastrointestinal tract, bacteria must overcome several biological barriers. Microorganisms that survive the gastric acidity of the stomach are able to transit to the intestine, where they encounter stresses associated with variations in pH, low oxygen availability, elevated osmolarity, nutrient limitations and relatively high concentrations of host-produced detergents [61-63]. Environmental stress responses in *E. faecalis* have been well studied for more than a decade and have shown their exceptional ability to survive and persist in a variety of adverse environments. In these studies, several *E. faecalis* genes have been identified to play important roles in the survival to environmental stresses and thus enabling the infection process (Table 2).

The ability of most bacteria to monitor and adapt to changing conditions is often mediated through two-component signal transduction systems. Two-component systems (TCS) generally consist of a sensory histidine kinase and a response regulator. The histidine kinase senses the signal and transfers a phosphoryl group to the response regulator, which can then regulate gene expression [64]. These systems are usually involved in environmental stress responses and other cellular processes important for survival and for infection of the host. It has been described that the *E. faecalis* response regulators Err04, Err08 and Err18 are involved in heat shock response. Moreover, TCS Err-Ehk05 has a role in the regulation of the *sagA* gene, which is involved in various stress responses (see Table 2; [65]) ; TCS Err-Ehk10 is involved in heat and acid pH response (EtaRK; [49]), in bile salts and, to a lesser extent in hyperosmotic stress response, and has a potential role in the expression of the heat shock proteins DnaK and GroEL [65].

Table 2: Genes described to be involved in environmental stress responses in *E. faecalis*.

Genes	Related stress	Reference
sagA	Bile, NaCl, SDS, ethanol, oxidative, heat, alkaline, acid	[66]
gsp62	Bile, SDS, acid, oxidative, heat, ethanol, tert-butyl hydroperoxide, sodium chloride	[67]
gsp65	SDS, acid, oxidative, heat, ethanol, sodium chloride	[68]
gls24	Bile, carbohydrate and complete starvation, $CdCl_2$	[69]
clpPBCEX	Heat	[70]
ctsR	Heat (Clp cluster regulator)	[71]
dnaK	Bile, heat	[63]
groEL	Bile, heat	[63]
sigV	Glucose and complete starvation, heat, ethanol, acid	[7]
rsiV	Glucose and complete starvation, heat, ethanol, acid (*sigV* regulator)	[7]
relA	Alarmone synthesis and degradation. Alarmone accumulates with heat, alkaline and vancomycin stress	[72]
hypR	Oxidative stress	[73]
sodA	Oxidative stress	[74]
katA	Oxidative stress	[75]
gor	Oxidative stress	[76]
npr	Oxidative stress	[77]
trxB	Oxidative stress	[77]
ahpF	Oxidative stress	[77]
ahpC	Oxidative stress	[77]
qacZ	Biocide – benzalkonium chloride	[78]

SDS – Sodium dodecyl sulfate

As discussed so far, bacteria have to be able to obtain the appropriate nutrients and adapt to the environmental conditions for survival and growth. One important requirement is the maintenance of metal ion homeostasis, particularly for the proper control of regulatory networks that govern gene expression and for virulence. In pathogenic bacteria, the mechanisms for metal ion homeostasis or, more specifically, metal ion transport may be the key to major adaptations to intracellular survival, colonization and infection [79].

In the next part of this Introduction chapter, the biological importance of metals and the bacterial mechanisms for metal regulation will be discussed with particular emphasis on zinc, manganese and copper ions, as these metals were the focus of the work presented in this thesis.

Metals

Transition metals are essential for all organisms, from bacteria to man. Approximately a quarter to a third of all proteins in any organism are metalloproteins [80]. They play important roles in the regulation of gene expression and in the activity of biomolecules. They are able to function as catalysts for biochemical reactions, as stabilizers of protein structures and bacterial cell walls, and can serve in maintaining osmotic balance [81, 82].

It is known that transition metals are also crucial for microbial invasion and infection, as bacterial pathogens must acquire metal nutrients in order to cause disease. The strict requirement for these elements during pathogenesis is due to their involvement in numerous processes, ranging from bacterial metabolism to accessory virulence factor function [83]. As metals are required for essential cellular processes, in both the host and the bacteria, vertebrates tend to frustrate this bacterial requirement by sequestering these elements [84].

Metal concentrations in the host can vary tremendously (Fig. 1) and for colonization and infection, bacteria need to have the proper mechanisms that will enable them to cope with such variations.

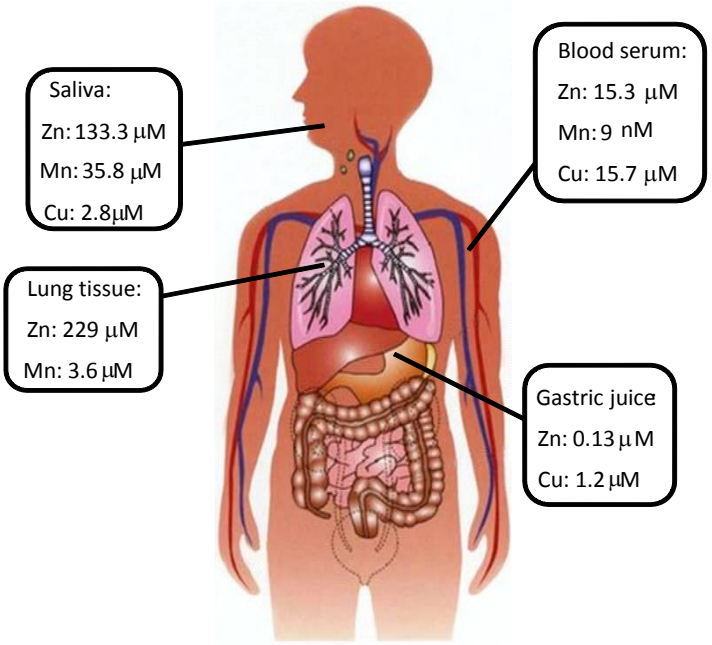

Figure 1 – Concentrations of zinc (Zn) [85-87], manganese (Mn) [85, 86] and copper (Cu) ions [85-87] in different parts of the human body (figure adapted from http://www.humanillnesses.com).

Metal ions are most often required in trace amounts. Suboptimal or elevated intracellular metal concentrations have severe, pleiotropic effects on many aspects of bacterial cellular metabolism. This obliges cells to be capable of scavenging trace metal ions from their environment to meet cellular requirements and, on the other hand, to have resistance mechanisms for when metal concentrations exceed physiological needs [88]. The existence of such resistance mechanisms is very important because high metal concentrations can cause severe problems such as damage to cell membranes, alteration of enzyme specificity, disruption of cellular functions and damage to the structure of DNA. Toxicity occurs through displacement of metals from their native binding sites or through ligand interactions, which may lead to alterations in the conformational

structure of nucleic acids and proteins, interference with oxidative phosphorylation and osmotic balance [89, 90].

Tight regulation of metal transport systems is the purview of metalloregulators that sense cytosolic metal levels and regulate the transcription of genes that maintain metal homeostasis accordingly [91-93], while metallochaperones storage metals or deliver them to proteins that need metal ions for function (Fig. 2; [92, 94-96]).

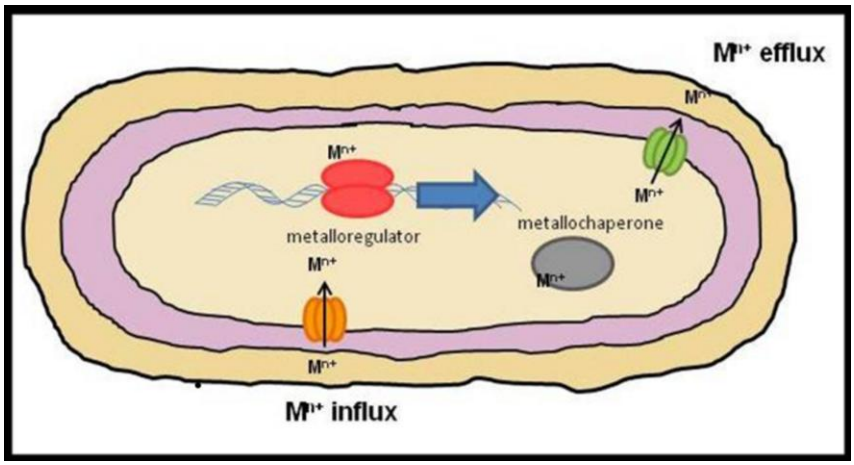

Figure 2 - Cellular processes for metal homeostasis. Membrane protein transport systems are responsible for the uptake (orange) and efflux (green) of metal ions according to cell's needs; metalloregulators (red) regulate transcription of the referred metal transport system genes (blue arrow); metallochaperones (grey) help with proper metal trafficking and/or storage. M^{n+}; n-valent metal ion.

For maintaining metal homeostasis, specific metalloregulatory proteins must be capable of discriminating the right ligand from a pool of transition metals that often have similar sizes (ionic radii) and net charges (often 2+; [88]). The fidelity of their discrimination controls the abundance of different metals within cells with consequences for metal occupancy of other metalloproteins. The affinities of proteins for the different trace metals are

substantially determined by the Irving– Williams series, for divalent metals (Mg^{2+} and Ca^{2+} (weakest binding) < Mn^{2+} < Fe^{2+} < Co^{2+} < Ni^{2+} < Cu^{2+} > Zn^{2+}, and Cu^{+} is also highly competitive) [83].

Metallochaperones have an important role in metal sequestration; however in prokaryotes, metal ion homeostasis is mostly maintained by metalloregulatory proteins. These proteins are able to bind metal ions directly and repress, derepress or activate the transcription of operons that encode metal-specific efflux pumps and/or membrane bound transporters, metal reductases, soluble cytoplasmic or periplasmic metal transport proteins, metal-sequestering proteins, as well as the metal-responsive transcriptional regulator itself [97].

Metal sensor proteins

Recent structural studies have contributed to the differentiation of four main distinct families of metal sensor proteins (Table 3). The Fur and DtxR families regulate most of the genes encoding proteins involved in metal ion uptake; in these cases, generally, the metal ion functions as a co-repressor in turning off uptake-related genes under metal-replete conditions. The MerR and ArsR/SmtB families regulate the expression of many genes required for metal ion detoxification and efflux; for these regulators, metal binding may lead to activation (MerR) or to derepression (ArsR/SmtB) of the resistance operon [98].

As more and more studies on metal transport and regulation are being reported, regulators from other families have been revealed as also playing roles as metalloregulators, which is suggestive of constant evolution and

adaptation of bacterial genomes to changing conditions and different environments.

Table 3: Examples of Gram positive metalloregulatory proteins.

Regulator	Metal	Organism	Targets	Description	References
Fur family					
Fur	Fe	*B. subtilis*	*fhuD*	Ferrichrome-binding protein	[99]
		S. aureus	*fhu* operon	Ferric-uptake system	[100]
Zur	Zn	*B. subtilis*	*ycdHI yceA*	Zinc ABC transporter	[101]
PerR	Fe	*B. subtilis*	*katA*	Catalase	[102]
DtxR family					
DtxR	Fe	*Corynebacterium diphtheria*	*Tox, hmuO*	Diphteriae toxin, Heme oxygenase	[103] [104, 105]
IdeR	Fe	*Mycobacterium smegmatis*	*fxbA*	Putative formyl transferase	[106]
MntR	Mn	*B. subtilis*	*mntH, mntABCD*	Manganese NRAMP transporter, Manganese ABC transporter	[107]
ScaR	Mn	*Streptococcus gordonii*	*scaBCA*	Manganese ABC transporter	[108]
SloR	Mn/Fe	*Streptococcus mutans*	*sloABCR*	Manganese and iron ABC transporter	[109]
EfaR	Mn	*E. faecalis*	*efaCBA*	Manganese ABC transporter	[110]
MerR family					
CueR	Cu/Cd/Ag	*B. subtillis*	*copA*	Copper efflux pump	[111]
ArsR/SmtB family					
ZntR	Zn	*S. aureus*	*zntA*	Zinc efflux pump	[112]
NmtR	Ni/Co	*Mycobacterium tuberculosis*	*nmtA*	P-type ATPase efflux pump	[112]
CzrA	Co/Zn	*S. aureus*	*crzAB*	Resistance determinant for zinc	[113]
CadC	Cd/Pb/Bi	*S. aureus*	*cadCA*	Cadmium ATPase efflux pump	[114]
Other					
CopY	Cu	*E. hirae*	*copYZAB*	P type ATPase copper transporter	[115]
SczA	Zn/Co/Ni	*S. pneumoniae*	*czcD*	Zinc efflux transporter	[116]
AdcR	Zn	*S. pneumoniae*	*adcCBA*	Zinc uptake system	[117]

NRAMP – Natural Resistance-Associated Macrophage Protein

Metal transport

Metal transport is basically performed by uptake and efflux systems, which will be further detailed below.

Uptake Systems

Metal uptake systems mostly rely on ATP-binding cassette (ABC) transporters. In Gram positive bacteria, the prototypical ABC transporter consists of a lipoprotein, a hydrophobic membrane protein, and an ATPase, with the two latter present as homo- or heterodimers. The lipoprotein is tethered to the outer side of the cell membrane and functions as a ligand binding protein [118]. Different ABC transporters translocate different substrates, ranging from small ions to large polypeptides, and they therefore play a wide variety of physiological roles. Mutations in ABC transporter genes are the underlying cause of a number of human genetic disorders. This fact contributes to their particular economic and medical importance as they can pump cytotoxic molecules from cells, thus conferring resistance to antibiotics, herbicides and chemotherapeutic drugs [119]. Here ABC transporters will be referred to only when they play a role in metal ion homeostasis (Table 3).

Efflux Systems

Microorganisms use efflux systems to export toxic metals from the cytoplasm of their cells. The various metal efflux systems belong to known protein families such as: the resistance nodulation (RND) family, which are proton-driven antiporters; the cation diffusion facilitators (CDF) family driven

by a chemiosmotic gradient or a potassium gradient [120]; and P-type ATPases that are driven by ATP hydrolysis [121].

The RND family of proteins is less common in bacteria. P-type ATPases and CDF proteins can be found in eukaryotes and in bacteria. The P-type ATPases are mostly metal cation transporters. Prokaryotic P-type ATPases transport a range of divalent metal cations that include Cu^{2+}, Ag^{2+}, Cd^{2+}, Zn^{2+} and Mn^{2+} (Table 3; [122]).

The study of the roles of essential metal ions and the regulation of their homeostasis has gained more and more relevance, mostly because of their involvement in bacterial pathogenesis.

In this thesis we focused on zinc, manganese and copper, three essential metal ions, that are crucial in many life processes across all kingdoms and that have particularly relevant roles in bacterial colonization and pathogenesis. The next sections will deal with each of these metals separately, referring mainly to their biological roles, their benefits and their associated diseases, and discussing the mechanisms involved in their regulation and in homeostasis maintenance.

Zinc

Zinc is an essential element for all living organisms. It is the second most abundant transition metal in seawater and in humans. This metal has been suggested to interact with as many as 10% of host proteins [123]. Factors that contribute to zinc's prominence among metal ions include its chemical characteristics as a relatively strong Lewis acid in enzymes and the fact that it is the only essential transition metal that lacks biological redox activity [124]. These factors make it well suited as a structural

cofactor [125]. Hence, zinc serves as a cofactor in all six classes of enzymes (oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases) as well as several classes of regulatory proteins [126, 127]. Zinc concentrations are highly variable in the human body, as shown previously in Fig. 1. High concentrations of zinc are toxic as this metal ion can interact with thiols and block essential reactions in the cell.

Zinc is known to be essential for all highly proliferating cells in the human body, especially the immune system. In addition, its immunosuppressive properties might be used for therapy in non-toxic concentrations [128]. A role for zinc in neurodegenerative diseases such as Alzheimer's disease has also been suggested (Table 4; [129]).

Zinc may be used by the host as a weapon against bacterial infections. In fact, zinc levels in the human body are increased during inflammation [130-132]. Curiously, many of us probably do not acknowledge that zinc toxicity is used every day to fight the development of dental plaque with zinc-containing toothpaste [83].

Table 4: Impact and role of zinc in the human host.

Beneficial properties of zinc	**Reference**
Immunosuppressant	[128]
Antioxidant	[133]
Cardiovascular protector	[134]
Zinc depletion	
Effects	
Decreased immune function	[128]
Symptoms (some examples)	
Gastrointestinal problems, frequent infections, dermatitis, endocrine disorders, cancer, degenerative diseases	[128, 135]
Zinc toxicity	
Effects	
Damage immune cells, microbicidal	[134, 136]
Symptoms (some examples)	
Nausea, vomiting, renal failure	[134]
Zinc unbalance	
Alzheimer's disease	[129]

Bacteria are predicted to incorporate zinc into approximately 4–6% of all of their proteins [137]. Whereas the total cellular zinc concentration is in the millimolar range, femtomolar concentrations of free Zn^{2+} trigger transcription of genes involved in zinc uptake or efflux machinery. This suggests an extraordinary intracellular zinc-binding capacity and shows that cells exert tight control over cytosolic metal concentrations, even for relatively low-toxicity metals such as zinc [138].

Zinc Regulation

Prokaryotes contain diverse mechanisms of zinc uptake and efflux and carry different metalloregulators from distinct protein families to control these systems (Table 3).

The Fur homologue Zur, a zinc uptake regulator, is a major regulator responsible for guaranteeing the necessary uptake of zinc in several bacteria, including *B. subtillis* [101] and *S. aureus* [139]. Fur is the prototype for a large family of regulators that sense iron (Fur), zinc (Zur), manganese (Mur) and nickel (Nur) sufficiency and peroxide stress (PerR) [140, 141]. The emerging consensus is that most of these proteins are likely to function as metal-dependent, DNA-binding repressors [142]. Zur controls zinc transport by binding to a Zur box in the presence of Zn^{2+} and thus repressing transcription of ABC transporter genes [143-145]. Although Zur regulates potential virulence determinants such as metalloproteases in *S. suis*, the effect of *zur* inactivation on virulence appears to be minimal [139, 143].

The ArsR/ SmtB family of metalloregulators, which negatively regulates genes involved in metal efflux, includes zinc-dependent members. They repress transcription of efflux pump genes namely those of

P-type ATPases and CDFs, in the absence of bound metal. Allosteric binding of specific metals regulates binding to DNA, triggering derepression [113, 141]. One of the members, *S. aureus* ZntR, regulates the expression of its own gene and the adjacent gene, *zntA.* Together they form an operon involved in the resistance to Zn^{2+} and Co^{2+} [112].

E. coli ZntR, on the other hand, is a member of the MerR family of regulators. It binds to the *zntA* promoter region leading to repression of *zntA* transcription. In the presence of metals, namely Zn^{2+}, Cd^{2+} or Pb^{2+}, ZntR becomes an activator of *zntA* expression, so that ZntA efflux system can expel the excess metals [146, 147].

Manganese

Manganese is the 12th most abundant element on the surface of the earth and is naturally present in rocks, soil, water and food such as cereals, fruits, vegetables and tea [148]. This metal is an essential micronutrient for all forms of life [149]. Manganese is necessary in vertebrates for a multitude of functions such as skeletal system development, energy metabolism, activation of certain enzymes, nervous system function, reproductive hormone function; it is also an antioxidant that protects cells from damage by free radicals (Table 5; [148]). Unlike zinc, there is little information regarding the effects of manganese deficiency on immune development and function [149].

The importance of Mn^{2+} for cellular physiology of bacteria has only recently been better revealed. By virtue of its redox activity and ability to function as a Lewis acid catalyst, manganese participates in several vital metabolic processes in bacteria.

Table 5: Impact and role of manganese in the host.

Beneficial properties of manganese	Reference
Antioxidant	[148]
Manganese depletion	
Neurological and behavioral effects	[150-152]
Epilepsy, Down's syndrome, osteoporosis	[151]
Manganese toxicity	
Manganism	[153-155]
Parkinson's disease	[155, 156]

Mn^{2+} has a primary role in the protection against oxidative stress and a variety of roles in lipid, protein and carbohydrate metabolism, biosynthesis, and signal transduction [157]. Mn^{2+}-dependent bacterial proteins include phosphoglyceromutase, enolase, pyruvate kinase, type I protein phosphatases, Mn^{2+}-dependent superoxide dismutases and catalases [158, 159]. Mn^{2+} also influences spore composition, structure and germination in some bacteria [160]. Mn^{2+} is particularly critical for the LAB, many of which have been shown to require Mn^{2+}, instead of Fe^{2+}, for survival [161-163]. LAB lack catalase but contain superoxide dismutases that require Mn^{2+} as a cofactor [161, 164]; Mn^{2+} itself can also act directly to detoxify superoxide and hydrogen peroxide [161, 165, 166]. There are also descriptions of this metal acting catalytically as an antioxidant in bacteria by associating with anions including phosphate, and metabolic intermediates such as lactate or malate [161, 165]. As an essential transition element, Mn^{2+} is extremely limited within the human body, where it may be complexed with carrier proteins. The concentration of this metal is 1000-fold higher in secretions than in internal body sites, as shown in Fig. 1. The change in the external Mn^{2+} concentration is one factor that signals the bacterium to change its expression of virulence factors in response to its environment [166].

Emerging data have revealed that vertebrates resist bacterial infections through manganese sequestration. Corbin *et al.* showed that

calprotectin, a well-known mammalian calcium-binding protein, chelates manganese and zinc within abscesses [167]. The inhibition of bacterial metal uptake represents a promising alternative area of research for the design of new antimicrobials [167].

Vertebrates also seem to limit metal availability to intracellular pathogens through the expression of the protein NRAMP-1 (Natural Resistance-Associated Macrophage Protein 1). This kind of proteins is expressed by many cell types including neutrophils and macrophages [168]; they have been suggested to transport iron and manganese out of the lysosome [168, 169]. Depletion of divalent metals, namely Mn^{2+}, from the bacteria-containing phagosome by NRAMP-1 might have a simple and species-general bacteriostatic effect by removing an essential element from the ecological niche of intracellular pathogens. This might enhance the bactericidal activity of macrophages by rendering the pathogen more sensitive to killing by oxygen radicals [170].

A hallmark of the susceptibility of a host cell to an invading pathogen is the relative susceptibility of the host cell and the pathogen to reactive oxygen and nitrogen species [158]. The use of Mn^{2+} complexes by bacteria to provide protection against reactive oxygen species (ROS) might increase their fitness. In fact, the involvement of complexes of Mn^{2+} with low molecular-weight anions such as lactate, bicarbonate or malate in the defense against ROS could form the basis of a widespread and so far overlooked defense mechanism [165]. In addition, a recent study on antibiotics has shown that exposure of Gram positive (*E. faecalis* and *S. aureus)* and Gram negative (*E. coli)* bacteria to bactericidal antibiotics induces the production of ROS by an oxidative damage cellular death pathway involving the tricarboxylic acid cycle, a transient depletion of NADH, destabilization of iron-sulfur clusters, and stimulation of the Fenton

reaction [171, 172]. These findings emphasize the central role of Mn^{2+}-dependent SodA in the intrinsic ability of *E. faecalis* to withstand drug-induced killing [171].

Manganese regulation

Primary transcriptional control of manganese transport genes in Gram positive bacteria is mediated by regulators from the DtxR family. Genes coding for this type of regulators have been found in the genome sequences of *Streptomyces* spp. [173], *Streptococcus mutans* [174], *Staphylococcus epidermidis* [175], *S. aureus* [176], *E. faecalis* and others. On the basis of protein structures, three groups can be distinguished within the DtxR family [177]. One group contains *Corynebacterium diphtheriae* DtxR and its closely related homologue IdeR from *M. tuberculosis*. Another group includes MntR from *B. subtilis* while the remaining group, referred to as the Mn/Fe group, comprises ScaR from *S. gordonii* and EfaR from *E. faecalis*. Proteins from the first group (e.g. DtxR and IdeR) are responsive *in vivo* only to iron. Proteins from the second group respond to manganese (e.g. MntR), and the third group is more diverse, with proteins responding to Fe^{2+} and/or Mn^{2+} (e.g. SirR and ScaR) [177]. There is evidence to suggest that the DtxR regulators are important in regulating genes encoding metal ion transport systems related to virulence and oxidative stress defense [178].

MntR modulates the expression of genes of ABC transporters involved in Mn^{2+} uptake and/or MntH, the bacterial NRAMP homologue. MntR functions as a typical repressor. In *B. subtilis*, when adequate Mn^{2+} is present, MntR binds to the promoters of *mntH* and *mnt/sitABCD*, repressing the expression of these genes [179]. When manganese levels are low,

MntR releases the promoter, allowing transcription of the transporter genes [107, 158, 180]. *S. aureus* MntR is also important for modulation of the PerR-mediated oxidative stress response [176] and for virulence [176, 181]. Addition of Mn^{2+} leads to repression of the *B. subtilis* MntR and Fur genes and of at least some members of the PerR regulon, and to the activation of the large regulons controlled by the general stress response sigma factor σ^B, and TnrA, a regulator of nitrogen metabolism [157].

In *S. mutans,* a member of the oral streptococci, *sloABCR* encodes a putative Fe^{2+}/Mn^{2+} uptake system suggested to be required for endocarditis [109]. A recent genome-wide study on SloR, the DtxR family regulator of this uptake system, led to the proposition that it plays a role as a bifunctional regulator of *S. mutans* gene expression, depending on the conditions of metal repletion/depletion [182]. Moreover, it has been suggested that SloR is involved in adherence, biofilm formation, genetic competence, metal ion homeostasis, oxidative stress tolerance and antibiotic gene regulation, all of which contribute to *S. mutans*- induced disease [183].

Manganese is also a cofactor of members of the Fur family of regulators. The *B. subtilis* Fur homologue PerR, which responds to peroxide, has been purified in an inactive and an active, metal-containing form. It was shown that active PerR contains Zn^{2+} and Fe^{2+} ions; Mn^{2+} is able to compete with Fe^{2+}, decreasing PerR sensitivity to H_2O_2 [184]. This competition between the two metal ions may have a relevant role by allowing different responses of PerR to low and high H_2O_2 stress levels [102, 184, 185].

Copper

Copper is an essential element for all living organisms, from bacteria to man. It acts as a cofactor for several enzymes that carry out fundamental biological functions required for growth and development [186]. Copper has been used since ancient times for its antiseptic properties. Egyptians used copper to sterilize chest wounds and drinking water [187]. Greeks, Romans, Aztecs and others continued to use copper or copper compounds for the treatment of headaches, burns, intestinal worms, ear infections and for hygiene in general. The use of copper as an antimicrobial agent was only surpassed with the appearance of antibiotics. Nevertheless, the emergence of antibiotic resistance in bacteria created the need for different approaches against pathogenic microorganisms. Some copper-utilizing alternatives have emerged lately, such as the use of copper surfaces in hygiene-sensitive areas and copper vessels to make water drinkable in developing countries [188]. Currently, there is a great interest in the use of copper as a self-sanitizing material, and several recent publications deal with mechanistic aspects of “contact killing” (contact-mediated killing) by this metal [187]. Understanding how cells deal with copper is fundamental to clarify the role of copper in health and disease [189]. Copper is an essential micronutrient but can be toxic when present in excess, with the most noticeable chronic effect in humans being liver damage. This metal has also been related to genetic and neurodegenerative diseases (Table 6) [190-192].

Table 6: Impact and role of copper in the human host.

Beneficial properties of copper	Reference
Antimicrobial	[188]
Copper related diseases	
Wilson's disease	[193]
Menkes' disease	[190]
Alzheimer's disease	[191, 194, 195]
Parkinson's disease	[192, 196]

The biological functions of copper are closely related to its properties as a transition metal. Second only to iron, copper is the biologically most used transition metal. There are more than 30 known types of copper-containing proteins [187]. Some examples are cytochrome c oxidase, lysyl oxidase and superoxide dismutase. In such copper-containing enzymes, copper serves as an electron donor/ acceptor by alternating between the redox states Cu^{+} and Cu^{2+} [197]. Copper can also be a prosthetic group of microbial enzymes involved in the reduction of nitrite and nitrous oxides [189]. The two oxidation states of copper, Cu^{+} and Cu^{2+}, not only allow this metal to participate in essential redox reactions but can also lead to the formation of ROS, causing cellular damage. Reactive hydroxyl radicals can be generated in a Fenton- type reaction:

$$Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + OH^{-} + OH^{\cdot}$$

The extremely reactive hydroxyl radical ($OH^{\cdot}$) can participate in a number of reactions that are detrimental to cellular molecules, such as the oxidation of proteins and lipids [198], leading to cell damage and death. Cells try to keep H_2O_2 at very low levels, and the latter reaction may not be the main toxic mechanism. Copper ions may also compete with zinc or other metal ions for important binding sites on proteins [187].

$2\ Cu^{2+} + 2\ RSH \rightarrow 2\ Cu^{+} + RSSR + 2H^{+}$ (RSH, RSSR: sulphydryl molecules)

$2\ Cu^{+} + 2H^{+} + O_2 \rightarrow 2\ Cu^{2+} + H_2O_2$

These combined reactions cause redox cycling of copper at the expense of glutathione (GSH) or other thiols and O_2 to produce their reduced form. Thus, copper homeostasis by controlling uptake, accumulation, detoxification, and removal of copper is critical to living organisms [199]. As copper catalyses the production of ROS, it is vital to bind cytoplasmic copper in order to keep it out of metal-binding sites destined for other metals. Copper ions are sent to cytoplasmic destinations by metallochaperones [200-202]. Furthermore, all copper-dependent enzymes known to date in *E. coli* are found in the cell envelope [203-205]. The reason for this may be that available cytosolic copper ions, bound or free, are detected by copper sensors and rapidly transported to the cell envelope for incorporation or expulsion [206].

Copper regulation

Bacteria have developed a number of mechanisms for controlling cellular copper levels. An illustrative case is that of *S. aureus*, in which a genome-wide study revealed four different mechanisms for adapting to high levels of environmental copper: induction of direct copper homeostasis mechanisms; increased oxidative stress resistance; expression of the misfolded protein response; and repression of a number of transporters and global regulators such as Agr and Sae, two major virulence regulators [207].

Increased oxidative stress resistance has also been associated with copper resistance in other bacteria. Most Gram negative bacteria encode a

multicopper oxidase (MCO), which is required for the periplasmic oxidation of Cu^{+} to Cu^{2+} following P-type ATPase transport of Cu^{+} from the cytoplasm [208].

The *cop* operon is the main known system responsible for copper regulation in Gram positive bacteria. It is best characterized in *E. hirae*, as will be discussed in the next section. A broad study on the *cop* operon in the *Lactobacillale* order detected the *cop*-like operon in 14 strains that corresponded to 9 species from this order. A *copB*-like gene was detected in only one case. The apparent absence of a CopB-like protein suggests that the efflux function belonging to CopB might be provided by paralogs of the CopA ATPase, such as those found in *E. faecalis, S. agalactiae, L. plantarum,* and *L. lactis*. Hence, CopA-like ATPases might have a double function by participating in the uptake and efflux of copper ions in the strains that possess only one gene coding for such ATPases [209].

Metal regulation in enterococci

As mentioned previously, in several Gram positive pathogens, metal regulation has been linked to colonization, virulence and establishment of infection. In the Enterococcus genus not much is known concerning metal regulation. In *E. faecalis*, the *efaCBA* operon encoding an ABC-type transporter system is regulated by EfaR, a Mn^{2+}-responsive transcriptional regulator from the DtxR family [110], which will be further discussed in Chapter 3.

The *cop* operon in *E. hirae* is the best known copper regulatory system. It regulates copper uptake, availability, and export. CopA and CopB are P-type ATPases, CopY is a copper-responsive repressor, and

CopZ is a chaperone which is able to catalyze intracellular copper routing [98]. According to the theory, CopA takes up copper when it is limiting and CopB extrudes copper when it reaches toxic concentrations. CopY, the copper responsive repressor, uses Zn^{2+} as a cofactor when bound to the promoter, suppressing the transcription of *cop* operon. CopZ transfers copper to CopY, releasing zinc and thereby releasing also this regulator from the DNA and allowing expression of the downstream *cop* genes. A recent global gene expression analysis revealed new putative components of copper homeostasis such as the transcription elongation factor GreA, and members of Rrf2, Cro/CI and SorC/DeoR transcription factor families that might complement the role of CopY in the regulatory network activated by copper ions [210]. Another recent study showed that the *E. hirae* stress response protein Gls24 also interacts with CopZ *in vitro*. Moreover, the *gls24* operon was induced in the presence of copper. It would be interesting to further explore this link and elucidate the molecular role of Gls24 and other Gls24-like proteins in copper homeostasis [211].

The plasmid-located *tcrYAZB* (transferable copper-resistance) operon from *E. faecium* has a genetic organization similar to that of *copYZAB*. In response to toxic levels of copper, plasmid-borne copper resistance mechanisms are often used by bacteria. The *tcrB* gene has been described in *E. faecium* [212] and *E. faecalis* [213], where it confers copper resistance [214].

Scope of this thesis

In recent years, several studies on *E. faecalis* have contributed as to how it transitions from a commensal to pathogen. Some studies have been conducted in which *E. faecalis* genome-wide expression was observed in different conditions mimicking the environments in the host. Conditions mimicking the ones found in the gastrointestinal tract were represented with SDS and/or bovine bile and examined the stresses sensed in *E. faecalis* strain V583 [61]; other environments such as blood and urine were also studied [54, 215]. Even though *E. faecalis* is a clinically significant pathogen implicated in different types of infections, knowledge on the molecular mechanisms that enable its adaptation to divergent conditions in the host environments, namely how it copes with changes in metal concentrations in the host, is scarce. The poor knowledge on how enterococci cope with different metal environments, being able to survive and cause infection, was the incentive of the work presented in this thesis.

This first chapter provides the readers with an updated overview of the *Enterococcus* genus, its distinctive characteristics and the factors involved in its survival in diverse environments, including in the host, some of which may be correlated with the enterococcal transition from being commensal to pathogenic. Chapter 1 also gives information on the importance of metals in biological processes in all living organisms, with a particular emphasis on the relation between metal regulation and bacterial pathogenicity; in addition it also brings to the attention our poor knowledge on *E. faecalis* metal transport and regulation and their relation with pathogenesis. In Chapter 2, we present the results of DNA microarray experiments on *E. faecalis* V583 grown in the presence of excess Zn^{2+}, Mn^{2+} and Cu^{2+} and give the first description of *E. faecalis* V583

transcriptomes under high concentrations of these metal ions. These studies aimed to identify (more) metal transport systems in this organism and contribute to the unraveling of some important mechanisms that are involved in metal regulation. Chapter 3 describes the study of a Mn^{2+}-dependent regulator, EfaR and its role in the regulation of particular putative metal transporters of which the genes carry a specific motif, *ebm* (EfaR binding motif), in their promoter regions. In addition, its involvement in processes contributing to colonization and infection of the host is described. Chapter 4 reports a study of two proteins, ZntAEf and EF0759, related with zinc stress response in *E. faecalis* and their influence on biologic processes relevant for bacterial infection and virulence. This thesis ends with a General Discussion chapter, where the main results are summarized and pointfull ideas of this work are debated, giving emphasis to the contribution of this work to the comprehension of the mechanisms of metal transport and regulation in *E. faecalis,* and acknowledging the link between metal homeostasis and the ability of *E. faecalis* to colonize and become virulent.

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Chapter 2

Impact of Manganese, Copper and Zinc Ions on the Transcriptome of the Nosocomial Pathogen *Enterococcus faecalis* V583

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Author Contributions

All experiments were performed by the author of this thesis. Planning the experiments, data analysis and manuscript preparation were done by the author and the supervisors Dr. Fátima Lopes and Prof. Dr. Jan Kok.

Table of Contents

Abstract 57
Introduction 59
Materials and Methods 62
Media and growth conditions 62
Transcriptome experiments 62
DNA microarray data analysis 63
Semi-quantitative Reverse Transcriptase (sqRT)-PCR 63
Transcriptional *lacZ* fusion construction 64
β-galactosidase assays 65
Results 65
Zn^{2+} regulated genes 67
Mn^{2+} regulated genes 70
Cu^{2+} regulated genes 71
Common genes 72
Discussion 75
Transport and binding 75
Metabolism and cellular processes 76
Cell envelope 78
Signal transduction 79
Summary 79
Acknowledgements 82
References 83
Supplementary Information 88

Abstract

Mechanisms that enable *Enterococcus* to cope with different environmental stresses and their contribution to the switch from commensalism to pathogenicity of this organism are still poorly understood. Maintenance of intracellular homeostasis of metal ions is crucial for survival of these bacteria. In particular Zn^{2+}, Mn^{2+} and Cu^{2+} are very important metal ions as they are cofactors of many enzymes, are involved in oxidative stress defense and have a role in the immune system of the host. Their concentrations inside the human body vary hugely, which makes it imperative for *Enterococcus* to fine-tune metal ion homeostasis in order to survive inside the host and colonize it. Little is known on metal regulation in *Enterococcus faecalis*. Here we present the first genome-wide description of gene expression of *E. faecalis* V583 growing in the presence of high concentrations of zinc, manganese or copper ions. The DNA microarray experiments revealed that mostly transporters are involved in the responses of *E. faecalis* to prolonged exposure to high metal concentrations although genes involved in cellular processes, in energy and amino acid metabolisms and genes related to the cell envelope also seem to play important roles.

Introduction

Maintenance of intracellular homeostasis of metal ions is crucial for survival of bacteria, particularly for appropriate transcriptional control of regulatory networks that govern gene expression and for virulence. Thus, mechanisms for metal ion homeostasis or, more specifically, metal ion transport may constitute major adaptations to intracellular survival and replication among pathogenic bacteria [3]. Of distinct relevance are zinc, copper and manganese ions, not only as components of many proteins and cofactors in enzymatic reactions, but also for their toxicity to bacterial cells when present above certain concentrations. Within the host, pathogens can come across variable concentrations of these metals which demands a precise transcriptional control of genes coding for transporters (responsible for metal uptake and efflux) or proteins involved in metal ion storage. In fact, the total zinc concentration in serum and in gastric juice is similar (13.8 µM and 13 µM, respectively [4]; [5]), but in saliva and in the lungs the total concentration of this metal can reach 133.3 µM and 229 µM, respectively [4, 6]. Zn^{2+} has a strong influence on the immune function of the human body [7-9]. In general, low levels of zinc lead to decreased performance of the immune system, while physiologically normal concentrations secure its normal functioning [8, 10]. High dosages of Zn^{2+} (0.1 mM) may even activate certain immune cells [10]. Moreover, zinc levels in the human body are increased during inflammation [7, 9]. Manganese is another important trace metal required in numerous cellular processes, including metabolism and oxidative stress defense [11]. Manganese may protect against reactive oxygen species (ROS) and increase the fitness of cells by minimizing energy expenditure on the synthesis of a defense regulon [12]. The total concentration of this metal is more than 1000-fold higher in secretions such as saliva (36.2 µM, [6]) than it is inside the human body, for example in blood (11.6 nM, [4]) or in urine, where Mn^{2+} levels are also in the nanomolar

range [13]. Thus, manganese ions become a potential signal by which bacteria can sense a shift from a mucosal environment to a more invasive site. Copper is an essential trace metal required by most organisms as a cofactor for many catabolic pathways and electron transport. However, copper is toxic to cells at concentrations higher than physiological levels (16 μM in serum, [4]) and excess copper avidly binds to many biomolecules such as proteins, lipids and nucleic acids, regardless of its valence state [14]. Thus, exposure to metals with redox properties such as copper [15] is a double-edged sword, for these properties render them highly toxic through interference with the functioning of intracellular macromolecules and because they can generate toxic free radicals through the Fenton reaction [16].

Enterococcus faecalis is a Gram positive bacterium with a dual nature, as it is present in the human digestive tract as a commensal organism, but is also frequently the cause of nosocomial infections. Mechanisms and factors involved in the switch from commensalism to pathogenicity of these bacteria remain unclear despite the fact that some virulence-associated genes have been identified [17-20]. Information about environmental stresses and their contribution to the switch to pathogenicity is still scarce. Since bacterial responses to stress often coincide with increased virulence [21, 22], a number of studies have been conducted recently where *E. faecalis* V583 gene expression was examined in different conditions mimicking the various environments in the host. Conditions such as the ones found in the gastrointestinal tract were represented with sodium dodecyl sulfate (SDS) and bovine bile (BB) stresses in V583 strain [23]; other environments such as blood and urine were also studied [1, 2]. Another study probed the expression of virulence related genes in *E. faecalis* OG1RF submitted to several sub-lethal stresses [24].

Nevertheless, little is known on how *E. faecalis* is able to cope with changes in metal concentrations in the host and assure its own metal

regulation. In other Gram positive pathogens, several transcriptional studies with metal ions have been conducted to gain more knowledge on metal homeostasis in those organisms [25-29]. Different studies in *Enterococcus hirae* led to the description and characterization of copper regulation by the *cop* operon [30-32]. More recently, a transcriptomic study on copper stress was performed in *E. faecalis* OG1RF strain, which helped to identify other regulators putatively involved in copper homeostasis through the *cop* operon [33]. Regarding manganese homeostasis in *E. faecalis* JH2-2, it was suggested that the *efaCBA* operon, encoding the virulence factor EfaA, is regulated by EfaR in a manganese-dependent way [34]. Nothing has yet been described on zinc regulation in this species. The poor knowledge on how these bacteria cope with different metals in the environment, being able to survive and cause infection, propels our work on the transcriptional response to metal stresses of *E. faecalis* V583, a vancomycin resistant clinical isolate and the first *E. faecalis* strain to have its genome sequenced. We performed DNA microarray experiments on *E. faecalis* V583 grown in the presence of Zn^{2+}, Mn^{2+} and Cu^{2+} and give the first description of *E. faecalis* V583 transcriptomes under high concentrations of these metal ions. Such studies will help unravel some important mechanisms that are involved in metal regulation in this organism.

Materials and Methods

Media and growth conditions

E. faecalis strain V583 [15] was grown in M17 with 0.5% glucose (GM17) and metal solution when required. GM17 contains 2.0 µM Mn^{2+}, 8.1 µM Zn^{2+} [25] and 2.1 mM SO_4^{2-} [35]. Data for Cu^{2+} are not available. Metal sensitivity tests were performed as follows: growth of *E. faecalis* V583 in the presence of various concentrations of each metal was followed in Schott GL18 glass tubes (Schott, Elmsford, NY, USA) and OD_{600} was read using a Spectronic 21D (Milton Roy, Pont-Saint-Pierre, France). Sterile metal stock solutions were prepared in water and stored at -20ºC.

Transcriptome Experiments

DNA microarray experiments were performed essentially as described previously [36, 37]. RNA was isolated from 30 ml of *E. faecalis* V583 culture grown until mid-exponential phase in GM17 with either (added-metal concentrations): $ZnSO_4$ (0, 4 mM), $MnSO_4$ (0, 0.4 mM) or $CuSO_4$ (0, 0.05 mM). Cells were harvested by centrifugation for 1 min at 10000 rpm at room temperature. Cell pellets were immediately frozen in liquid nitrogen and stored at -80°C. Initial steps of RNA isolation were performed as previously described [37]. Isolation was completed with the Roche High Pure RNA isolation kit (Roche Diagnostics GmbH, Mannheim, Germany). cDNA synthesis and indirect labeling with Cy-3-dCTP and Cy-5-dCTP were performed as reported [37]. Labeled cDNA was mixed and hybridized on glass slides carrying 70-mer oligonucleotides for 3160 genes of *E. faecalis* V583 [38]. Hybridization (16 h at 45°C) was performed with Ambion Slidehyb #1 hybridization buffer (Ambion Europe Ltd., Huntingdon, United Kingdom). Slides were scanned in a GeneTac LS IV confocal laser scanner (Genomics Solutions, Huntingdon, United Kingdom).

DNA Microarray Data Analysis

Slide images were analyzed using GenePix Pro 6.0 software. Processing and normalization (LOWESS spotpin-based) of slides was done with the in-house developed *MicroPrep* software [36]. DNA microarray data were obtained from at least two independent biological replicates and a dye swap hybridized to three glass slides. Expression ratios were calculated from the measurements of nine spots per gene. Differential expression of genes was determined as previously described [37]. A gene was considered differentially expressed when the *p* value was ≤0.0001 and the ratio ≥ |1.5|. Data obtained were further analyzed and grouped in functional categories according to the JCVI (John Craig Venter Institute) website (http://cmr.jcvi.org). A Venn diagram was used to visualize and compare the results obtained for the three metal experiments.

Data were uploaded in GEO with accession numbers # GSE30947, GSE30948 and GSE30949.

Semi-quantitative Reverse Transcriptase (sqRT)-PCR

Expression of selected genes in *E. faecalis* V583 was studied by sqRT-PCR, to confirm results obtained in the DNA microarray experiments. The strain was grown in GM17, with and without one of the following metal solutions: $ZnCl_2$ (at 4 mM added-metal concentration), $MnCl_2$ (at 0.4 mM added-metal concentration) and $CuSO_4$ (at 0.05 mM added-metal concentration). Cells were grown until mid-exponential phase; RNA was then extracted with Qiagen RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany), following the manufacturers' procedure. cDNA synthesis was done with the Roche Transcriptor High Fidelity cDNA synthesis Kit (Roche Diagnostics GmbH). For these reactions, cDNA dilutions 10^0, 10^{-1} and 10^{-2}

were used. PCR products were obtained with Finnzymes® Taq DNA polymerase MasterMix (Finnzymes OY, Espoo, Finland). Primers used are described in Table 1.

Transcriptional *lacZ* fusion construction

In order to observe the effect of addition of metals in gene expression through β-galactosidase assays, a transcriptional fusion with the *E. coli lacZ* gene was constructed in plasmid pILORI4 [39]. Primer pair P*ef0575*-1/P*ef0575*-2 was used to generate a PCR fragment spanning the whole upstream region of *ef0575* (Table 1). The fragment was cloned in EcoRI/BamHI site of pILORI4, which was subsequently introduced in the strain VE14089, corresponding to strain V583 cured of its plasmids [40].

Table 1. List of primers used in this study.

Gene	Sequence (5' to 3')	Amplicon size (bp)
ef0575	TTTTGTGTACCATACAATCG	813
	AAGTGAAAGTCATACAGACC	
ef0758	AGGCTTTGGAGATACGTATCG	1549
	TTGAGACACGGTGAGCATAGC	
mntH2	TATTGCAAAACGAAAGAAGG	1620
	TAACCTCCTCTACTTGTTGC	
ef1400	GACACTGGAAGTTGAATCAGG	1911
	ACCAACATCAGCAAACACTGC	
efaC	CCTTATACTGATTTTAAGGC	814
	AAACCATCAATAAATGCAGC	
ef0575 promoter region	GAGAAGAATTCAATGGTCTTCCCATGTATTTAGG	548
	AAGCAGGATCCATTTTCCAGCACCATTTGGACC	

β-galactosidase assays

The procedure was based on the assay described by Miller [41]. Cells were grown in GM17 with erythromycin 50 µg/ml and metals. Metal solutions were used in the following added-metal concentrations $ZnCl_2$: 0 and 4 mM, $MnCl_2$: 0 and 0.4 mM, $CuSO_4$: 0 and 0.05 mM. Mid-log cells were spun down and frozen in liquid nitrogen to be used later. Frozen cell pellets were resuspended in the same volume of Z buffer (Na_2HPO_4 0.06 M, NaH_2PO_4 0.04 M, KCl 0.01 M, $MgSO_4$ 0.001 M and β-mercaptoethanol 0.05 M, pH 7.0), and the OD_{600} was measured. Diluted cells were permeabilized with 100 µl of chloroform and 50 µl of 0.1% SDS. Reactions were started with the addition of 200 µl of 2-Nitrophenyl β-D-galactopyranoside (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) (4 mg/ml in 0.1M phosphate buffer, pH 7.0) and stopped with 500 µl of 1 M Na_2CO_3. Cells were centrifuged for 5 min at 14000 rpm and the OD at 420 and 550 nm were recorded. Activity of LacZ (in Miller units) was calculated according to Miller [41]. These assays were performed as three independent replicates.

Results

We have studied the transcriptional response of *E. faecalis* V583 to different metal stresses using a genome-wide DNA microarray approach. By allowing the cells to grow until mid-exponential phase in the presence of high concentrations of metals, we were able to investigate the effects of prolonged exposure to these stresses. To simplify, we will use the term “metal stress” when referring to the prolonged exposure to high concentration of metals. In each metal experiment, the transcriptomes of V583 grown in GM17 with added metal and in GM17 without added metal

were compared. To find the appropriate concentrations of metal solutions to use in these experiments, growth of *E. faecalis* V583 in GM17 with different metal concentrations was recorded. For DNA microarray experiments added-metal concentrations of 4 mM Zn^{2+}, 0.4 mM Mn^{2+} and 0.05 mM Cu^{2+} were chosen as these only slightly affected cell growth, causing no more than a 30 min delay in growth, as shown in Figure 1.

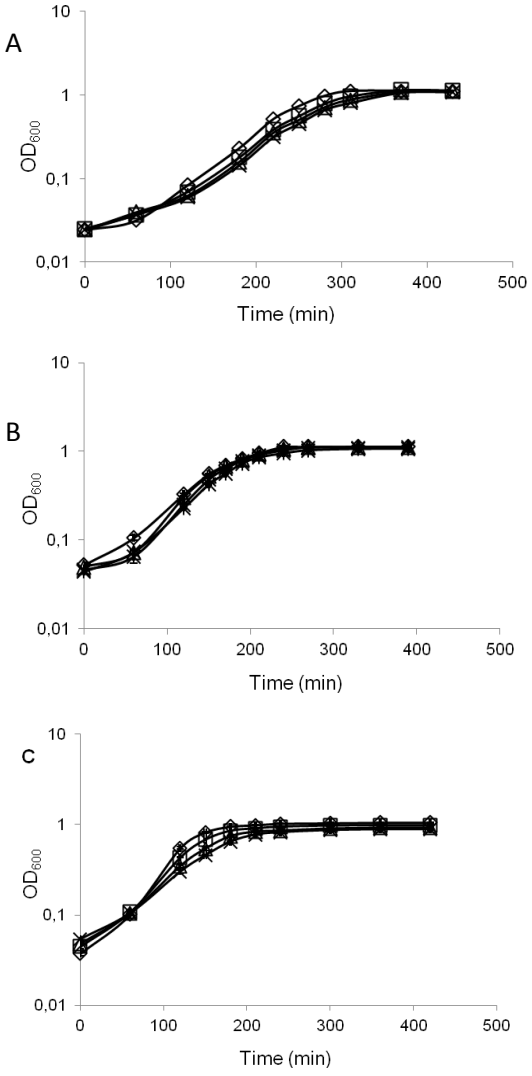

Figure 1. *E. faecalis* V583 growth in GM17 with and without added metals. **A**. *E. faecalis* V583 growth in GM17 (empty diamonds) and GM17 with 4 mM $ZnSO_4$ (empty squares), 5 mM $ZnSO_4$ (empty triangles) or 6 mM $ZnSO_4$ (crosses); **B**. *E. faecalis* V583 growth in GM17 (empty diamonds) and GM17 with 0.4 mM $MnSO_4$ (empty triangles), 1 mM $MnSO_4$ (crosses) or 2 mM $MnSO_4$ (asterisks); **C.** *E. faecalis* V583 growth in GM17 (empty diamonds) and GM17 with 0.05 mM $CuSO_4$ (empty squares), 0.075 mM $CuSO_4$ (empty triangles) and 0.100 mM $CuSO_4$ (crosses). All experiments were performed in triplicate.

The DNA microarray results using the three metal concentrations are represented in Figure 2. In order to see the relevance of each gene functional category in response to the imposed metal stresses, we have represented the weight of each category by dividing the number of genes differentially expressed in each category by the total number of predicted genes in that category in the genome of *E. faecalis* V583. Most of the genes that were differentially expressed in the presence of any of the three metals code for hypothetical proteins. However, this group of genes does not assume a particular relevance in response to the imposed metal stresses, as they represent less than 4% of all hypothetical proteins in the V583 genome. Other functional categories, particularly those including genes encoding transport and binding proteins, reaching weight values of 14%, are more important for growth in the presence of these high metal concentrations.

Genes encoded by the plasmids pTEF1, pTEF2 or pTEF3 present in *E. faecalis* V583 [15] were not differentially expressed in any of these DNA microarray experiments.

Zn^{2+}-regulated genes

Genes differentially expressed in the presence of 4 mM $ZnSO_4$ represent ca. 4.8% of all V583 genes (Table S1). Most of them are up-regulated (87 genes). Genes putatively involved in transport represent approximately 25% of all differentially expressed genes; most of these are induced in the presence of Zn^{2+}. Of these transport related genes 45% encode proteins described as metal transporters, according to the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov). About 16% of all differentially expressed genes are present in mobile genetic regions, including ten up-regulated genes in the pathogenicity island (PAI).

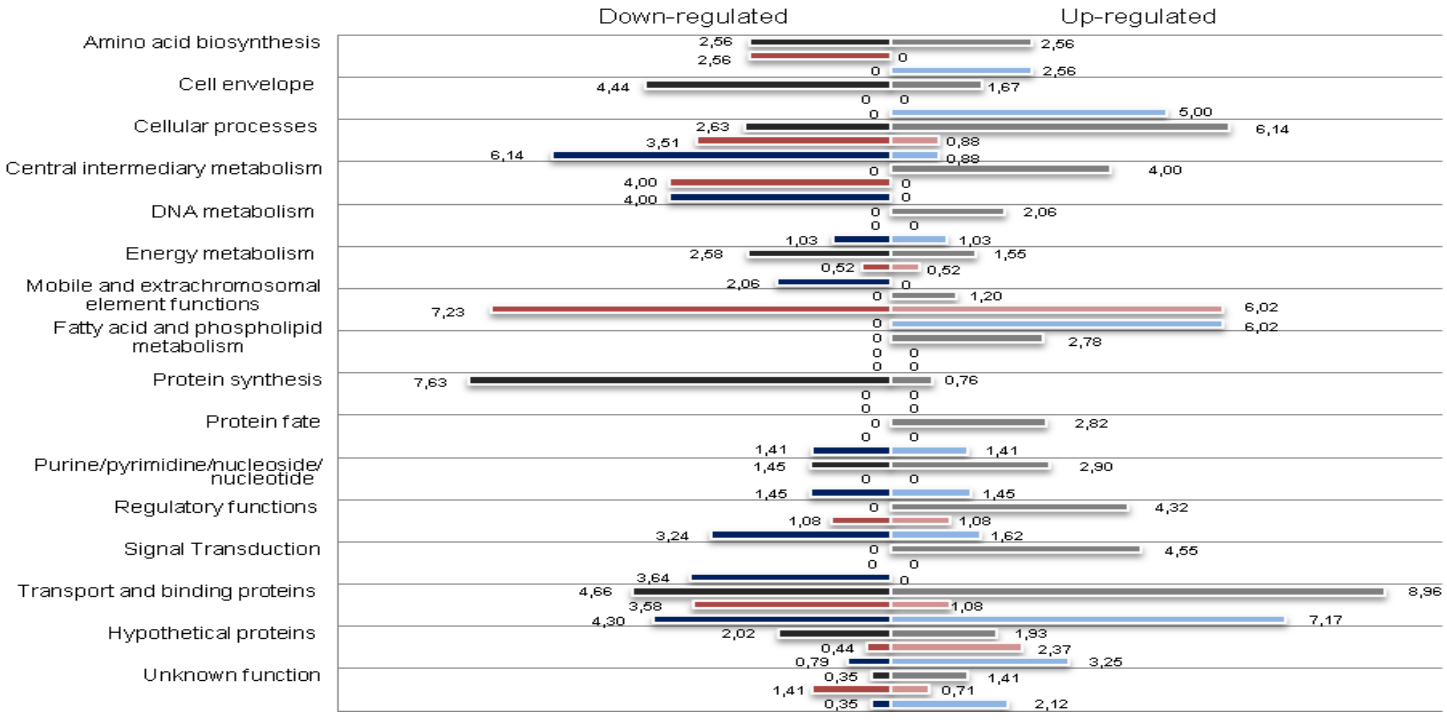

Figure 2. Representation of *E. faecalis* V583 transcriptome responses to metal stress by functional categories. Representation of the weight of each functional category in the transcriptome responses of *E. faecalis* V583 to high concentrations of Zn^{2+}, Mn^{2+} or Cu^{2+}. The weight represents the percentage of genes differentially expressed in each category relative to the total number of predicted genes in that category in the genome of V583. Zinc experiments – black/grey bars; manganese experiments - dark pink/light pink; copper experiments – dark blue/light blue. The weight in percentage is given at the sides of the bars.

Several metabolic pathways (amino acid, purine, glycerophospholipid, energy and vitamin metabolism and also peptidoglycan biosynthesis) were affected by zinc stress, mostly by up-regulation of some of the genes involved in those pathways. Seven genes related to the cell envelope were repressed in the presence of zinc stress; three of these genes are involved either in teichoic acid biosynthesis (*ef2486* and *ef2487*) or in peptidoglycan biosynthesis (*ef0993*). In addition, genetic information processing was altered, mainly by repression of nine ribosomal genes, which may be an indication of a slight difference in growth in the presence of zinc. Ten genes involved in cellular processes were also differentially expressed. Amongst these are genes encoding stress related proteins (Gls24, EF0781, EF1058 and EF1084), adhesion lipoproteins (EF0055, EF0577 and EfaA) and a putative aggregation substance (EF0149) proposed to be related to virulence [42]. Most of these genes were up-regulated, suggesting an important role in adjustment to this stress. All putative regulatory genes that showed differential expression under zinc stress were up-regulated. Six genes involved in signal transduction were up-regulated in the presence of zinc. These include genes encoding a phosphotransferase (PTS) system involved in the mannose pathway (EF0552-3), KdpDE (constituting the two-component system Ehk-Err12 reported to be involved in potassium transport [43]), and the two-component system Err-Ehk06 (EF1260-1). Overall, eight genes differentially expressed in the presence of zinc stress were proposed to be involved in virulence.

ZnuABC is a zinc uptake system first described in *E. coli* [44]. Its homologue in V583 (EF0055-EF0057) is encoded by the most down-regulated operon in the experiments with zinc. An operon encoding a cadmium translocating P-type ATPase and a putative SapB family protein (*ef0758, ef0759*), together with another cadmium translocating P-type ATPase encoding gene (*ef1400)*, were the highest up-regulated genes under zinc stress. The *glnA* and *glnR* genes, involved in amino acid

metabolism, were induced. The *fatB-ceuDCB* cluster (*ef3085-ef3082*), proposed to be involved in iron transport and virulence [42], was up-regulated.

Mn^{2+}-regulated genes

During growth in the presence of 0.4 mM manganese, approximately 2.4% of all genes of *E. faecalis* V583 (Table S2) were differentially expressed, most of them being up-regulated (41 genes). Phage encoding genes are apparently very sensitive to manganese stress as they constitute 64% of all differentially expressed genes and 88% of the up-regulated genes under the conditions tested. Approximately 73% of all genes (55 genes) showing differential expression are present within mobile genetic regions. Manganese induced more changes in gene expression of mobile genetic regions than zinc or copper.

Transporters also seem to play important roles in manganese homeostasis. Most of the transport systems are probably related to Mn^{2+} uptake under normal conditions, as the corresponding genes were mainly down-regulated in the presence of the high concentration of manganese applied in this experiment. More than half of the transporter genes differentially expressed in these manganese experiments are described as encoding metal transporters. Other transport-related genes were down-regulated, namely an operon coding for two ABC transporters (*ef0583-4*). One cation efflux family protein showing high homology with the Mn^{2+} efflux system protein MntE in *Streptococcus pneumoniae* [45, 46] is encoded by *ef0859* and it was up-regulated. Four genes that code for transcriptional regulators (*ef0107*, *ef0578*, *ef0579* and *ef2138*) were differentially expressed. Four genes involved in cellular processes were down-regulated, of which two, *ef0577* and *efaA*, encode adhesion lipoproteins and are putatively involved in virulence [42]; the remaining two encode a universal

stress protein mentioned above (EF1058) and AhpC, an alkyl hydroperoxide reductase potentially involved in oxidative stress response [42].

Cu^{2+}-regulated genes

Genes differentially expressed in the presence of 0.05 mM $CuSO_4$ represent ca. 4.1% of all V583 genes (Table S3); most of them were up-regulated (85 genes). In the presence of copper the lowest percentage of differentially expressed genes in mobile genetic regions was seen, namely approximately 10% down-regulated genes.

Transport related genes apparently also play an important role during growth in the presence of this metal as they represent 24% of all affected genes. Most of these were up-regulated. Nine genes related to the cell wall, including the *dlt* operon and *ef0559* which are involved in cell wall biosynthesis, were induced. Genes involved in cellular processes and signaling were mostly down-regulated. In this group are genes that encode two adhesion lipoproteins (EF0577 and EfaA) and one putative aggregation substance (EF0149) proposed to be related to virulence [42] and also seen responding to the other two metals, and four stress response related proteins (EF0298, EF1058, EF1076 and KatA). The *cop* operon (*ef0297-9*), described as being responsible for copper uptake, availability and export in *E. hirae* [30], was the highest induced operon, as previously observed for *E. faecalis* OG1RF [33]. The genes *kdpA* and *kdpB*, involved in potassium transport [43], were also up-regulated. An operon coding for V-type ATP enzymes (EF1492-EF1500), proposed to be related to osmotic stress [42], was also up-regulated. Another operon that codes for two ABC transporters, a hypothetical protein and a GntR family transcriptional regulator (*ef1673-76*) was highly up-regulated. Three genes proposed to be related to virulence [42], encoding a LemA family protein with unknown

function (EF0468), a cell-envelope associated acid phosphatase (EF3245) and a cell wall surface anchor family protein (EF3314) were up-regulated.

In order to confirm the transcriptome results, the expression in GM17 with or without added metals of five genes, *ef0575*, *ef0758*, *mntH2*, *ef1400* and *efaC*, was studied by sqRT-PCR. In these experiments the expression of these five genes showed the same tendency as observed in the DNA microarray experiments with high concentrations of zinc, manganese and copper ions. Results for genes *ef0758* and *mntH2* are shown in Figure S1. The same effects of addition of metals were also observed on β-galactosidase assays with a P*ef0575*::*lacZ* fusion, represented on Figure S2.

Common genes

The Venn diagram in Figure 3 shows that most of the genes that were differentially expressed in the presence of one of the metals were specific for that metal; only a few genes respond to the presence of two metals while a small number of genes respond to all three metals.

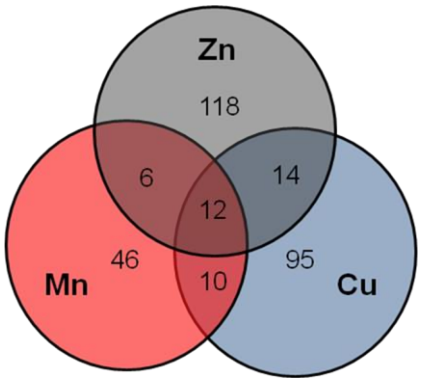

Figure 3. Distribution of differentially expressed genes in the presence of high concentrations of metals. Distribution of *E. faecalis* V583 genes differentially expressed in the presence of 4 mM Zn^{2+} (Zn), 0.4 mM Mn^{2+} (Mn) and 0.05 mM Cu^{2+} (Cu). Venn diagram showing the number of unique and common differentially expressed genes in the three metal DNA microarray experiments.

Differentially expressed genes common to the three metal DNA microarray experiments are shown in Table 2. Most of these genes code for putative metal transporters and some are involved in energy or amino acid metabolism. One such gene, *arcA* (*ef0104*), from the arginine metabolism, was repressed under zinc or copper stress and induced in the presence of manganese. In the same operon, *ef0107-108* were also down-regulated under copper stress and overexpressed with manganese. An operon encoding two ABC transporters and an aspartate aminotransferase (*ef0893-91*) was repressed under manganese stress and induced in the presence of copper; the first gene was also repressed under zinc stress. With the exception of *arcA* and *ef0893*, all genes common to the three metal stress conditions were overexpressed in the presence of zinc and down-regulated in the presence of manganese and copper. Two homologous systems, *efaCBA* and *ef0575-78,* were the most differentially expressed in this group of common genes. Each of these systems codes for two putative cationic ABC transporters and an adhesion lipoprotein. The last system also encodes a putative iron-dependent repressor family protein (EF0578) while immediately downstream another regulator is encoded, EF0579, a TetR family regulator of which the gene was also differentially expressed under all three metal conditions. The *efaCBA* operon has been described to code for a manganese scavenging system [34] and includes the virulence factor EfaA [17]. Also among the group of common genes are *mntH2*, coding for a putative Mn^{2+}/Fe^{2+} transporter, and *ef1058,* encoding a putative universal stress protein.

Table 2. Genes differentially expressed in *E. faecalis* V583 grown in the presence of $ZnSO_4$, $MnSO_4$ or $CuSO_4$.

Gene	Fold change [a]			Description [b]
	Zn	Mn	Cu	
ef0104 (arcA)	-1.59	1.99	-4.67	arginine deiminase
ef0575	2.64	-8.21	-1.85	cationic ABC transporter, ATP-binding protein
ef0576	3.97	-7.04	-1.86	cationic ABC transporter, permease protein
ef0577	3.85	-5.39	-1.67	adhesion lipoprotein
ef0578	6.40	-4.77	-1.49	helix-turn-helix, iron-dependent repressor family
ef0579	2.62	-1.90	-1.27	transcriptional regulator, putative
ef0893	-1.52	-1.72	2.18	amino acid ABC transporter, amino acid binding/ permease protein
ef1057 (mntH2)	2.97	-7.69	-3.10	Mn^{2+}/Fe^{2+} transporter, NRAMP family
ef1058	5.36	-5.80	-3.78	universal stress protein family
ef2074 (efaC)	2.94	-16.58	-2.24	ABC transporter, ATP-binding protein
ef2075 (efaB)	4.10	-13.14	-2.01	ABC transporter, permease protein
ef2076 (efaA)	4.02	-11.54	-2.03	endocarditis specific antigen

[a] Metals used (added-metal concentrations in GM17): 4 mM $ZnSO_4$, 0.4 mM $MnSO_4$ or 0.05 mM $CuSO_4$; fold change represents the gene expression in the presence of the indicated metal ion over the gene expression with no added metal. Genes were considered differentially expressed when fold changes were ≤ |1.5| and *p* values ≤ 10^{-4}.

[b] Annotation according to the NCBI website (http://www.ncbi.nlm.nih.gov).

Discussion

Zn^{2+}, Mn^{2+} and Cu^{2+} play crucial roles in bacterial cells and in the host. Moreover, both host and bacteria can modulate their responses according to concentrations of the three metal ions [47]. The purpose of this study was to obtain more knowledge on the genes involved in *E. faecalis* response to high metal ions concentrations. The analysis of the genome-wide transcriptional response of *E. faecalis* V583 to zinc, manganese and copper stresses revealed induction of genes involved in different functional categories. Particularly relevant functions were transport and binding, metabolism and cellular processes, cell envelope and signal transduction.

Transport and binding

Metal homeostasis in bacteria is achieved by metal export and uptake systems. Some of these transporter systems were highly up- or down-regulated in our experiments. The *cop* operon (*ef0297-99*) was the highest induced operon when *E. faecalis* was grown in the presence of copper. This was expected as the encoded transport system is the main factor in copper homeostasis in *Enterococcus* [30, 33]. Notwithstanding this, it has been suggested that additional putative genes and transcriptional regulators might be able to mediate the response of *E. faecalis* to copper [33]. In fact, we observed the up-regulation of other transport genes in the presence of this metal, namely *ef1673-76,* encoding two putative ABC transporters, a gene for a hypothetical protein (*ef1674*) and a transcriptional regulator gene (*ef1676*). Some of these genes were also affected by SDS and urine [2, 23]. The function of the *ef1673-76* operon has not been elucidated, but its function as encoding a putative metal transport system for Cu^{2+} would explain its significant up-regulation upon copper stress: it was the second highest up-regulated operon, after *cop*.

The most repressed operon when *E. faecalis* was grown in the presence of zinc was *ef0055-57* encoding ZnuABC orthologues. Since it is annotated as a putative zinc uptake system, its down-regulation under high zinc concentration is expected. An operon specifying a cadmium-translocating P-type ATPase and a putative SapB family protein (*ef0758-9*), together with another putative cadmium-translocating P-type ATPase encoding gene (*ef1400*), were highly up-regulated under zinc stress. The low concentration of metals in urine might explain the repression of *ef1400* in urine and its up-regulation with zinc [2].

Two operons, encoding transport proteins, were affected by the three metal ions, being up-regulated with zinc and down-regulated with manganese and copper. These operons, *efaCBA* and *ef0575-78*, encode homologous proteins and were also affected by growth in the presence of urine or blood [22-23]. The EfaCBA proteins constitute a manganese scavenging system [34]. Our results, showing the repression of *ef0575-78* in the manganese experiments, and the fact that the same genes were up-regulated in the transcriptome experiments with blood or with urine [1, 2], two Mn^{2+}-depleted environments, strengthen the supposition [34] that *ef0575-78* encode another manganese scavenging system.

Many transport systems which were differentially regulated in our experiments with metals, have not yet been proven to be involved in metal uptake or efflux. That is the case of *ef0082* gene, encoding a major facilitator ABC transporter, *ef0583-ef0584* and *fatB-ceuDCB* operons. These transport systems were also affected in urine and blood experiments [22-23].

Metabolism and cellular processes

The *glnR* and *glnA* genes, which are involved in glutamine regulation and synthesis, were induced at a high zinc concentration, in this work, and

repressed in blood and urine experiments [1, 2]. The fact that both genes are affected under different conditions, such as during growth in the presence of blood or urine or excess zinc, highlights the importance in *E. faecalis* of nitrogen metabolism and in particular of glutamine and glutamate levels in response to environmental changes.

The arginine deiminase (ADI) operon encodes three enzymatic steps of arginine breakdown, generating ATP. The *arcA* (*ef0104*) gene was down-regulated in zinc and copper stresses and induced in the presence of manganese. The *arcC1* (*ef0106*) gene was repressed in the presence of copper; *ef0107* and *ef0108* were overexpressed with manganese and repressed with copper stress. Arginine can lead to the formation of nitric oxide (NO) through the action of NO synthase. It has been described that *Giardia lamblia* ADI competes with human NO synthase by scavenging arginine from the intestinal environment in the host [48]. Even more, NO synthesis in the intestinal epithelium, which represents a host defense mechanism against pathogen infection, can be blocked by ADI [48, 49]. Therefore, ADI has a relevant role in host colonization and infection. The presence and relative concentration of metals may influence the levels of ADI operon expression, as shown from our results. One might speculate that this could ultimately influence colonization and infection of the host by *E. faecalis*.

Genes involved in the general stress response of *E. faecalis* include class-I heat shock genes *dnaK* and *groEL*, class-III heat shock genes *clpPBCEX*, the regulator gene *ctsR* and the general stress gene *gls24*. In our metal stress experiments, only the latter gene was slightly induced in the presence of zinc stress. Other genes encoding stress related proteins (EF0781, EF1058, EF1076, EF1084) were differentially transcribed under at least one of the metal stresses studied and *ef1058* and *ef1084* were also induced in blood [1]. The oxidative stress related gene *ef2739*, which encodes an alkyl hydroperoxide reductase, was down-regulated in the

presence of manganese suggesting that high concentrations of this metal ion reduces the oxidative burden related with H_2O_2. Of the known virulence factor-encoding genes such as *gelE, sprE,* the *fsr* cluster*, cylB, epa, cps, srtA, atlA and efaA*, only the last gene was differentially expressed in the presence of the studied metal cations, as discussed above.

Cell envelope

The bacterial cell wall prevents metal ions from passively entering the cell. Yet this protective barrier must be overcome in order for the cell to meet its metal requirements [50]. Interestingly, some genes involved in the cell envelope were affected in our experiments with copper or zinc, namely those involved in the teichoic acid pathway (*ef2486-87*), found to be repressed in the presence of zinc, blood and urine [1, 2], and the *dltABCD* operon, found to be up-regulated by copper. The *dltABCD* operon is responsible for incorporating D-alanine residues into cell-wall associated teichoic acids and lipoteichoic acids (LTA). D-alanylation of LTA helps regulate many bacterial processes including cation regulation, activity of autolysins, and resistance to antimicrobial compounds [51]. LTA together with peptidoglycans define the polyelectrolyte properties of the cell wall and provide anionic sites for binding of metal cations. Thus, these structures are responsible for cation homeostasis and assimilation [52]. The overexpression of the referred genes in the presence of copper may help sequester the excess of Cu^{2+}, preventing these ions to take part in redox reactions that would lead to the production of ROS able to damage the cell. Also noteworthy, *ef3314* encodes a cell wall surface anchor family protein recently shown to be important for the pathogenicity of *E. faecalis* [53]. It was up-regulated in copper stress. Overall, the cell envelope seems to have a relevant role in the response to high concentrations of copper.

Signal transduction

The genes *kdpDE* (*ef0570-1*), constituting the two-component system *Ehk12-Err12,* reported to be involved in regulation of potassium transport [43], and another two-component system, *Err06-Ehk06* (*ef1260-61*), with unknown function, were induced in the presence of zinc. In *E. faecalis* V583, the *ehk*12-*err*12 locus is located in the PAI, a transferable genetic element containing genes with known or potential roles in virulence, or adaptation and survival in different environments [54]. This is the first report on the influence of zinc on the expression of *err06-ehk06* and *ehk12-err12*.

Summary

In summary, mostly transporters are involved in the response of *E. faecalis* to high concentrations of the metal ions Cu^{2+}, Zn^{2+} or Mn^{2+}. Also, rearrangements in the expression of genes involved in signal transduction, amino acid metabolism and related to the cell envelope may play important roles. The added metals seem to mainly induce metal-specific transcriptional responses, as there were not many differentially expressed genes in common in the three DNA microarray experiments. Most genes affected under the conditions we applied seem to be part of an *E. faecalis* response to environmental stresses and do not constitute a general stress response. This is supported by the negligible number of genes involved in DNA metabolism and encoding general stress proteins. An overall comparison of our transcriptome results with the blood and urine transcriptomes [1, 2] is represented by the heat map in Figure 4a. This representation shows the parallels between *E. faecalis* gene expression in conditions that mimic host environments such as blood and urine and in conditions with high metal concentrations. *E. faecalis* V583 transcriptome responses to the metals studied showed many differentially expressed

genes in common with the referred host environments, as has been discussed throughout this paper. A clear example discussed in this paper is the low concentration of manganese ions in blood and urine, which induced the expression of Mn^{2+} transport systems and adhesion lipoproteins such as the virulence factor EfaA (Figure 4b). As shown here the same Mn^{2+} transporter systems were repressed when *E. faecalis* was confronted with a high Mn^{+2} concentration, adding support to the notion that these transporter systems are important in *E. faecalis* adaptation to the environment and for colonization and virulence. It is plausible that bacteria, in general, sense their environment in terms of its components. As mentioned before, metal ions are essential elements and bacteria need to monitor and control their levels and be able to quickly adapt to changes in these concentrations by readjusting their genome expression profile. In this sense, metal ion concentrations can be extremely important in certain environments as they can trigger or tone down the expression of genes necessary for *Enterococcus* colonization and virulence.

The broadening of our knowledge on the mechanisms that *E. faecalis* employs to maintain metal homeostasis will certainly help understand how this important human pathogen is able to adapt to diverse environments, colonize and become virulent.

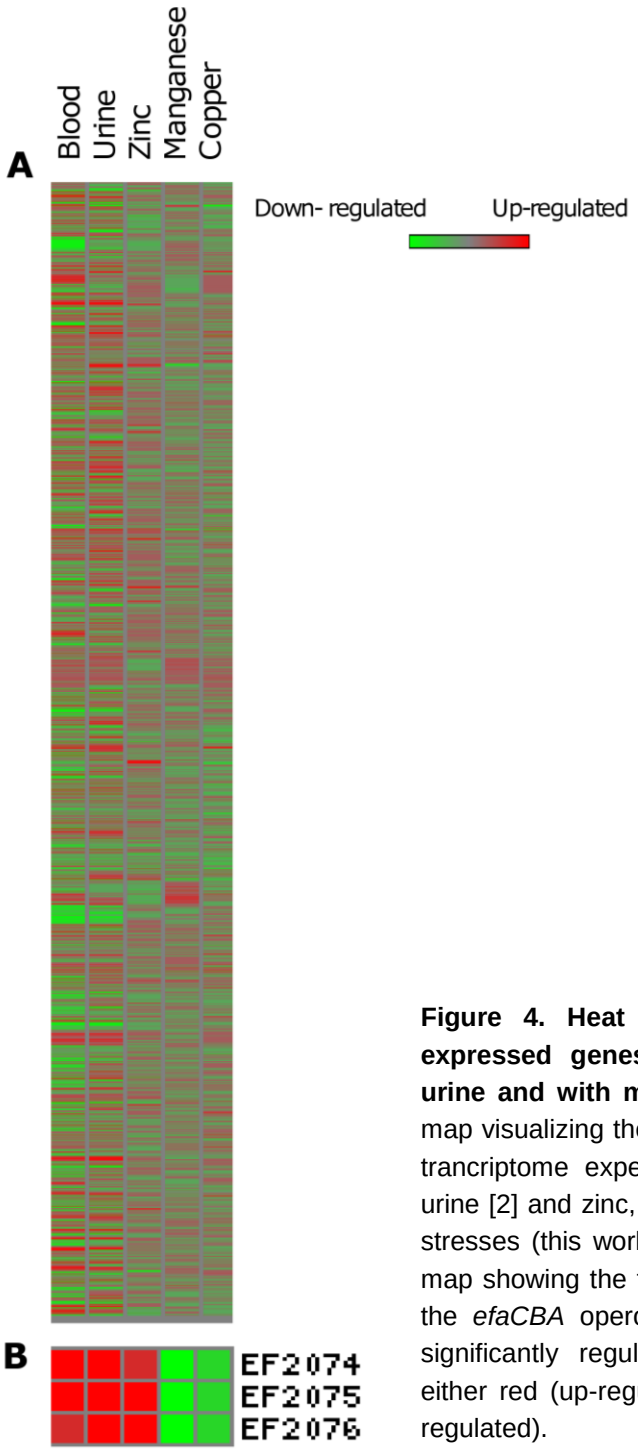

Figure 4. Heat map of differentially expressed genes grown in blood, in urine and with metal stresses. **A**. Heat map visualizing the regulated genes in the trancriptome experiments with blood [1], urine [2] and zinc, manganese and copper stresses (this work). **B**. Detail of the heat map showing the transcriptome results for the *efaCBA* operon. Genes found to be significantly regulated are indicated by either red (up-regulated) or green (down-regulated).

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Supplementary Information

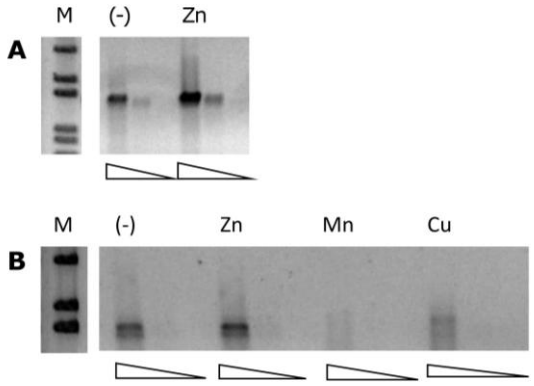

Figure S1. Expression of genes *ef0758* and *mntH2* in the presence of metals by sqRT-PCR. The effect of metal addition on the transcription of the *E. faecalis* V583 genes *ef0758* (**A**) and *mntH2* (**B**), by semi-quantitative Reverse Transcriptase-PCR. Cells were grown in GM17 in the absence (-) or presence of added $ZnCl_2$ 4 mM (Zn), $MnCl_2$ 0.4 mM (Mn) or $CuSO_4$ 0.05 mM (Cu). Triangles represent the decrease in cDNA concentration used in the PCR reactions (dilutions 10^0, 10^{-1} and 10^{-2}). M indicates 1kb plus DNA ladder (Gibco, Invitrogen).

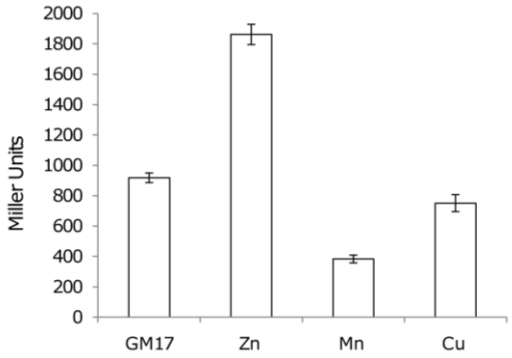

Figure S2. Representation of the effect of metal addition on an *ef0575* promoter::*lacZ* fusion by β-galactosidase assays. Representation of β-galactosidase assays showing the expression of a plasmid-encoded P*ef0575-lacZ* fusion in *E. faecalis* VE14089 grown in the presence of metal ions. The strain was grown in GM17 with or without one of the following added metals: $ZnCl_2$, 4 mM (Zn); $MnCl_2$, 0.4 mM (Mn) or $CuSO_4$, 0.05 mM (Cu).

Table S1. Gene expression data of *E. faecalis* V583 grown to mid-exponential phase in the presence of 4 mM $ZnSO_4$, relative to that during growth in GM17. Significantly differentially expressed genes have a *p* value ≤ 0.0001 and a ratio ≥ |1.5|. "NA" denotes genes for which the *p* value was not available due to the lack of one (or more) replicate(s).

Gene	Fold change	*p* value	Description	Functional category
ef0009	-1,5	3,89E-05	rpsR 30S ribosomal protein S18	Protein synthesis
ef0055	-3,8	1,12E-11	adhesion lipoprotein	Cellular processes
ef0056	-3,4	3,05E-11	ABC transporter, ATP-binding protein	Transport and binding proteins
ef0057	-2,4	1,21E-05	ABC transporter, permease protein	Transport and binding proteins
ef0079	1,6	6,04E-06	gls24 protein	Cellular processes
ef0082	-1,6	1,13E-06	major facilitator family transporter	Transport and binding proteins
ef0083	-2,0	1,63E-09	hypothetical protein	Hypothetical proteins
ef0095	-1,9	2,59E-05	lipoprotein, putative	Cell envelope
ef0100	-1,9	2,53E-05	serS-1 seryl-tRNA synthetase	Protein synthesis
ef0104	-1,6	2,85E-06	arcA arginine deiminase	Energy metabolism
ef0112	-1,9	6,15E-05	hypothetical protein	Hypothetical proteins
ef0113	-1,5	5,79E-06	hypothetical protein	Hypothetical proteins
ef0131	-1,7	4,91E-06	hypothetical protein	Hypothetical proteins
ef0132	-2,3	1,02E-05	hypothetical protein	Hypothetical proteins
ef0133	-2,3	3,93E-06	hypothetical protein	Hypothetical proteins
ef0140	-1,8	5,92E-06	conserved domain protein	Hypothetical proteins
ef0141	-2,1	7,92E-07	hypothetical protein	Hypothetical proteins
ef0142	-1,8	3,53E-05	conserved hypothetical protein	Hypothetical proteins

Gene	Fold change	*p* value	Description	Functional category
ef0149	-1,9	2,79E-05	aggregation substance, putative	Cellular processes
ef0214	-2,0	5,76E-06	rpmC ribosomal protein L29	Protein synthesis
ef0220	-2,0	6,24E-05	rpsH 30S ribosomal protein S8	Protein synthesis
ef0224	-1,6	3,09E-05	rpsE ribosomal protein S5	Protein synthesis
ef0228	-1,9	2,91E-05	adk adenylate kinase	Purines, pyrimidines, nucleosides, and nucleotides
ef0231	-1,6	6,35E-06	rpsM 30S ribosomal protein S13	Protein synthesis
ef0234	-1,6	3,43E-05	rplQ 50S ribosomal protein L17	Protein synthesis
ef0252	1,5	2,57E-06	N-acetylmuramoyl-L-alanine amidase	Cell envelope
ef0288	1,7	1,92E-05	hypothetical protein	Hypothetical proteins
ef0335	1,5	3,16E-05	minor head protein	Mobile and extrachromosomal element functions
ef0386	2,8	9,91E-05	arcC-2 carbamate kinase	Energy metabolism
ef0390	2,4	8,63E-05	N-acyl-D-amino-acid deacylase family protein	Unknown function
ef0399	-1,6	6,93E-06	hypothetical protein	Hypothetical proteins
ef0552	1,6	7,49E-05	PTS system, IIC component	Signal transduction
ef0553	1,8	4,42E-05	PTS system, IID component	Signal transduction
ef0567	1,9	2,81E-06	kdpA potassium-transporting ATPase subunit A	Transport and binding proteins
ef0570	1,7	0,026314	kdpD sensor histidine kinase	Signal transduction
ef0571	1,5	1,09E-04	kdpE DNA-binding response regulator	Regulatory functions
ef0575	2,6	3,59E-10	cationic ABC transporter, ATP-binding protein	Transport and binding proteins
ef0576	4,0	1,41E-11	cation ABC transporter, permease protein	Transport and binding proteins
ef0577	3,9	4,58E-11	adhesion lipoprotein	Cellular processes

Gene	Fold change	*p* value	Description	Functional category
ef0578	6,4	1,87E-05	helix-turn-helix protein, iron-dependent repressor family	Regulatory functions
ef0579	2,6	1,78E-10	transcriptional regulator, putative	Regulatory functions
ef0633	1,8	6,51E-05	tryS-1 tyrosyl-tRNA synthetase	Protein synthesis
ef0685	1,6	5,10E-08	rotamase family protein	Protein fate
ef0739	1,6	8,11E-06	nicotinamide mononucleotide transporter PnuC, putative	Transport and binding proteins
ef0744	1,7	1,52E-06	sodium/dicarboxylate symporter family protein	Transport and binding proteins
ef0758	40,5	1,52E-12	cadium-translocating P-type ATPase	Transport and binding proteins
ef0759	48,9	1,11E-16	sapB protein, putative	Unknown function
ef0778	6,9	4,44E-16	hypothetical protein	Hypothetical proteins
ef0779	5,9	7,41E-13	glycerophosphoryl diester phosphodiesterase family protein	Fatty acid and phospholipid metabolism
ef0780	6,4	2,22E-16	MutT/nudix family protein	DNA metabolism
ef0781	1,8	9,51E-09	cold shock domain family protein	Cellular processes
ef0789	-1,6	2,03E-06	ABC transporter, ATP-binding/permease protein	Transport and binding proteins
ef0790	-1,6	2,38E-05	ABC transporter, ATP-binding/permease protein	Transport and binding proteins
ef0797	1,8	1,73E-08	conserved domain protein	Hypothetical proteins
ef0798	1,6	1,27E-06	hypothetical protein	Hypothetical proteins
ef0803	-1,6	8,40E-05	hypothetical protein	Hypothetical proteins
ef0891	-1,4	0,00056	aspartate aminotransferase	Amino acid biosynthesis
ef0892	-1,3	0,009561	amino acid ABC transporter, ATP-binding protein	Transport and binding proteins
ef0893	-1,5	1,43E-05	amino acid ABC transporter, amino acid-binding/permease protein	Transport and binding proteins
ef0900	-2,0	2,16E-06	aldehyde-alcohol dehydrogenase	Energy metabolism

Gene	Fold change	*p* value	Description	Functional category
ef0907	-1,7	1,82E-07	peptide ABC transporter, peptide binding protein	Transport and binding proteins
ef0932	2,1	4,84E-07	hypothetical protein	Hypothetical proteins
ef0987	1,6	4,19E-05	lipoprotein, putative	Cell envelope
ef0993	-1,5	8,20E-05	murD UDP-N-acetylmuramoyl-L-alanyl-D-glutamate synthetase	Cell envelope
ef1019	1,7	1,22E-05	PTS system, IIC component	Signal transduction
ef1024	1,6	7,23E-05	ppdK pyruvate phosphate dikinase	Energy metabolism
ef1057	3,0	3,05E-10	Mn^{2+}/Fe^{2+} transporter, NRAMP family	Transport and binding proteins
ef1058	5,4	9,55E-11	universal stress protein family	Cellular processes
ef1067	2,1	3,37E-05	hypothetical protein	Hypothetical proteins
ef1082	10,0	NA	hypothetical protein	Hypothetical proteins
ef1083	7,8	1,04E-14	hypothetical protein	Hypothetical proteins
ef1084	7,3	1,65E-14	universal stress protein family	Cellular processes
ef1085	6,9	2,55E-15	conserved domain protein	Hypothetical proteins
ef1192	-1,6	4,67E-05	aquaporin Z	Transport and binding proteins
ef1218	2,6	1,83E-07	spermidine/putrescine ABC transporter, permease protein	Transport and binding proteins
ef1219	2,1	3,68E-04	spermidine/putrescine ABC transporter, permease protein	Transport and binding proteins
ef1221	4,8	7,52E-08	spermidine/putrescine ABC transporter, spermidine/putrescine-binding protein	Transport and binding proteins
ef1228	1,9	1,03E-09	hypothetical protein	Hypothetical proteins
ef1229	1,9	4,19E-09	hypothetical protein	Hypothetical proteins
ef1230	1,8	1,31E-07	hypothetical protein	Hypothetical proteins
ef1260	1,5	0,00022	DNA-binding response regulator	Regulatory functions

Gene	Fold change	*p* value	Description	Functional category
ef1261	1,5	4,94E-06	sensor histidine kinase	Signal transduction
ef1262	6,6	4,96E-13	hypothetical protein	Hypothetical proteins
ef1263	6,7	9,60E-10	hypothetical protein	Hypothetical proteins
ef1301	1,6	2,87E-05	cell division protein, FtsW/RodA/SpovE family	Cellular processes
ef1302	1,5	7,88E-05	transcriptional regulator, putative	Regulatory functions
ef1304	1,9	1,19E-07	magnesium translocating p-type ATPase	Transport and binding proteins
ef1353	1,6	1,62E-05	pdhA pyruvate dehydrogenase complex E1 component, alpha subunit	Energy metabolism
ef1358	1,5	2,74E-06	gldA glycerol dehydrogenase	Central intermediary metabolism
ef1368	1,8	3,50E-06	hypothetical protein	Hypothetical proteins
ef1400	44,0	0	cadmium-translocating P-type ATPase	Transport and binding proteins
ef1402	1,5	2,53E-06	hypothetical protein	Hypothetical proteins
ef1533	2,0	1,65E-06	conserved hypothetical protein	Hypothetical proteins
ef1693	-1,7	3,19E-05	KH domain protein	Unknown function
ef1752	1,8	9,78E-07	hypothetical protein	Hypothetical proteins
ef2074	2,9	4,70E-12	ABC transporter, ATP-binding protein	Transport and binding proteins
ef2075	4,1	1,36E-13	ABC transporter, permease protein	Transport and binding proteins
ef2076	4,0	2,93E-11	endocarditis specific antigen	Cellular processes
ef2094	-1,8	4,81E-05	hypothetical protein	Hypothetical proteins
ef2102	-1,8	6,72E-06	hypothetical protein	Hypothetical proteins
ef2125	-1,7	8,70E-06	hypothetical protein	Hypothetical proteins
ef2126	-1,8	1,59E-06	hypothetical protein	Hypothetical proteins

Gene	Fold change	*p* value	Description	Functional category
ef2131	-1,9	5,70E-07	hypothetical protein	Hypothetical proteins
ef2134	-1,8	4,70E-06	hypothetical protein	Hypothetical proteins
ef2159	1,9	3,32E-07	glnA, glutamine synthetase, type I	Amino acid biosynthesis
ef2160	1,7	3,30E-06	glnR, regulatory protein GlnR	Regulatory functions
ef2364	2,4	1,38E-09	xanthine permease	Transport and binding proteins
ef2365	2,4	4,92E-07	xpt xanthine phosphoribosyltransferase	Purines, pyrimidines, nucleosides, and nucleotides
ef2429	2,8	5,56E-09	guaC guanosine 5'-monophosphate oxidoreductase	Purines, pyrimidines, nucleosides, and nucleotides
ef2430	2,4	3,00E-09	xanthine/uracil permease family protein	Transport and binding proteins
ef2431	2,0	5,73E-07	chlorohydrolase family protein	Unknown function
ef2442	1,5	1,85E-06	phosphate transporter family protein	Transport and binding proteins
ef2484	-1,9	2,02E-05	hypothetical protein	Hypothetical proteins
ef2486	-1,7	5,30E-06	ABC transporter, ATP-binding protein	Transport and binding proteins
ef2487	-1,7	2,89E-06	UDP-galactopyranose mutase	Cell envelope
ef2488	-1,9	1,26E-06	lipoprotein, putative	Cell envelope
ef2490	-1,8	2,62E-07	hypothetical protein	Hypothetical proteins
ef2491	-1,6	8,34E-06	glycosyl transferase, group 2 family protein	Cell envelope
ef2556	-2,3	1,61E-10	fumarate reductase flavoprotein subunit	Energy metabolism
ef2641	-2,2	6,53E-06	glycine betaine/L-proline ABC transporter , ATP-binding subunit	Transport and binding proteins
ef2642	-3,3	4,72E-06	glycine betaine/L-proline ABC transporter , glycine betaine/L-proline-binding/	Transport and binding proteins
ef2702	2,2	3,13E-07	hypothetical protein	Hypothetical proteins
ef2703	2,2	1,76E-08	transcriptional regulator	Regulatory functions

Gene	Fold change	*p* value	Description	Functional category
ef2711	1,7	8,28E-06	transcriptional regulator, AraC family	Regulatory functions
ef2713	-2,2	3,89E-08	cell wall surface anchor family protein	Cell envelope
ef2715	-1,6	6,34E-05	rplL ribosomal protein L7/L12	Protein synthesis
ef3006	1,5	1,07E-05	hypothetical protein	Hypothetical proteins
ef3007	1,7	1,96E-07	hypothetical protein	Hypothetical proteins
ef3027	3,4	1,46E-13	serine protease DO	Protein fate
ef3050	1,6	4,30E-05	tag-2 DNA-3-methyladenine glycosylase I	DNA metabolism
ef3082	1,6	6,59E-05	iron compound ABC transporter, substrate-binding protein	Transport and binding proteins
ef3083	1,8	3,39E-06	iron compound ABC transporter, ATP-binding protein	Transport and binding proteins
ef3084	2,0	3,33E-08	iron compound ABC transporter, permease protein	Transport and binding proteins
ef3085	2,0	6,48E-08	iron compound ABC transporter, permease protein	Transport and binding proteins
ef3104	-1,5	4,70E-05	ABC transporter, ATP-binding protein	Transport and binding proteins
ef3105	-1,7	6,52E-07	hypothetical protein	Hypothetical proteins
ef3170	1,5	4,28E-06	HD/KH domain protein	Unknown function
ef3173	-2,0	1,86E-07	hypothetical protein	Hypothetical proteins
ef3198	1,4	5,67E-05	lipoprotein, YaeC family	Cell envelope
ef3199	1,5	2,16E-06	ABC transporter, permease protein	Transport and binding proteins
ef3200	1,9	3,96E-06	ABC transporter, ATP-binding protein	Transport and binding proteins
ef3230	-1,5	2,64E-06	rpsI 30S ribosomal protein S9	Protein synthesis
ef3255	-1,6	9,39E-05	thiamin biosynthesis lipoprotein ApbE, putative	Cell envelope
ef3256	-1,7	1,59E-06	pheromone cAD1 precursor lipoprotein	Cell envelope
ef3287	-1,7	4,40E-07	tRNA-Pro-2	Hypothetical proteins

Gene	Fold change	*p* value	Description	Functional category
ef3303	1,7	4,14E-08	hypothetical protein	Hypothetical proteins
ef3312	-2,2	5,99E-05	trmE tRNA modification GTPase TrmE	Cellular processes
ef3324	-1,8	8,49E-06	sodium ion-translocating decarboxylase, beta subunit	Energy metabolism
ef3325	-2,4	2,02E-06	acetyl-CoA carboxylase biotin carboxyl carrier protein subunit	Energy metabolism
ef3326	-2,4	1,58E-06	conserved hypothetical protein	Hypothetical proteins

Table S2. Gene expression data of *E. faecalis* V583 grown to mid-exponential phase in the presence of 0.4 mM $MnSO_4$, relative to that during growth in GM17. Significantly differentially expressed genes have a *p* value ≤ 0.0001 and a ratio ≥ |1.5|. "NA" denotes genes for which the *p* value was not available due to the lack of one (or more) replicate(s).

Gene	Fold change	*p* value	Description	Functional category
ef0104	2,0	1,48E-05	arcA, arginine deiminase	Energy metabolism
ef0107	2,0	5,74E-05	transcriptional regulator, Crp/Fnr family	Regulatory functions
ef0108	2,2	3,26E-07	C4-dicarboxylate transporter, putative	Transport and binding proteins
ef0333	-1,6	5,32E-07	conserved hypothetical protein TIGR01630	Hypothetical proteins
ef0335	-1,6	9,85E-06	minor head protein	Mobile and extrachromosomal element functions
ef0339	-1,5	7,74E-05	major capsid protein, putative	Mobile and extrachromosomal element functions
ef0341	-1,6	3,24E-05	hypothetical protein	Hypothetical proteins
ef0343	-1,5	1,13E-05	conserved hypothetycal protein TIGR01725	Hypothetical proteins
ef0344	-1,7	7,86E-07	hypothetical protein	Hypothetical proteins
ef0347	-1,7	5,37E-07	peptide methionine sulfoxide reductase domain protein	Unknown function
ef0348	-1,8	2,50E-08	tail protein	Mobile and extrachromosomal element functions
ef0350	-1,5	1,50E-06	hypothetical protein	Hypothetical proteins
ef0351	-1,5	3,63E-05	structural protein, putative	Mobile and extrachromosomal element functions
ef0354	-1,6	2,15E-05	holin, putative	Mobile and extrachromosomal element functions
ef0355	-1,5	3,18E-07	endolysin, putative	Mobile and extrachromosomal element functions
ef0575	-8,2	1,10E-09	cationic ABC transporter, ATP-binding protein	Transport and binding proteins

Gene	Fold change	*p* value	Description	Functional category
ef0576	-7,0	5,24E-11	cation ABC transporter, permease protein	Transport and binding proteins
ef0577	-5,4	3,59E-11	adhesion lipoprotein	Cellular processes
ef0578	-4,8	9,34E-06	helix-turn-helix, iron-dependent repressor family	Regulatory functions
ef0579	-1,9	7,30E-07	transcriptional regulator, putative	Regulatory functions
ef0583	-1,5	2,49E-06	ABC transporter, ATP-binding/permease protein	Transport and binding proteins
ef0584	-1,5	2,47E-06	ABC transporter, ATP-binding/permease protein	Transport and binding proteins
ef0859	3,2	5,49E-08	cation efflux family protein	Transport and binding proteins
ef0891	-2,2	1,50E-05	aspartate aminotransferase	Amino acid biosynthesis
ef0892	-2,1	2,38E-05	amino acid ABC transporter, ATP binding protein	Transport and binding proteins
ef0893	-1,7	4,02E-06	amino acid ABC transporter, amino acid binding/permease protein	Transport and binding proteins
ef0895	-1,7	3,32E-07	glycerol dehydrogenase, putative	Central intermediary metabolism
ef1057	-7,7	7,51E-12	Mn^{2+}/Fe^{2+} transporter, NRAMP family	Transport and binding proteins
ef1058	-5,8	NA	universal stress protein	Cellular processes
ef1108	-1,6	1,15E-05	oxidoreductase, putative	Unknown function
ef1110	-1,6	2,32E-06	YkgG family protein	Unknown function
ef1425	2,0	1,88E-08	hypothetical protein	Hypothetical proteins
ef1426	1,8	1,24E-06	vrlI protein, putative	Cellular processes
ef1428	1,9	2,19E-06	hypothetical protein	Hypothetical proteins
ef1429	2,0	3,08E-05	hypothetical protein	Hypothetical proteins
ef1431	2,0	1,92E-06	hypothetical protein	Hypothetical proteins
ef1432	2,0	2,09E-07	hypothetical protein	Hypothetical proteins

Gene	Fold change	*p* value	Description	Functional category
ef1436	1,9	2,76E-06	hypothetical protein	Hypothetical proteins
ef1440	1,7	1,41E-05	conserved hypothetical protein TIGR01671	Hypothetical proteins
ef1441	1,8	4,94E-08	hypothetical protein	Hypothetical proteins
ef1442	1,5	2,21E-05	DNA topoisomerase domain protein	Unknown function
ef1447	1,9	3,30E-07	hypothetical protein	Hypothetical proteins
ef1448	1,8	9,74E-07	hypothetical protein	Hypothetical proteins
ef1489	1,8	5,72E-06	hypothetical protein	Hypothetical proteins
ef2074	-16,6	1,20E-13	ABC transporter, ATP-binding protein	Transport and binding proteins
ef2075	-13,1	1,78E-10	ABC transporter, permease protein	Transport and binding proteins
ef2076	-11,5	4,79E-13	endocarditis specific antigen	Cellular processes
ef2084	1,6	7,69E-05	hypothetical protein	Hypothetical proteins
ef2088	1,7	3,05E-06	hypothetical protein	Hypothetical proteins
ef2092	2,0	2,98E-06	hypothetical protein	Hypothetical proteins
ef2096	1,9	1,23E-05	tail protein	Mobile and extrachromosomal element functions
ef2121	2,2	7,44E-08	hypothetical protein	Hypothetical proteins
ef2123	2,3	1,82E-07	hypothetical protein	Hypothetical proteins
ef2124	2,2	1,84E-08	methyltransferase, putative	Mobile and extrachromosomal element functions
ef2125	2,3	5,12E-09	hypothetical protein	Hypothetical proteins
ef2126	2,1	9,48E-09	hypothetical protein	Hypothetical proteins
ef2127	2,1	1,37E-07	hypothetical protein	Hypothetical proteins
ef2128	2,3	6,80E-08	conserved hypothetical protein	Hypothetical proteins

Gene	Fold change	*p* value	Description	Functional category
ef2129	2,1	6,41E-08	DNA replication protein, putative	Mobile and extrachromosomal element functions
ef2130	2,0	5,78E-08	DnaD domain protein	Unknown function
ef2131	2,3	3,99E-09	hypothetical protein	Hypothetical proteins
ef2132	2,3	4,94E-09	recT protein, putative	Mobile and extrachromosomal element functions
ef2133	2,2	2,11E-08	hypothetical protein	Hypothetical proteins
ef2134	2,0	3,73E-07	hypothetical protein	Hypothetical proteins
ef2135	2,0	6,71E-07	hypothetical protein	Hypothetical proteins
ef2136	2,1	4,22E-07	hypothetical protein	Hypothetical proteins
ef2137	2,0	4,41E-08	hypothetical protein	Hypothetical proteins
ef2138	2,2	1,18E-08	transcriptional regulator, Cro/CI family	Regulatory functions
ef2140	2,1	1,33E-09	conserved hypothetical protein	Hypothetical proteins
ef2141	2,2	3,01E-08	transcriptional regulator, Cro/CI family	Mobile and extrachromosomal element functions
ef2364	2,8	7,86E-05	xanthine permease	Transport and binding proteins
ef2738	-1,6	1,55E-07	thioredoxin reductase/glutathione related protein	Unknown function
ef2739	-1,5	2,46E-05	ahpC alkyl hidroperoxide reductase	Cellular processes
ef2961	-1,5	3,32E-07	rbsK ribokinase	Energy metabolism

Table S3. Gene expression data of *E. faecalis* V583 grown to mid-exponential phase in the presence of 0.05 mM $CuSO_4$, relative to that during growth in GM17. Significantly differentially expressed genes have a *p* value ≤ 0.0001 and a ratio ≥ |1.5|. "NA" denotes genes for which the *p* value was not available due to the lack of one (or more) replicate(s).

Gene	Fold change	*p* value	Description	Functional category
ef0081	1,6	3,21E-06	hypothetical protein	Cell envelope
ef0104	-4,7	3,33E-16	arginine deiminase	energy metabolism
ef0106	-6,6	7,62E-14	carbamate kinase	Energy metabolism
ef0107	-4,6	0	transcriptional regulator, Cro/CI family	Regulatory functions
ef0108	-4,6	4,44E-16	C4-dicarboxylate transporter, putative	Transport and binding proteins
ef0125	1,5	1,06E-05	IS256, transposase	Mobile and extrachromosomal element functions
ef0131	-1,8	1,96E-07	hypothetical protein	Hypothetical proteins
ef0139	-1,8	1,97E-05	FtsK/SpoIIIE family protein	Cellular processes
ef0140	-1,6	7,41E-06	hypothetical protein	Hypothetical proteins
ef0141	-1,6	1,94E-05	hypothetical protein	Hypothetical proteins
ef0143	-2,0	7,55E-06	transcriptional regulator, Cro/CI family	Regulatory functions
ef0144	-1,6	4,53E-05	hypothetical protein	Hypothetical proteins
ef0149	-1,5	2,19E-05	aggregation substance, putative	Cellular processes
ef0151	-1,6	6,47E-05	hypothetical protein	Hypothetical proteins
ef0247	-1,5	4,35E-07	amino acid ABC transporter, amino acid-binding/permease protein	Transport and binding proteins
ef0297	12,9	0	transcriptional repressor CopY	Regulatory functions
ef0298	9,6	0	copper-translocating P-type ATPase	Cellular processes
ef0299	6,1	3,25E-11	copper transport protein CopZ	Transport and binding proteins

Gene	Fold change	*p* value	Description	Functional category
ef0318	1,6	1,70E-06	hypothetical protein	Hypothetical proteins
ef0322	1,7	3,14E-05	hypothetical protein	Hypothetical proteins
ef0324	1,6	6,69E-05	hypothetical protein	Hypothetical proteins
ef0332	1,8	1,27E-08	hypothetical protein	Hypothetical proteins
ef0333	1,5	4,28E-06	conserved hypothetical protein TIGR01630	Hypothetical proteins
ef0334	1,6	2,27E-06	portal protein	Mobile and extrachromosomal element functions
ef0337	1,7	1,32E-09	hypothetical protein	Hypothetical proteins
ef0338	1,5	3,31E-08	scaffold protein	Mobile and extrachromosomal element functions
ef0340	1,6	1,65E-08	hypothetical protein	Hypothetical proteins
ef0341	1,6	2,23E-08	hypothetical protein	Hypothetical proteins
ef0342	1,5	4,57E-08	hypothetical protein	Hypothetical proteins
ef0343	1,7	2,27E-08	conserved hypothetical protein TIGR01725	Hypothetical proteins
ef0345	1,6	7,65E-09	hypothetical protein	Hypothetical proteins
ef0347	1,5	4,14E-07	peptide methionine sulfoxide reductase domain protein	Unknown function
ef0348	1,5	4,80E-07	tail protein	Mobile and extrachromosomal element functions
ef0349	1,5	1,09E-07	tail protein, putative	Mobile and extrachromosomal element functions
ef0352	1,6	1,10E-07	hypothetical protein	Hypothetical proteins
ef0443	1,6	2,45E-07	LysM domain protein	Unknown function
ef0468	2,3	2,05E-11	LemA family protein	Unknown function
ef0469	2,5	6,95E-14	hypothetical protein	Hypothetical proteins

Gene	Fold change	*p* value	Description	Functional category
ef0478	1,6	1,55E-06	hypothetical protein	Hypothetical proteins
ef0559	1,6	6,28E-05	polysaccharide biosynthesis family protein	Cell envelope
ef0562	1,8	6,18E-07	hypothetical protein	Hypothetical proteins
ef0563	1,8	1,56E-06	hypothetical protein	Hypothetical proteins
ef0564	2,1	3,23E-08	hypothetical protein	Hypothetical proteins
ef0567	1,7	1,13E-06	kdpA - potassium-transporting ATPase subunit A	Transport and binding proteins
ef0568	1,6	1,69E-07	kdpB - potassium-transporting ATPase subunit B	Transport and binding proteins
ef0575	-1,9	5,67E-07	cationic ABC transporter, ATP-binding protein	Transport and binding proteins
ef0576	-1,9	6,25E-07	cationic ABC transporter, permease protein	Transport and binding proteins
ef0577	-1,7	NA	adhesion lipoprotein	Cellular processes
ef0578	-1,5	NA	helix-turn-helix, iron-dependent repressor family	Regulatory functions
ef0579	-1,3	0,01406	transcriptional regulator, putative	Regulatory functions
ef0634	1,7	5,65E-10	decarboxylase, putative	Unknown function
ef0635	1,5	9,80E-09	amino acid permease protein	Transport and binding proteins
ef0798	1,6	9,86E-09	hypothetical protein	Hypothetical proteins
ef0809	1,6	9,13E-06	hypothetical protein	Cell envelope
ef0853	-1,6	7,46E-06	hypothetical protein	Hypothetical proteins
ef0854	-1,5	3,49E-05	signal peptidase	Protein fate
ef0891	2,1	3,65E-08	aspartate aminotransferase	Amino acid biosynthesis
ef0892	2,3	3,46E-09	amino acid ABC transporter, ATP-binding protein	Transport and binding proteins
ef0893	2,2	1,25E-11	amino acid ABC transporter, amino acid binding/permease protein	Transport and binding proteins

Gene	Fold change	*p* value	Description	Functional category
ef0932	2,7	1,25E-06	hypothetical protein	Hypothetical proteins
ef0938	-1,7	3,30E-06	ABC transporter, ATP-binding/TOBE domain protein	Transport and binding proteins
ef0972	1,6	6,06E-06	DNA repair exonuclease family protein	DNA metabolism
ef1017	-2,2	4,84E-11	PTS system, IIB component	Signal transduction
ef1018	-3,7	2,85E-09	PTS system, IIA component	Signal transduction
ef1019	-3,2	2,23E-05	PTS system, IIC component	Signal transduction
ef1028	1,8	4,14E-10	hydrolase, alpha/beta hydrolase fold family	Unknown function
ef1033	-1,7	1,01E-06	6-aminohexanoate-cyclic-dimer hydrolase, putative	Central intermediary metabolism
ef1057	-3,1	3,85E-05	Mn^{2+}/Fe^{2+} transporter, NRAMP family	Transport and binding proteins
ef1058	-3,8	NA	universal stress protein	Cellular processes
ef1076	-1,6	4,68E-05	streptomycin 3''-adenylyltransferase, putative	Cellular processes
ef1190	-2,1	9,61E-09	hypothetical protein	Hypothetical proteins
ef1191	-1,8	8,67E-07	DegV family protein	Unknown function
ef1263	1,7	6,56E-07	hypothetical protein	Hypothetical proteins
ef1304	1,6	2,16E-06	magnesium-translocating P-type ATPase	Transport and binding proteins
ef1412	2,0	8,23E-07	conserved domain protein	Hypothetical proteins
ef1492	1,9	5,48E-12	V-type ATPase, subunit F	Transport and binding proteins
ef1493	1,9	1,77E-09	V-type ATP synthase subunit I	Transport and binding proteins
ef1494	1,9	2,32E-08	V-type ATP synthase subunit K	Transport and binding proteins
ef1495	1,9	5,72E-09	V-type ATPase, subunit E	Transport and binding proteins
ef1496	1,8	4,27E-09	V-type ATPase, subunit C	Transport and binding proteins
ef1497	1,7	1,00E-09	V-type ATP synthase subunit F	Transport and binding proteins

Gene	Fold change	*p* value	Description	Functional category
ef1498	1,8	8,35E-11	V-type ATP synthase subunit A	Transport and binding proteins
ef1499	1,9	2,22E-11	V-type ATP synthase subunit B	Transport and binding proteins
ef1500	1,9	1,50E-09	V-type ATP synthase subunit D	Transport and binding proteins
ef1501	1,9	5,73E-10	hypothetical protein	Hypothetical proteins
ef1533	2,4	4,63E-10	conserved hypothetical protein	Hypothetical proteins
ef1541	-3,2	2,21E-12	hypothetical protein	Hypothetical proteins
ef1597	-1,5	4,59E-06	katA catalase/peroxidase	Cellular processes
ef1673	3,3	6,86E-09	ABC transporter, ATP-binding protein	Transport and binding proteins
ef1674	11,1	1,67E-12	hypothetical protein	Hypothetical proteins
ef1675	5,9	NA	ABC transporter, ATP-binding protein	Transport and binding proteins
ef1676	9,4	1,51E-06	transcriptional regulator, GntR family	Regulatory functions
ef1718	-2,3	7,60E-09	pyrC dihydroorotase	Purines, pyrimidines, nucleosides, and nucleotides
ef1752	1,9	2,67E-09	conserved hypothetical protein	Hypothetical proteins
ef1796	1,5	1,78E-05	lipoprotein, putative	Cell envelope
ef1903	1,7	3,82E-09	conserved hypothetical protein	Hypothetical proteins
ef2074	-2,2	5,47E-06	ABC transporter, ATP-binding protein	Transport and binding proteins
ef2075	-2,0	2,36E-05	ABC transporter, permease protein	Transport and binding proteins
ef2076	-2,0	4,38E-05	endocarditis specific antigen	Cellular processes
ef2211	2,2	2,76E-08	conserved hypothetical protein TIGR01655	Hypothetical proteins
ef2213	-1,7	2,39E-06	PTS system, IIBC components	Signal transduction
ef2440	-1,6	2,50E-05	celC-related protein	DNA metabolism

Gene	Fold change	*p* value	Description	Functional category
ef2441	-1,7	1,23E-06	hypothetical protein	Hypothetical proteins
ef2606	1,8	6,56E-06	hypothetical protein	Hypothetical proteins
ef2703	1,6	2,58E-07	transcriptional regulator	Regulatory functions
ef2720	-1,7	4,04E-07	ABC transporter, ATP-binding protein	Transport and binding proteins
ef2730	1,6	2,60E-05	secE preprotein translocase, SecE subunit	Protein fate
ef2746	2,6	1,51E-14	dltD protein	Cell envelope
ef2747	2,7	2,37E-13	D-alanine--poly(phosphoribitol) ligase subunit 2	Cell envelope
ef2748	2,6	1,33E-12	basic membrane protein DtlB	Cell envelope
ef2749	2,1	2,58E-10	D-alanine--D-alanyl carrier protein ligase	Cell envelope
ef2750	2,1	1,21E-07	hypothetical protein	Hypothetical proteins
ef2771	2,7	3,44E-10	hypothetical protein	Hypothetical proteins
ef2774	1,5	3,32E-05	hypothetical protein	Hypothetical proteins
ef2942	1,6	5,67E-06	hypothetical protein	Hypothetical proteins
ef2943	1,7	4,59E-08	hypothetical protein	Hypothetical proteins
ef2944	1,7	1,34E-08	hypothetical protein	Hypothetical proteins
ef2960	-2,3	5,15E-05	ribose transporter protein RbsD	Transport and binding proteins
ef2961	-1,6	5,27E-05	rbsK ribokinase	Energy metabolism
ef2966	-2,0	1,55E-06	transcriptional antiterminator, bglG family	Regulatory functions
ef2985	3,3	7,43E-13	permease, putative	Transport and binding proteins
ef2986	2,8	2,34E-10	ABC transporter, ATP-binding protein	Transport and binding proteins
ef2987	3,0	4,00E-11	hypothetical protein	Hypothetical proteins

Gene	Fold change	*p* value	Description	Functional category
ef3001	-1,6	7,04E-08	protease synthase and sporulation negative regulatory protein pai 1	Regulatory functions
ef3110	-1,5	9,42E-07	peptide ABC transporter, ATP-binding protein	Transport and binding proteins
ef3239	1,9	2,44E-08	conserved hypothetical protein	Hypothetical proteins
ef3244	1,9	3,17E-08	hypothetical protein	Hypothetical proteins
ef3245	1,6	4,76E-07	cell-envelope associated acid phosphatase	Unknown function
ef3293	1,7	1,41E-05	guaB inositol-5-monophosphate dehydrogenase	Purines, pyrimidines, nucleosides, and nucleotides
ef3314	1,6	1,34E-05	cell wall surface anchor family protein	Cell envelope
ef3325	-1,6	8,71E-05	acetyl-CoA carboxylase biotin carboxyl carrier protein subunit	Energy metabolism

Chapter 3

EfaR is a major regulator of *Enterococcus faecalis* manganese transporters and influences processes involved in host colonization and infection

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Author Contributions

All experiments were performed by the author of this thesis. Planning the experiments, data analysis and manuscript preparation were done by the author and the supervisors Dr. Fátima Lopes and Prof. Dr. Jan Kok.

Table of Contents

Abstract 113
Introduction 115
Materials and Methods 117
Bacterial strains, plasmids and growth conditions 117
DISCLOSE analysis 117
Mutant construction 119
Construction of transcriptional *lacZ* fusions 119
β-galactosidase assays 120
DNA microarray experiments and data analysis 120
Intramacrophage survival assay 121
Oxidative stress assay 122
Biofilm formation assay 122
Results 123
Genes carrying an *ebm* site respond to metals 123
EfaR regulates different transporters encoded by genes with an *ebm* 125
EfaR contributes to *E. faecalis* virulence 129
Discussion 131
Acknowledgements 136
References 137
Supplementary Information 141

Abstract

Metal ions, in particular manganese, are important modulators of bacterial pathogenicity. However, little is known about the role of manganese dependent proteins in the nosocomial pathogen *Enterococcus faecalis,* a major cause of bacterial endocarditis. The present study demonstrates that the DtxR/MntR family metalloregulator EfaR of *E. faecalis* controls the expression of several of its regulon members in a manganese-dependent way. We also show that *efaR* inactivation impairs *E. faecalis* in its ability to form biofilms, to survive inside macrophages and to tolerate oxidative stress. Our results reveal that EfaR is an important modulator of *E. faecalis* virulence, and link manganese homeostasis to enterococcal pathogenicity.

Introduction

Enterococci are nosocomial opportunistic pathogens that can cause infections of the urinary tract, the bloodstream, intra-abdominal and pelvic regions and surgical sites [1, 2]. They are of important concern in the hospital environment because of their ability to bind to, colonize and produce biofilms on medical devices such as stents, catheters, artificial cardiac pacemakers and prosthetic heart valves [3]. Primarily, these bacteria are human commensals that have adapted to complex environments rich in nutrients and low in oxygen such as the gastrointestinal tract and the oral cavity. For enterococci to act as pathogens they must start by adhering to and then invading host tissues. During the process of tissue invasion, enterococci encounter an environment that differs greatly from the site of colonization, with higher redox potentials, limited essential nutrients, phagocytic leukocytes and other host defenses. A potentially limiting factor for bacterial growth is the scarcity of various metals that are also required by the host and which are needed for various essential cellular processes. Metal homeostasis has important implications for enzymatic function and for appropriate transcriptional control of regulatory networks governing gene expression under diverse environmental conditions [4]. In particular, acquisition of manganese plays an important role in the pathogenesis of a number of bacterial species [5]. This metal ion plays a role as a cofactor of enzymes involved in metabolism, in signal transduction and in protection against oxidative stress [5, 6]. Mn^{2+} is present at about 36 μM in saliva [7], but at only nanomolar concentrations at internal sites of the host [8, 9]. Manganese could be an important cue by which the new environment is sensed by invading bacteria, functioning as a signal of the transition to internal body sites [10]. The same has been proposed for iron depletion as

a signal for many bacterial pathogens to sense that they are within a vertebrate host [11].

Little is known on how *E. faecalis* achieves manganese homeostasis and on the impact of this ion on *E. faecalis* biology. In the only study relating manganese to *E. faecalis* pathogenicity, the *efaCBA* operon of *E. faecalis* JH2-2 was described to be dependent on EfaR, a regulator belonging to the DtxR/MntR family of transcriptional regulators [12]. Binding of EfaR to the promoter region of *efaCBA* was shown to be promoted by manganese, which acted as a co-repressor. The *efaCBA* operon encodes the adhesion lipoprotein EfaA, first isolated from serum of a patient and described as an endocarditis-associated antigen [13] and later demonstrated to be expressed during enterococcal endocarditis [14]. Its role as a virulence factor was established when infection with an *E. faecalis* OG1RF∆*efaA* mutant led to delayed mortality in mice [15]. EfaCB encode two ATP-binding cassette (ABC) transporters [12].

Previously we have reported the transcriptomic response of *E. faecalis* V583 to manganese, zinc and copper stresses [16]. Here, we analyzed the promoter regions of genes differentially expressed as a consequence of excess metal added to the medium in which *E. faecalis* V583 was grown. A DNA motif, here called EfaR binding motif (*ebm*), was identified in the promoter regions of 30 genes, five of which were previously shown to be repressed by manganese and copper and induced by zinc stress [16]. Among this group of genes was the *efaCBA* operon. Thus, we further investigated the role of EfaR as a regulator of genes preceded by an *ebm* and the involvement of EfaR, and some of the genes it regulates, in a number of processes important for virulence, namely resistance to oxidative stress, biofilm formation and survival inside macrophages.

Materials and Methods

Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *E. faecalis* strains were grown as standing cultures at 37 ºC in M17 medium with 0.5% glucose (GM17) or in metal-depleted (Chelex-treated) GM17. Chelated medium was prepared by autoclaving M17 medium with 2% Chelex 100 sodium form (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), followed by 2 h of stirring. Glucose (0.5%) and 20 μM $MgSO_4$ were added to allow *E. faecalis* growth in this medium (chelGM17). *Escherichia coli* was grown in LB (Luria-Bertani) broth or 2xYT (Yeast Extract and Tryptone) broth in a shaking incubator at 37 ºC. Strains with thermo-sensitive plasmids were grown at 28ºC. Chloramphenicol was used at a concentration of 30 μg/ml for *E. faecalis* VE14412; tetracycline was used at concentrations of 15 μg/ml for *E. coli* VE14192 and at 10 μg/ml for *E. faecalis* strains. Kanamycin, for *E. coli* strains, and erythromycin, for *E. faecalis* strains, were used at 50 μg/ml. When necessary, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (VWR International Ltd., Leicestershire, UK) was added to the growth medium at 250 μg/ml. BHI (Brain Heart Infusion) plates were used to count viable bacterial cells in the intramacrophage survival and the oxidative stress assays.

DISCLOSE analysis

DISCLOSE software [17] was used on DNA microarray data previously obtained [16] in order to determine clusters of genes that exhibit similar expression patterns and identify overrepresented DNA binding sites in the upstream DNA sequences of genes from those clusters.

Table 1: Strains and plasmids used in this study.

Strains/Plasmids	Description [a]	Reference or source
Enterococcus faecalis		
VE14089	V583 cured of pTEF1, pTEF2 and pTEF3	[18]
SAVE20	ery^r; VE14089 (pSAVE10);	This work
SAVE21	ery^r ; VE14089 (pSAVE11) ;	This work
VE14412	cm^r; VE14089 (pGhost 3);	[18]
SAVE22	tet^r; VE14089 with *tet* insertion in *efaR*	This work
SAVE23	ery^r; tet^r; SAVE22 (pSAVE10)	This work
SAVE24	ery^r; tet^r; SAVE22 (pSAVE11)	This work
EF0577_S1_CA	tet^r; VE14089 with *tet* insertion in *ef0577* gene	[18]
EF0579_S1_CA	tet^r; VE14089 with *tet* insertion in *ef0579* gene	[18]
SAVE25	tet^r; ery^r; EF0579_S1_CA (pSAVE10)	This work
SAVE26	tet^r; ery^r; EF0579_S1_CA (pSAVE11)	This work
EF2076_S1_JO	tet^r; VE14089 with *tet* insertion in *efaA*	[18]
Escherichia coli		
VE14188	kan^r; GM1674 (*dam*$^-$*dcm*$^-$ *repA*$^+$)	[18]
VE14192	kan^r; tet^r; GM1674 (pVE14218)	[18]
pILORI4	ery^r; pIL252 with MCS and promoterless *lacZ* of pORI13	[19]
pSAVE10	ery^r; pILORI4 P*ef0575* :: *lacZ* fusion	This work
pSAVE11	ery^r; pILORI4 P*mntH2:: lacZ* fusion	This work
pG$^+$host 3	cm^r; *repA*$^+$; thermo-sensitive	[20]
pVE14218	tet^r; derived from p3TETTery and pOrinew	[18]
pSAVE12	tet^r; pVE14218 with ca. 80% of *efaR* in MCS	This work

[a] kan^r, kanamycin resistance; ery^r, erythromycin resistance; cm^r, chloramphenicol resistance; MCS, multiple cloning site; tet^r, tetracycline resistance.

Mutant construction

Single crossover insertion mutagenesis was performed to create an *E. faecalis* VE14089 *efaR* mutant using the two-vector system essentially described by Law and co-workers [21]. Plasmid pG$^+$host 3 and the integrative plasmid pVE14218 were used in this strategy [18]. Primers used

for construction of the integration vector are presented in Table 2. The *efaR* mutant was confirmed by Southern hybridization.

Table 2: Primers used in this study.

Primers	Nucleotide sequence 5'- 3'
***EF1005*-1**	TACCTACCTGCAGGAGGGGACTGCTGCTTTAAAGCTGACGG
***EF1005*-2**	TACTACCCTAGGGTGGGCAGTTCTGGACTCGATGTTTCGG
OEF102	GGCGATCGGACTAAACAATTGAACACGGC
OEF106	TGATGAAACGGCACGGATAG
P*EF0575*fw	GAGAAGAATTCAATGGTCTTCCCATGTATTTAGG
P*EF0575*rev	AAGCAGGATCCATTTTCCAGCACCATTTGGACC
P*mntH2*fw	ATTTCGAATTCCTTTAAGACCGCACATTTACG
P*mntH2*rev	GCATAGGATCCCAAATGATGTCTTTGCTTTGG
pILORI4-1	CCATTCGCCATTCAGGCT
pILORI4-2	CCGCTACGGATCACATCT

Construction of transcriptional *lacZ* fusions

Transcriptional fusions with the *E. coli lacZ* gene were constructed in plasmid pILORI4 [19]. Primer pairs P*EF0575*fw/P*EF0575*rev and P*mntH2*fw/P*mntH2*rev (Table 2) were used to generate PCR fragments spanning the upstream regions of *ef0575* and *mntH2*, respectively. The fragments were cloned in EcoRI/BamHI sites of pILORI4, leading to pSAVE10 and pSAVE11, respectively. Each plasmid was introduced in *E. faecalis* strains VE14089, SAVE22 and EF0579_S1_CA. Confirmation of these constructs was done by PCR with primers pILORI4-1 and pILORI4-2 (Table 2) and subsequent nucleotide sequencing.

β-Galactosidase assays

Cells were grown in GM17 or chelGM17 with 50 μg/ml erythromycin and metal ions. Metal solutions were used in the following added metal concentrations $ZnCl_2$: 0, 0.5 mM, 2 mM, 4 mM and 6 mM, $MnCl_2$: 0, 0.2 mM, 0.4 mM, 0.6 mM and 1 mM, $CuSO_4$: 0, 0.025 mM and 0.05 mM, $CoCl_2$: 0, 0.1 mM, 0.5 mM and 1 mM, $FeCl_2$: 0, 0.1 mM, 0.5 mM and 1 mM, $NiSO_4$: 0, 0.1 mM, 0.5 mM and 1 mM, and $MgCl_2$: 0, 0.1 mM and 0.5 mM. Cells from the mid-logarithmic growth phase were spun down and frozen in liquid nitrogen to be used later. Frozen cell pellets were resuspended in the same volume of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$ and 50 mM β-mercaptoethanol, pH 7.0), and the OD_{600} was measured. Cells in diluted suspensions were permeabilized with 100 μl of chloroform and 50 μl of 0.1% Sodium Dodecyl Sulfate (SDS). Reactions were started with the addition of 200 μl of 2-nitrophenyl β-D-galactopyranoside (Sigma-Aldrich Chemie GmbH) (4 mg/ml in 0.1 M phosphate buffer, pH 7.0) and stopped with 500 μl of 1 M Na_2CO_3. The reaction mixtures were centrifuged for 5 min at 14000 rpm and the OD at 420 and 550 nm were recorded. Activity of LacZ (in Miller units) was calculated according to Miller [22]. For each assay, three independent replicates were performed.

DNA microarray experiments and data analysis

DNA microarray experiments were performed essentially as described previously [16]. RNA was isolated from 30 ml of *E. faecalis* VE14089 and SAVE22 cultures grown until mid-exponential phase in GM17, following the procedure previously described [16]. cDNA synthesis and indirect Cy-3/Cy-5-dCTP labeling and hybridization procedures were performed as reported [16]. Glass slides carrying 70-mer oligonucleotides for 3160 genes of *E.*

faecalis V583 [16] were scanned in a GeneTac LS IV confocal laser scanner (Genomics Solutions, Huntingdon, United Kingdom). DNA microarray data were obtained from three independent biological replicates hybridized to glass slides with gene oligos spotted in triplicates.

Slide images were analyzed using the GenePix Pro 6.0 software (Axon Instruments, Union City, CA, USA). Data processing was performed as described elsewhere [16]. Expression ratios were calculated from the measurements of at least seven of nine spots. Differential expression tests were performed as previously described [23].

Data were uploaded in GEO, accession number #GSE33698.

Intramacrophage survival assay

The macrophage survival assay was basically performed as described previously [24] with some modifications. Confluent monolayers of the murine macrophage cell line J774.A1, established from a tumor that arose in a female BALB/c mouse, were infected with cultures of *E. faecalis* strains VE14089, SAVE22, EF0577_S1_CA, EF0579_S1_CA or EF2076_S1_JO. Approximately $4*10^6$ bacteria were added to J774.A1 monolayers, to yield a multiplicity of infection of approximately 10 bacteria per cell. The cell cultures were incubated at 37°C in 5 % CO_2 atmosphere for 1h to allow bacterial adherence and entry. After this, 250 µg/ml gentamycin and 60 µg/ml penicillin G were added to the cultures followed by an incubation of 1h to kill extracellular bacteria. Triton X-100 (Fluka Analytical, Sigma-Aldrich, Buchs, Switzerland) at 1% in phosphate-buffered saline, pH 7.4, (Gibco, Invitrogen, Paisley, UK) was used to lyse macrophage cells at 0, 2, 4, 6, 8 and 24 h post infection. Lysates were then diluted and plated on BHI plates to count viable intracellular bacteria. Assays were performed in three independent replicates and results are reported as intracellular Survival

Index (SI) *i.e.*, the percent (mean) of the internalized CFUs at 0 h post infection that survived after phagocytosis.

Oxidative stress assay

The oxidative stress assay was performed on *E. faecalis* strains VE14089, SAVE22, EF0577_S1_CA and EF2076_S1_JO grown in BHI until an OD_{600} of 0.5 was reached. Then, the cell cultures were exposed to hydrogen peroxide stress (20 mM H_2O_2 in 0.9% NaCl) as described by Verneuil and co-workers [25]. Each data point is the average of the data from three independent experiments with triplicate plating. The percentage of survival at a given time point was calculated by determining the ratio between the number of CFUs at a given time after treatment and the number of CFUs at time zero.

Biofilm formation assay

E. faecalis VE14089, SAVE22, EF0577_S1_CA, EF0579_S1_CA and EF2076_S1_JO were grown as standing cultures for 16 h in 2xYT medium with 0.5% glucose, at 37°C. The cultures were subsequently diluted 1:100 (v/v) in the same fresh medium. 200 μl of the diluted cell suspensions were inoculated in sterile 96-well polystyrene microtiter plates (Sarstedt AG & Co., Nümbrecht, Germany). Biofilm formation on polystyrene was quantified after 24 h of incubation at 37ºC with the crystal violet-staining method, as previously described [26]. For each assay, three independent experiments were performed in hexuplicates. All experiments included blank wells (medium without inoculum).

Results

Genes carrying an *ebm* site respond to metals

Analysis with DISCLOSE [17] of our previous data on the *E. faecalis* V583 transcriptomic response to high metal concentrations [16] revealed the presence of a putative DNA binding motif, EfaR binding motif (*ebm*), in the upstream regions of several genes of which expression was affected by manganese, zinc or copper addition (Fig. 1). This motif was present in the promoter regions of thirty genes in the V583 genome (Table S1).

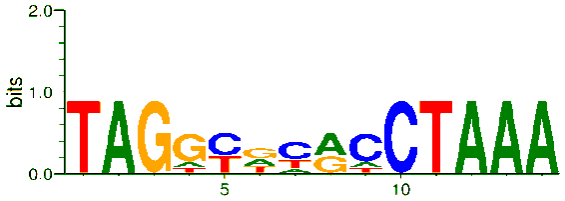

Figure 1 – *ebm*, present in the promoter regions of several genes differentially expressed in the presence of excess Zn^{2+}, Mn^{2+} and Cu^{2+} ions.

To examine the role of the *ebm* in sensing metal stress, the activity was measured of plasmid-encoded *lacZ* fusions to the *ebm*–containing promoter regions of *ef0575* (pSAVE10; data not shown) and *mntH2* (pSAVE11; Fig. 2A). Expression of *lacZ* was only significantly affected upon addition to the medium of Zn^{2+}, Mn^{2+}, Cu^{2+} and Fe^{2+} (strain VE14089(pSAVE11)). The results confirm the up-regulation in the presence of Zn^{2+} and down-regulation in the presence of Mn^{2+} and Cu^{2+} of the *mntH2* and *ef0575* promoter activities; Fe^{2+} also affected the activity of the *mntH2* promoter, which is not surprising considering that the downstream gene is annotated as a putative Mn^{2+}/Fe^{2+} transporter gene. Other metal ions, namely Mg^{2+}, Co^{2+} and Ni^{2+}, showed no significant effects (data not shown).

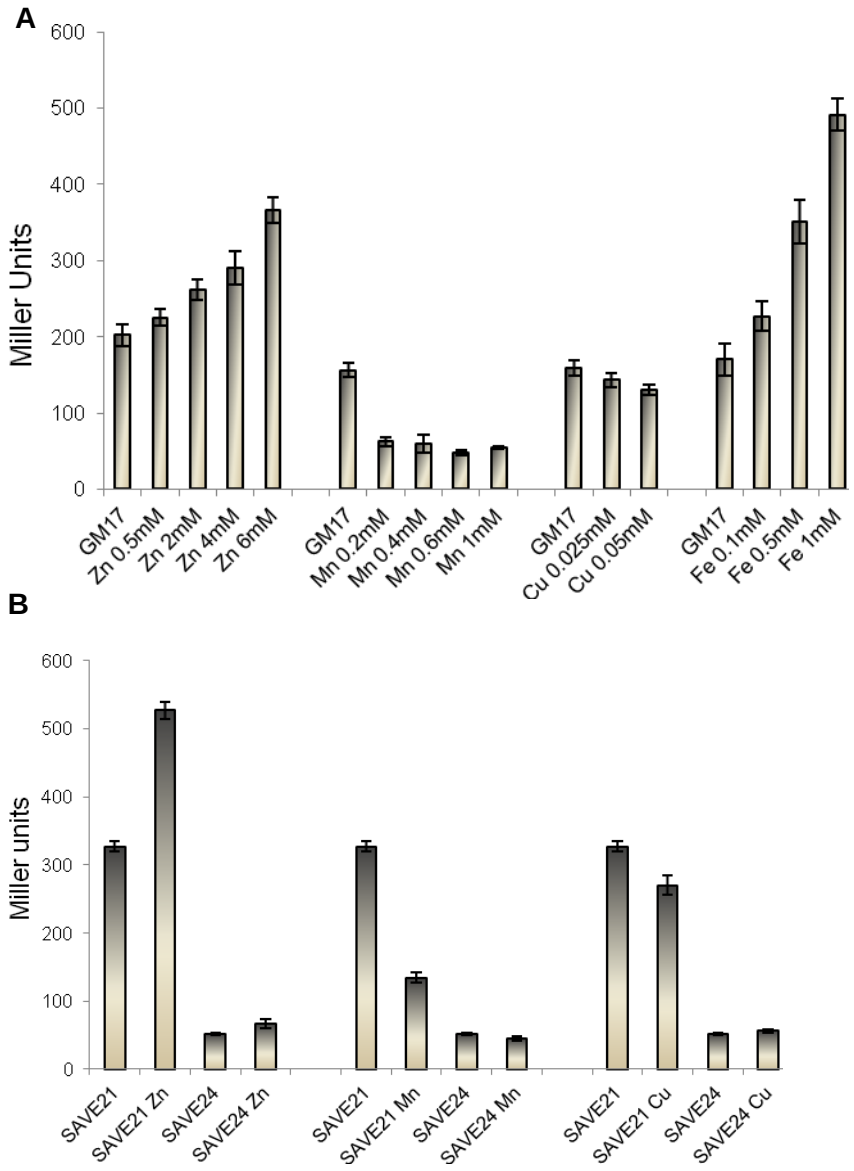

Figure 2 – Effect of metal addition on *E. faecalis* strains carrying a plasmid with a fusion of a promoter containing *ebm* to *lacZ*. A. *lacZ* expression in *E. faecalis* VE14089(pILORI4::P_{mntH2}) (SAVE21) grown in GM17 with the indicated added metal concentrations of $ZnSO_4$, $MnCl_2$, $CuSO_4$ or $FeCl_2$. **B**. *lacZ* expression in *E. faecalis* VE14089 (pILORI4::P_{mntH2}) and *E. faecalis* VE14089 *efaR::tet* (pILORI4::P_{mntH2}) (SAVE24) grown in GM17 with $ZnSO_4$ (0 mM or 4 mM), $MnCl_2$ (0 mM or 0.4 mM) or $CuSO_4$ (0 mM or 0.05 mM).

EfaR regulates different transporters encoded by genes with an *ebm*

Five of the genes/operons that contain an *ebm* in their promoter region were previously shown to be up-regulated in the presence of a high concentration of Zn^{2+} and down-regulated with high concentrations of Mn^{2+} and Cu^{2+} ions [16]. These genes and their operons, presented in Table 3, are mostly (predicted to be) involved in transport and regulation and include the *efaCBA* operon and the cluster *ef0575-ef0578*, which is present in the pathogenicity island of E*. faecalis* V583. The genes *ef0575-ef0577* encode homologues of the EfaCBA proteins (identities of 52%, 60% and 60%, respectively). This group of genes also includes a putative TetR family regulator gene (*ef0579*), *mntH1* and *mntH2*, both annotated as being putatively involved in manganese transport, and a universal stress protein-encoding gene, *ef1058*, of which function and role in metal homeostasis, if any, have not been studied yet.

In a previous study of the *efaCBA* operon, a bioinformatic approach was used to search the genome of *E. faecalis* V583 for an earlier described DtxR consensus binding box [12, 27]. Two DNA binding boxes, Box 1 and Box 2, were identified in the promoter regions of 13 genes [12]. Predicted Box 1 fits our empirically determined *ebm*, but only so for those upstream of the *ef0575*, *efaC*, *mntH1* and *mntH2* genes.

Table 3: *E. faecalis* V583 genes preceded by an *ebm*[a].

Gene*	Motif sequence	Description **
ef0575	taggcttgactaaa	cationic ABC transporter, ATP-binding protein
ef0576		cationic ABC transporter, permease protein
ef0577		adhesion lipoprotein
ef0578		helix-turn-helix, iron-dependent repressor family
ef0579	tagactcatctaaa	transcriptional regulator, putative
mntH2	taggtgtacctaaa	Mn^{2+}/Fe^{2+} transporter, NRAMP family
ef1058		universal stress protein family
mntH1 (ef1901)	taggtgtgcctaaa	manganese transport protein MntH
efaC	taggtgcgcctaaa	ABC transporter, ATP-binding protein
efaB		ABC transporter, permease protein
efaA		endocarditis specific antigen

[a] Genes carrying *ebm* in their promoter regions that were up-regulated in the presence of zinc excess and down-regulated in the presence of manganese or copper.

*The motifs were found in the promoter regions of genes in **bold**.

** According to the National Center for Biotechnology Information (NCBI).

We wondered whether *E. faecalis* V583 EfaR, which is identical to EfaR of strain JH2-2, regulates not only the expression of *efaCBA* but also of the other genes containing an *ebm* site in their promoter regions. Thus, DNA microarrays were performed to compare the expression of the genomes of VE14089 and its isogenic *efaR* mutant, SAVE22 (data not shown). Indeed, some genes carrying a copy of *ebm* in their promoter region were differentially expressed in the *efaR* mutant *e.g., ef0575* and *efaA* were down-regulated. However, not all genes present in Table 3 were differentially expressed in these microarray experiments. One of the genes not significantly down-regulated in the *efaR* mutant was *mntH2*. In order to clarify whether expression of *mntH2* is dependent on EfaR or not, β-galactosidase activities driven by the P_{mntH2}-*lacZ* transcriptional fusion on plasmid pSAVE11 were compared in *E. faecalis* VE14089 and its *efaR*

mutant, strain SAVE 24 (Fig. 2B). In the absence of added zinc, manganese or copper ions, β-galactosidase activity was lower in the mutant than in the parent strain, suggesting that EfaR has a regulatory role in *mntH2* expression. When zinc, manganese or copper ions were added to the *efaR* mutant carrying pSAVE11 (strain SAVE24), no significant induction or repression of P_{mntH2}-*lacZ* was seen, as opposed to the response in the parent strain VE14089(pSAVE11), suggesting that these metal ions function as effectors of EfaR regulation of *mntH2* expression.

To examine the effect of each metal ion individually, the same assays were performed in metal depleted medium (Fig. 3). Under these conditions, a huge increase in P_{mntH2}-driven LacZ activity was observed in both the wild type and the *efaR* strain: *lacZ* expression was much lower in metal repleted conditions (Fig. 2B). Mn^{2+} was still able to repress P_{mntH2}- driven expression in the *efaR* mutant in the metal depleted environment (Fig. 3), suggesting that under such conditions yet another regulator may be involved.

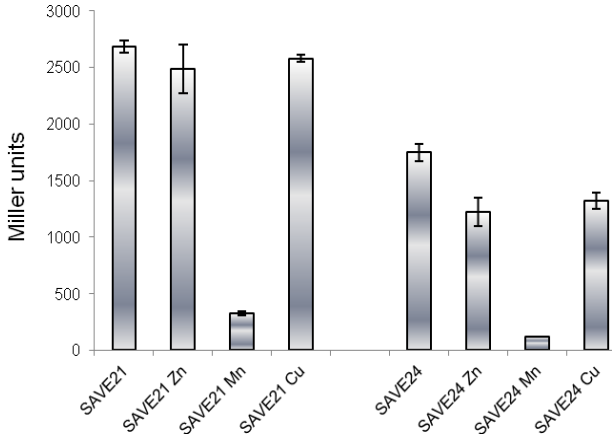

Figure 3 – Effect of metal addition on *E. faecalis lacZ* expression, in the wild type and *efaR* mutant strains, under metal depleted conditions. Indicated are *lacZ* expression in VE14089 (pILORI4::P_{mntH2}) (SAVE21) and VE14089 *efaR::tet* (pILORI4::P_{mntH2}) (SAVE24) grown in chelGM17 with $ZnSO_4$ (0 mM or 4 mM), $MnCl_2$ (0 mM or 0.4 mM) or $CuSO_4$ (0 mM or 0.05 mM).

Since one of the genes in Table 3, *ef0579*, encodes a putative regulator, we tested its role in metal sensing. To this end, the activities of the P_{mntH2}::*lacZ* and P_{ef0575}::*lacZ* fusions were measured in the absence of EF0579, using an insertional mutant of *ef0579* [18]. The results presented in Fig. 4A and 4B clearly show that EF0579 does not play a role in the metal-dependent regulation of *ef0575* or *mntH2*, as the β-galactosidase activities in the wild type strain and the *ef0579* mutant were similar.

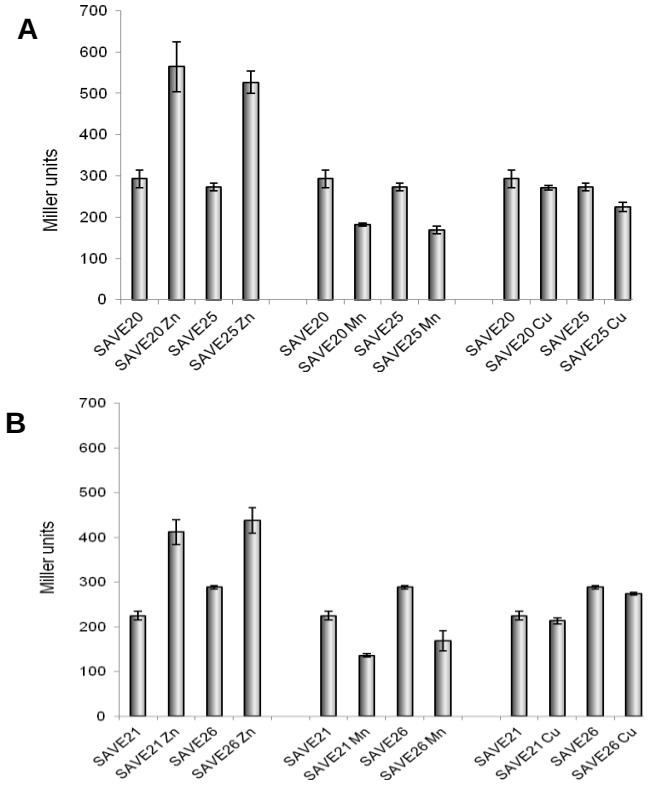

Figure 4 - **Effect of metal addition in the wild type strain or the *ef0579* mutant carrying a plasmid with *ebm* promoter-*lacZ* fusion. A**. *lacZ* expression in VE14089 (pILORI4::P_{ef0575}) and *ef0579* mutant (pILORI4::P_{ef0575}) strains grown in GM17 with $ZnSO_4$ (Zn; 0, 4mM), $MnCl_2$ (Mn; 0, 0.4mM) and $CuSO_4$ (Cu; 0, 0.05 mM); **B**. *lacZ* expression in VE14089 (pILORI4::P_{mntH2}) and *ef0579* mutant (pILORI4::P_{mntH2}) strains grown in GM17 with $ZnSO_4$ (Zn; 0, 4mM), $MnCl_2$ (Mn; 0, 0.4mM) and $CuSO_4$ (Cu; 0, 0.05 mM).

EfaR contributes to *E. faecalis* virulence

As the results presented above show that EfaR is a relevant player in metal-dependent gene regulation in *E. faecalis*, we studied its further role(s) in the biology of this bacterium. Several EfaR homologues have been implicated in processes such as virulence, survival in macrophages, biofilm formation and oxidative stress in *Mycobacterium tuberculosis, Streptococcus gordonii, Streptococcus pneumoniae* and *Streptococcus mutans* [10, 28-30]. The *E. faecalis efaR* mutant and several isogenic strains carrying mutations in genes regulated by EfaR were examined with respect to their capacity to survive inside macrophages. As shown in Fig. 5, the *efaR* mutant was significantly impaired in this ability when compared to the wild type VE14089 (*p* value of $4.24 * 10^{-5}$). The latter was even able to divide to some extent inside the macrophages for the first two hours and only later succumbed to macrophageal defenses. The other tested mutants, namely *ef0577*, *ef0579* and *efaA* mutants behaved as the wild type strain with respect to intramacrophage survival (data not shown).

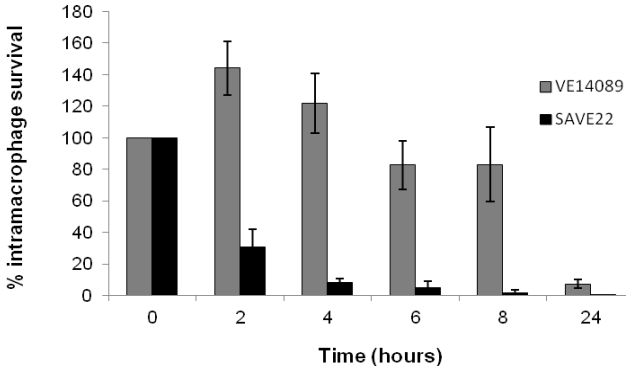

Figure 5 – **Intramacrophage survival of *E. faecalis*.** Percentage of survival of *E. faecalis* VE14089 (grey bars) and its *efaR* mutant SAVE22 (black bars) inside macrophages (J774 A1 cells) at the indicated time points after addition of the bacteria to a confluent layer of macrophages. Bacterial survival was measured by plating on BHI agar plates. Results represent the mean percent of three independent experiments.

The same strains were also tested for their resistance to stress resulting from treatment with 20 mM H_2O_2. Again, the *efaR* mutant but not the other strains showed a decreased capacity to deal with oxidative stress (Fig 6).

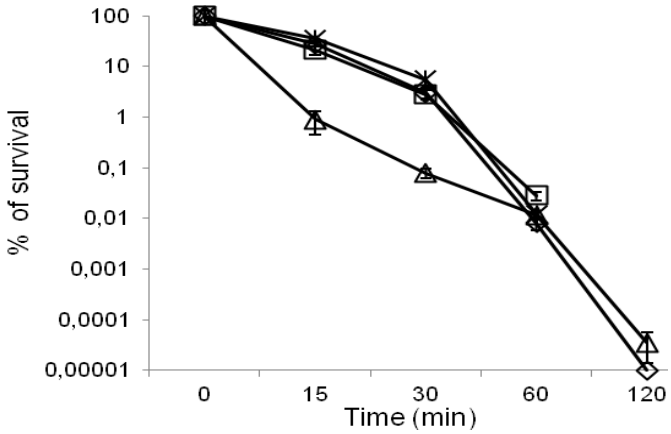

Figure 6 – *E. faecalis* resistance to oxidative stress. Survival of *E. faecalis* VE14089 (diamonds) and its isogenic insertion mutants *efaA* (squares), *efaR* (triangles) and *ef0577* (asterisks) at 15, 30, 60 and 120 min after challenge with 20 mM H_2O_2. A value of 100% corresponds to the number of CFUs immediately prior to the additions of H_2O_2 (0 min). The values are the averages of three independent experiments.

The *efaR* and *efaA* mutants were significantly impaired in biofilm forming ability (*p* values of 2.9E-11 and 8.3E-06, respectively) in comparison to their parental strain VE14089 (Fig 7). In fact, the *efaR* mutant appeared to have completely lost the ability to form biofilms.

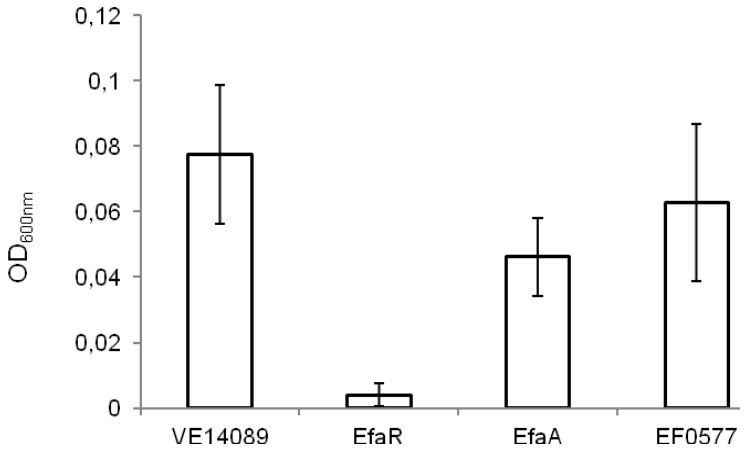

Figure 7 – *E. faecalis* biofilm formation. The biofilm-forming ability of *E. faecalis* strains VE14089, *efaR* mutant (EfaR), *efaA* mutant (EfaA) and *ef0577* mutant (EF0577). Values are averages of results obtained in three independent experiments, performed in hexuplicates.

Discussion

In this work we establish that EfaR is a major regulator in *E. faecalis*; it modulates the expression of several Mn^{2+}-dependent systems using Mn^{2+} as a cofactor. We show that the operon *ef0575-ef0578*, of which the products of the first three genes are homologous to those of the *efaCBA* operon, is part of the EfaR regulon. Both operons respond to metals in a similar way: they were down-regulated in the *efaR* mutant and both carry the *ebm* site in their promoter regions. Our results clearly suggest that EF0575 and EF0576, similar to EfaCB and annotated as a putative cationic ABC transporter, form a second Mn^{2+} transport system. As EF0577 is similar to EfaA, it might encode a putative virulence factor involved in sensing and binding to host cells. EF0578 is a putative small iron-dependent repressor with homology to part of EfaR and its DtxR-type

homologs. Such a small protein is not encoded by *efaCBA*. Twenty-one of thirty-three *E. faecalis* genome sequences, including hospital and food strains, contain both *efaCBA* and *ef0575-ef0578* (data not shown). As both operons seem to be broadly distributed and are commonly present together, it is likely that their concomitant presence in *E. faecalis* cells grants these bacteria with some advantage.

Previous DNA microarray results [16], the presence in their upstream sequences of the *ebm* site and their annotation as Mn^{2+} transporter genes all suggest that the *mntH* genes are part of the EfaR regulon. Using the P_{mntH2}-*lacZ* fusion we were able to demonstrate that EfaR indeed has a regulatory role in *mntH2* expression, particularly under metal-repleted conditions. It is also clear from our experiments that under metal-depleted conditions, expression of *mntH2* is much higher than in metal-repleted medium. Most likely, genes in Table 3 are expressed with the purpose of scavenging manganese ions in environments where this metal is extremely scarce. In *Lactobacillus plantarum*, the genes of the homologs of *E. faecalis* MntH1 and MntH2 were expressed upon Mn^{2+} limitation, supporting the role of both proteins in Mn^{2+} homeostasis [31]. Although the role of these transporters in *E. faecalis* has not been studied, the fact that they are part of the EfaR regulon reinforces the theory that they are involved in manganese transport.

In the presence of a low concentration of metals (in chelated medium) another regulator seems to be involved, which action was only visible in the absence of EfaR and in metal-depleted medium (Fig. 3). In the genome of *E. faecalis* V583, there are several predicted Mn^{2+}- transport systems. It would not be surprising if more than a single regulator would affect the regulation of these different systems. Although EfaR has been shown here to have a major metalloregulatory role, in environments as complex as the ones found in the host, manganese regulation in *E. faecalis* must be fine-

tuned to be able to quickly adjust to changing conditions. Thus, it is likely that other players are involved, complementing the major function of EfaR in the maintenance of manganese homeostasis. The putative regulator EF0579, a member of the EfaR regulon, was ruled out to perform such a role, at least with respect to controlling *ef0575* and *mntH2* expression. Another candidate regulator would be EF0578, as it is also part of the EfaR regulon and shares 45% homology with EfaR. However, none of the residues that probably constitute the metal binding site in EfaR, by homology with the residues described for ScaR [32] discussed below, are present in EF0578 and thus, its function as a regulator has yet to be proven.

Structural information on EfaR is necessary to fully understand the interaction of metal ions with this member of the DtxR/MntR family of metalloregulators. A phylogenetic tree of these regulators indicates that ScaR from *S. gordonii* and EfaR from *E. faecalis* belong to the same group, sharing 41% homology [32]. Based on structural studies on ScaR [32] it seems that the key residues involved in metal binding are also present in EfaR. ScaR responds selectively to manganese *in vivo*, despite being activated *in vitro* by other metal ions as well. Similarly, EfaR was found to bind *in vitro* to a box, which is part of the *ebm* defined here, in the promoter region of *efaC* in the presence of different metal ions [12]. Metal binding to metalloregulatory proteins is determined by affinity, allostery and access [33]. Based on the similarities in the (putative) metal binding sites in ScaR and EfaR, we presume that metal binding in both proteins occurs basically in the same way, with the manganese ion being the main co-regulator. Zn^{2+} and Cu^{2+} do not seem to be true co-regulators as they do not affect significantly the expression from an EfaR-regulated promoter in metal-depleted medium (Fig 3). Nevertheless, it has been shown that EfaR binds to P_{efaC} in the presence of Mn^{2+}, Zn^{2+} and Cu^{2+} [12], suggesting that Zn^{2+}

and Cu^{2+} can compete with Mn^{2+} for EfaR binding, impairing manganese co-repressor activity. Metal ions competing with each other for ligation to EfaR could strongly influence the maintenance of manganese homeostasis, which is essential for colonization and infection (see below).

Manganese has a major role in several cellular processes, among which is the response to oxidative stress. Destabilization of manganese homeostasis, in particular by the inability to scavenge this metal ion, could seriously affect a number of cellular processes and jeopardize the ability of a bacterium to survive in and colonize its host. As a key player in manganese-dependent gene expression in *E. faecalis*, EfaR could be involved in processes relevant for host-pathogen interactions. In fact, it has been observed in other pathogens that mutations in EfaR homologs affect biofilm formation (SloR, [28]), intramacrophage survival (IdeR, [29]) and oxidative stress resistance (ScaR, [30]). Indeed, a mutant of EfaR was highly impaired in the ability to survive inside macrophages and to form biofilms, while it also had a lower resistance to oxidative stress. Although alterations in biofilm architecture have been described for an *S. mutans sloR* mutant [28], this is the first description of a substantial decrease in biofilm formation by a DtxR/MntR family regulator mutant. An *efaA* mutant also showed a decrease in biofilm forming ability but not as pronounced as an *efaR* mutant. The other mutants did not show a phenotype different from that of the wild type, thus suggesting that the affected genes are not involved in oxidative stress response, intramacrophage survival or biofilm formation, or that Mn^{2+} scavenging systems may compensate for each other when one is not functional. The gene *sodA*, coding for Mn-superoxide dismutase, was down-regulated in the *efaR* mutant in our DNA microarray experiments. As SodA is crucial for oxidative stress resistance, it is possible that the role of EfaR in oxidative stress and intramacrophage survival is exerted through activation of SodA protein expression.

Overall, this work highlights the importance of metal homeostasis in the regulation of processes important for *E. faecalis* virulence and the role of EfaR therein. In particular, manganese appears as a key player in the regulation of EfaR activity. However, other metal ions most likely also contribute to *E. faecalis* virulence. In the case of zinc and copper this is probably through competition with manganese for EfaR binding. We show that EfaR is an important regulator of biofilm formation, intramacrophage survival and oxidative stress resistance. This is the first description of a DtxR/MntR regulator of which the absence has such severe implications in different cellular processes crucial for survival and host colonization. EfaR is thus a target to be considered for therapeutic intervention against enterococcal infections.

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Supplementary Information

Table S1: *E. faecalis* V583 genes that have *ebm* in their promoter regions.

Gene	Description[1]	*ebm* sequence
ef0049	sigma-70 factor family protein	tgc-tatgctctcctaaa-tca
ef0129	Cro/CI family transcriptional regulator	ata-tatgttaaactaaa-ata
ef0340	hypothetical protein	cag-taggcgcaactaag-aca
ef0575	cationic ABC transporter, ATP-binding protein	att-taggcttgactaaa-ata
ef0579	transcriptional regulator, putative	tagactcatctaaa
ef0603	hypothetical protein	cta-tagatgtgtttaaa-tta
ef0653	hypothetical protein	agc-taggtgtatcaaaa-tat
ef0716	hypothetical protein	cga-tagagttttctaaa-act
ef0815	PTS system, IIAB components	aag-tagggtcacctaca-tct
ef0872	potassium uptake system	ctg-ttggttaaactaaa-cga
ef1056	hypothetical protein	ttt-taggtacacctaaa-ata
ef1057	Mn^{2+}/Fe^{2+} transporter, NRAMP family mntH2	ttt-taggtgtacctaaa-ata
ef1193	DNA-binding response regulator VicR	tac-taggagaacctaaa-ggc
ef1408	ABC transporter, ATP-binding protein	cga-tatgagtaactaaa-cag
ef1901	manganese transport protein MntH1	ttt-taggtgtgcctaaa-agt
ef1902	glyoxylase family protein	ttt-taggcacacctaaa-aat
ef1929	glpK	aat-taggctttttaaa-caa
ef2015	minor head protein	att-tagatgtgtctaaa-att
ef2022	hypothetical protein	tag-taggtataattaaa-taa
ef2073	prsA-1 ribose-phosphate pyrophosphokinase	ttt-taggcgcacctaaa-tac
ef2074	ABC transporter, ATP-binding protein	att-taggtgcgcctaaa-aat
ef2186	hypothetical protein	gtc-tggacttgactaaa-gga
ef2205	hypothetical protein	cag-taggttcttttaaa-att
ef2238	sugar-binding transcriptional regulator, LacI family	caa-tagtttttgctaaa-aaa
ef2868	hypothetical protein	ttgggtcgactaaa
ef3087	hypothetical protein	caa-taggttatactaaa-gct
ef3088	hypothetical protein	caa-taggttatactaaa-act
ef3244	hypothetical protein	att-aaggctcttctaaa-aat
ef3251	hypothetical protein	gga-taggaatcactaaa-aaa
ef3280	peptidase, U32 family, putative	aga-taggctcttttaaa-gtt

[1] According to the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/).

Chapter 4

$ZntA_{Ef}$ mediates *E. faecalis* defense against zinc overload, lysozyme and oxidative stress

Abrantes M.C., Kok, J. and Lopes, M. de F. $ZntA_{Ef}$ mediates *Enterococcus faecalis* defense against zinc overload, lysozyme and oxidative stress. In preparation.

Author Contributions

All experiments were performed by the author of this thesis. Planning the experiments, data analysis and manuscript preparation were done by the author and the supervisors Dr. Fátima Lopes and Prof. Dr. Jan Kok.

Table of Contents

Abstract 147
Introduction 149
Materials and Methods 151
- Bacterial strains, plasmids and growth conditions 151
- MOODS analysis 151
- Mutant construction 151
- Construction of a transcriptional *lacZ* fusion 153
- β-galactosidase assays 153
- Zinc susceptibility assay 153
- Intramacrophage survival assay 154
- Oxidative stress assay 154
- Lysozyme assay 155

Results and Discussion 155
Ackowledgements 164
References 165
Supplementary Information 169

Abstract

Two *E. faecalis* genes encoding the P-type ATPase EF1400 and a putative SapB protein, EF0759, were previously shown to be strongly up-regulated in the presence of high zinc concentrations. In the present work we show that a Zn^{2+}-responsive DNA binding motif (*zim*) is present in the promoter regions of these genes. The encoded proteins were further studied with respect to their involvement in zinc homeostasis and invasion of the host. EF0759 contributed to intramacrophage survival by an as yet unknown mechanism(s). EF1400, here renamed ZntA$_{Ef}$, is an ATPase with specificity for zinc which plays a role in dealing with host defenses, namely zinc overload, oxidative stress and lysozyme; it provides *E. faecalis* cells with the ability to survive inside macrophages. As these three host defense mechanisms are important at several sites in the host, namely inside macrophages and in saliva, this work suggests that ZntA$_{Ef}$ is a crucial *E. faecalis* defense mechanism that is likely to contribute to the ability of this bacterium to endure life inside its host.

Introduction

Divalent metal cations are essential for all living cells although in high concentrations they can be toxic. Tight control of the cellular concentration of divalent metals prevents the formation of toxic metal complexes and the occurrence of redox reactions that are noxious to the cell [1]. One of these metals, zinc, is a component of many proteins, such as DNA polymerases, proteases, ribosomal proteins, and a cofactor of many enzymatic reactions [2]. Notwithstanding the importance of zinc, excess can be deleterious as it can compete with other metals for binding to the active centers of enzymes [2]. Zinc concentrations in the human body are variable, with levels in saliva and lungs [3, 4] being higher than those in serum or gastric juice [3, 5]. In particular, it has been shown that zinc accumulates in host immune cells such as macrophages upon infection, as a host defense mechanism [6]. Zinc levels can also increase through antimicrobial therapy, as some antibiotics are formulated as zinc salts. That is the case for bacitracin, the salt of which has been demonstrated to induce the expression of zinc efflux systems in *Bacillus subtilis* [7]. These efflux systems can be responsible for zinc resistance in bacteria. Others mechanisms involve proteins such as reductases and cysteine-rich metallothioneins [8]. They either expel the metal ions from the cells, detoxify or sequester them so that the cells can grow in an environment containing high levels of zinc [9]. Many efflux transporters involved in the expulsion of toxic amounts of zinc from the cell and in achieving zinc homeostasis are P-type ATPases. These enzymes constitute a large family of integral membrane transporters which generate and maintain crucial chemical gradients across cellular membranes [10]. Prokaryotic P-type ATPases transport a range of divalent metal cations, such as Cu^{2+}, Ag^{2+}, Cd^{2+}, Zn^{2+} and Mn^{2+} [11]. The genes encoding these

exporters are regulated in bacteria by MerR-like activators or by ArsR/SmtB-like repressors [12].

In a recent genome-wide study, we identified transporter systems likely to be involved in zinc homeostasis in *Enterococcus faecalis* [13]. *E. faecalis* is an important nosocomial agent which is also able to survive in a diversity of environments. Although some mechanisms involved in *E. faecalis* stress responses have been studied [14-18], there is still a major gap in our knowledge on how these bacteria succeed in adapting to environments with different metal concentrations and maintain metal homeostasis. In particular, nothing is known on *E. faecalis* mechanisms for dealing with high zinc concentrations. In this study we explore the specificity of an ATPase, EF1400, which was previously shown to be highly overexpressed (fold change of 44) in the presence of zinc stress [13] and investigate its role in biologic processes such as intramacrophage survival and resistance to oxidative stress or lysozyme. This is the first report on the involvement of an *E. faecalis* P-type ATPase in biologic processes relevant for bacterial infection and virulence; the work links zinc homeostasis to the outcome of host-pathogen interactions. The gene *ef0759*, which encodes a putative SapB protein, was also highly up-regulated in the presence of zinc stress (49 fold change), and was also investigated for its role in these processes.

Materials and Methods

Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *E. faecalis* strains were grown as standing cultures at 37 ºC in M17 (Difco, Becton, Dickison and Company, Le Pont de Claix, France) containing 0.5% glucose (GM17) or on Brain Heart Infusion (BHI; Oxoid, Hampshire, England) or Luria Bertani (LB; Sigma-Aldrich Chemie GmbH Steinheim, Germany) agar plates. *Escherichia coli* was grown in LB in a shaking incubator at 37 ºC. Strains with thermo-sensitive plasmids were grown at 30ºC. Chloramphenicol was used at a concentration of 30 μg/ml; tetracycline was used at concentrations of 15 μg/ml for VE14192 and 10 μg/ml for VE14089-derived strains. Kanamycin was used at 50 μg/ml for VE14188 and VE14192. Erythromycin was used at 50 μg/ml for SAVE27, SAVE32, SAVE33, SAVE34 and SAVE35. When necessary, 250 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (VWR International Ltd., Leicestershire, UK) was added to the growth medium.

MOODS analysis

MOODS software [19] was used on DNA microarray data previously obtained [13] in order to identify overrepresented DNA binding sites in the upstream DNA sequences of *E. faecalis* V583 genes.

Mutant construction

Single crossover insertion mutagenesis was performed to create strains SAVE28, SAVE29, SAVE30, SAVE31, SAVE36 and SAVE37 using the two-vector system essentially described by Law and co-workers [20].

Plasmid pG$^+$host3 and the integrative plasmid pVE14218 were used in this strategy [21]. Primers used for construction of the integration vectors are presented in Table S1.

Table 1: Strains and plasmids used in this work.

Strains	Description [a]	Reference or source
Enterococcus faecalis		
VE14089	V583 cured of its three plasmids	[21]
SAVE27	ery^r; VE14089 (pSAVE13)	This work
VE14412	cm^r; VE14089 (pG$^+$host 3)	[21]
SAVE28	tet^r; VE14089 with a *tet* insertion in *ef0421*	This work
SAVE29	tet^r; VE14089 with a *tet* insertion in *ef0966*	This work
SAVE30	tet^r; VE14089 with a *tet* insertion in *ef1699*	This work
SAVE31	tet^r; VE14089 with a *tet* insertion in *ef2225*	This work
SAVE32	tet^r; ery^r; SAVE28 *(*pSAVE13)	This work
SAVE33	tet^r; ery^r; SAVE29 *(*pSAVE13)	This work
SAVE34	tet^r; ery^r; SAVE30 *(*pSAVE13)	This work
SAVE35	tet^r; ery^r; SAVE31 *(*pSAVE13)	This work
SAVE36	tet^r; VE14089 with a *tet* insertion in *ef0759*	This work
SAVE37	tet^r; VE14089 with a *tet* insertion in *zntA$_{Ef}$*	This work
Escherichia coli		
VE14188	kan^r; GM1674 (*dam$^-$dcm$^-$ 152 ta$^+$*)	[21]
VE14192	kan^r; GM1674 (pVE14218)	[21]
Plasmids		
pILORI4	ery^r; pIL252 carrying the MCS and promoterless *lacZ* of pORI13	[22]
pSAVE13	ery^r; pILORI4 P*zntA$_{Ef}$*::*lacZ* fusion	This work
pG$^+$host 3	cm^r; thermo-sensitive replication	[23]
pVE14218	Derived from p3TETTery and pOrinew; integration vector	[21]
pSAVE14	tet^r; pVE14218 with ca. 80% of *ef0421* in the MCS	This work
pSAVE15	tet^r; pVE14218 with ca. 80% of *ef0966* in the MCS	This work
pSAVE16	tet^r; pVE14218 with ca. 80% of *ef1699* in the MCS	This work
pSAVE17	tet^r; pVE14218 with ca. 80% of *ef2225* in the MCS	This work
pSAVE18	tet^r; pVE14218 with ca. 80% of *ef0759* in the MCS	This work
pSAVE19	tet^r; pVE14218 with ca. 80% of *zntA$_{Ef}$* in the MCS	This work

[a] kan^r, kanamycin resistance; ery^r, erythromycin resistance; cm^r, chloramphenicol resistance; tet^r, tetracycline resistance; MCS, multiple cloning site

Construction of a transcriptional *lacZ* fusion

A transcriptional fusion to the *E. coli lacZ* gene was made in plasmid pILORI4 [22]. Primer pair EF1400-1/EF1400-2 (Table S1) was used to generate a PCR fragment spanning the upstream region of *ef1400* gene, which was then cloned in the EcoRI/BamHI sites of pILORI4, resulting in pSAVE13. Confirmation of this construct was done by PCR with primers pILORI4-1 and pILORI4-2 (Table S1) and subsequent nucleotide sequencing. The plasmid was subsequently introduced in various *E. faecalis* strains used in this study.

β-Galactosidase assays

Cells were grown in GM17 with erythromycin at 50 μg/ml, with and without added metal solution. Metals were used in the following (added) metal concentrations $ZnCl_2$: 0.5 mM, 2 mM, 4 mM or 6 mM, $MnCl_2$: 0.2 mM, 0.4 mM, 0.6 mM or 1 mM, $CuSO_4$: 0.025 mM or 0.05 mM, $CoCl_2$: 0.1 mM, 0.5 mM or 1 mM, $FeCl_2$: 0.1 mM, 0.5 mM or 1 mM, $NiSO_4$: 0.1 mM, 0.5 mM or 1 mM, and $MgCl_2$: 0.1 mM or 0.5 mM. Assays were performed as described elsewhere [13]. Activity of LacZ (in Miller units) was calculated according to Miller [24]. For each assay, three independent replicates were performed.

Zinc susceptibility assay

Overnight cultures of the *E. faecalis* strains VE14089, SAVE36 (VE14089 *ef0759::tet*) and SAVE37 (VE14089 *zntA$_{Ef}$::tet*) were adjusted to an OD_{600} of 1 in a 0.85% NaCl solution and diluted up to a million-fold. Subsequently, 20 µl of the 10^{-5} and 10^{-6} dilutions were spotted on BHI plates with and without added $ZnSO_4$ (0 to 20 mM). Plates were incubated for 24 h at 37ºC and later photographed.

Intramacrophage survival assay

The macrophage survival assay was essentially performed as described previously [25] with some modifications. The murine macrophage cell line J774.A1, established from a tumor that arose in a female BALB/c mouse [26], was grown to confluent monolayers in Medium 1 (Roswell Park Memorial Institute medium (RPMI) 1640 with L-Glutamine (Invitrogen/Gibco, Paisley, UK), 10% Fetal Bovine Serum (Invitrogen/Gibco), 1% Penicillin/Streptomycin (Invitrogen/Gibco)) at 37ºC and 5% CO_2. Subsequently, they were infected with cultures of *E. faecalis* VE14089, SAVE36 (VE14089 *ef0759::tet*) and SAVE37 (VE14089 *zntA$_{Ef}$::tet*). Approximately $4*10^6$ bacteria were added to J774.A1 monolayers, to yield a multiplicity of infection of approximately 10 bacteria per murine cell. The cell cultures were incubated at 37°C in 5% CO_2 atmosphere for 1 h to allow bacterial adherence and entry. Subsequently, 250 µg/ml gentamycin and 60 µg/ml penicillin G were added to the cultures, followed by an incubation of 1 h, to kill extracellular bacteria. Triton X-100 (Fluka Analytical, Sigma-Aldrich, Buchs, Switzerland) at 1% in phosphate buffered saline, pH 7.4, (Invitrogen/Gibco) was used to lyse macrophage cells at 0, 2 and 4 h post infection. Lysates were then diluted and plated on BHI plates to count viable intracellular bacteria. These assays were performed in three independent replicates and results are reported as intracellular Survival Index (SI) *i.e.*, the percent (mean) of the internalized CFUs at 0 h post infection that survived after phagocytosis.

Oxidative stress assay

E. faecalis strains VE14089, SAVE36 (VE14089 *ef0759::tet*) and SAVE37 (VE14089 *zntA$_{Ef}$::tet*), grown in BHI until an OD_{600} of 0.5, were submitted to a 20 mM H_2O_2 stress (in 0.9% NaCl) exactly as described by

Verneuil and co-workers [27]. Each data point is the average of the data from three independent experiments with triplicate plating. The percentage of survival at a given point in time was calculated by determining the ratio between the number of CFUs at that time point after treatment and the number of CFUs at zero time.

Lysozyme assay

Dilutions of VE14089, SAVE36 (VE14089 *ef0759::tet*) and SAVE37 (VE14089 *zntA$_{Ef}$::tet*) cultures were spot-plated on LB plates containing different concentrations (0 to 15 mg/ml) of egg white lysozyme (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) according to the procedure described by Le Jeune and co-workers [28]. Plates were incubated for 24h at 37ºC and photographed.

Results and Discussion

Analysis of our previous data on the *E. faecalis* V583 transcriptome response to high zinc concentrations [13] with MOODS software [19] revealed that two promoter regions of the highest up-regulated genes in the presence of 4 mM added Zn^{2+}, that of *ef0758*/*ef0759* and that of *ef1400*, contained a conserved DNA motif shown on Fig. 1. This motif, here denominated *zim* for Zinc Motif, is present upstream of several other *E. faecalis* genes, as shown in Table S2.

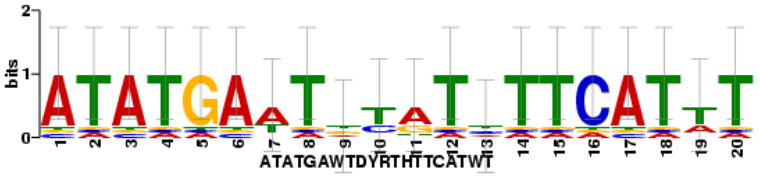

Figure 1 – Weight matrix of *zim*, as present in the promoter regions of *E. faecalis* V583 genes up-regulated in the presence of added Zn^{2+} [13].

The genes *ef0758* and *ef1400* encode two putative Cd^{2+}-translocating P-type ATPases and *ef0759* encodes a putative SapB protein. The two P-type ATPases have 32% homology and share certain similarities. Both have a multi-domain related to cation transport denominated ZntA, which constitutes the biggest part of the proteins, an E1-E2 ATPase domain and a Haloacid dehalogenase-like hydrolase domain (Fig S1). In addition, EF1400 contains a Heavy-metal-associated domain at its N-terminal (NCBI, http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi?INPUT_TYPE=live&SEQUENCE=AAO81191.1). On the basis of these data and the results presented below, we propose to rename EF1400 into $ZntA_{Ef}$. We chose to focus on one of the possible ATPases, $ZntA_{Ef}$ and on the putative SapB protein, EF0759, in order to explore the role, if any, of the two different proteins in the *E. faecalis* zinc stress response.

Metal specificity of *zim* was evaluated by measuring the activity of plasmid-encoded LacZ fused to the *zim*–containing *zntA*$_{Ef}$ promoter (pSAVE13) in *E. faecalis* VE14089 (strain SAVE27) in the presence of high concentrations of various metal ions (Fig 2).

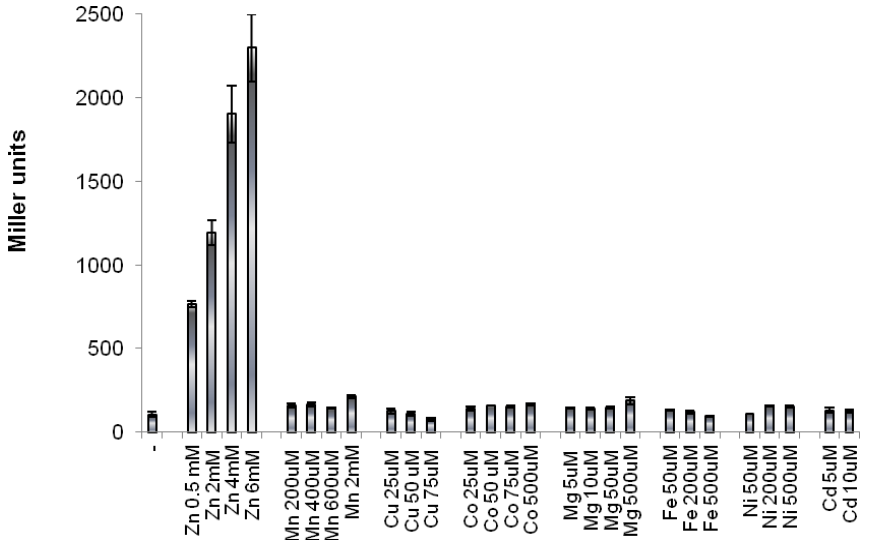

Figure 2 – Effect of metal addition on the *E. faecalis* VE14089 promoter P$_{zntA}$. The *lacZ* activity (in Miller Units) was recorded as described in Materials and Methods in *E. faecalis* VE14089 (pSAVE13; P$_{zntA}$:*lacZ*) grown in GM17 with the indicated -added- metal concentrations of $ZnSO_4$, $MnCl_2$, $CuSO_4$, $CoCl_2$, $MgCl_2$, $FeCl_2$, $NiCl_2$ or $CdCl_2$.

Expression of *lacZ* was only significantly affected by the addition of Zn^{2+}, demonstrating that regulation of P*zntA*$_{Ef}$ responds to this ion only, in these conditions. In order to evaluate the role of the EF0759 and ZntA$_{Ef}$ proteins in *E. faecalis* resistance to zinc, *ef0759* and *zntA*$_{Ef}$ mutants were constructed and their growth on zinc-rich BHI plates was compared to that of the parent strain VE14089. Strain SAVE37 (*zntA*$_{Ef}$*::tet*) showed an increased susceptibility only to high concentrations of zinc (20 mM) (Fig 3). These results suggest a role for the ZntA$_{Ef}$ ATPase in zinc expulsion.

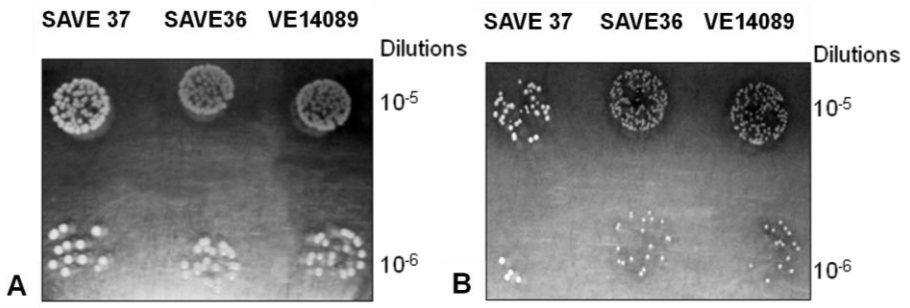

Figure 3 - *E. faecalis* growth in the presence of high concentrations of zinc. *E. faecalis* VE14089, SAVE36 (VE14089 *ef0759*::*tet*)) and SAVE37 (VE14089 *zntA*$_{Ef}$::*tet*) were grown overnight, adjusted to OD of 1, diluted up to a million fold and 20 µl drops were spotted on BHI plates with 0 mM $ZnSO_4$ (A) or 20 mM $ZnSO_4$ (B), and incubated for 24 h at 37ºC.

Although zinc P-type ATPases are still to be explored in most bacterial species, particularly in Gram positive bacteria, the few known P-type ATPases with ZntA domains are encoded by genes responsive to several metal ions, such as Zn^{2+} and Co^{2+} in *S. aureus* [9] and Zn^{2+}, Co^{2+}, Cd^{2+} and Pb^{2+} in *E. coli* [29]. Our results point to zinc specificity of $ZntA_{Ef}$ but more functional studies on the protein would be necessary to confirm these observations. This is the first annotated P-type ATPase responsive to zinc described in *E. faecalis*.

The gene *zntA* is described to be regulated by ZntR in *E. coli* and *S. aureus* [30, 31]. Since the sequence of *S. aureus* ZntR, referred to by Singh and co-workers [31], is not available, we used *E. coli* ZntR and other *S. aureus* ZntR-like sequences to search for homologs in the genome of *E. faecalis* V583. All *E. faecalis* ZntR homologues, namely EF0421, EF0966, EF1699 and EF2225 belong to the MerR family of regulators. *E. faecalis* strains carrying mutations in the genes encoding these MerR regulators were constructed in order to investigate their possible role in regulation of the *zntA*$_{Ef}$ gene. Plasmid pSAVE13 (P_{zntAEf}::*lacZ*) was introduced in the

various mutants and the effect of zinc addition on the activity of P_{zntAEf} was tested. LacZ expression in the mutants still responded to zinc addition (data not shown), indicating that none of the predicted MerR regulators in the genome of V583 is involved in the regulation of *zntA$_{Ef}$* expression. Another possibility to explore in future studies is that the ZntA$_{Ef}$ regulator belongs to the ArsR/SmtB family, as a number of other ZntR-like proteins belong to this family [31]. *E. faecalis* V583 carries two predicted copies of this type of regulator [32].

The protein EF0759, although highly expressed under zinc stress, seems to not be directly involved in zinc resistance, as suggested by the fact that the *ef0759* mutant is equally susceptible to high zinc concentrations as its parental strain (Fig 3). The *ef0759* gene is in operon with that of the P-type ATPase EF0758, which is also highly up-regulated and is putatively involved in cation transport (NCBI, http://www.ncbi.nlm.nih.gov/gene/1199654, [13]).

Phagocytic and antigen-presenting cells of the immune system, including macrophages, engulf bacteria into phagosomes, which then merge with lysosomes subjecting engulfed bacteria to an onslaught of antimicrobial factors, such as reactive oxygen species, metal stress [6] and lysozyme [33]. A recent study reported that *Mycobacterium tuberculosis* and *E. coli* use P-type ATPases in a strategy to avoid the toxic effects of zinc inside macrophages, in which a burst of free zinc occurs within a few hours post bacterial infection [34]. The roles of *E. faecalis* ZntA$_{Ef}$ and EF0759 in survival inside macrophages, resistance to oxidative stress and to lysozyme were investigated. As shown in Fig 4, the *E. faecalis zntA$_{Ef}$* and *ef0759* mutants were significantly impaired in their capacity to survive inside macrophages when compared to their parent *E. faecalis* VE14089. Both proteins seem to be, indeed, relevant for survival inside macrophages.

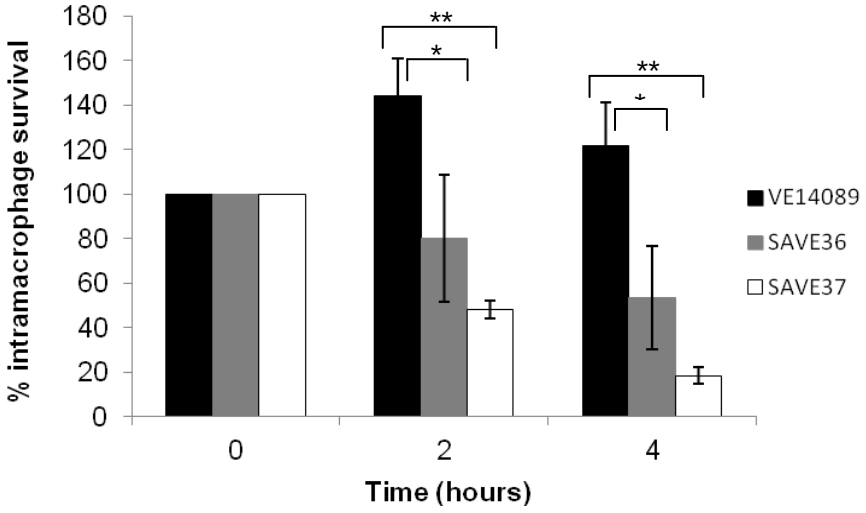

Figure 4 – Intramacrophage survival of *E faecalis*. Percentage of survival of *E. faecalis* strains VE14089 (black bars), SAVE36 (VE14089 *ef0759::tet)* (grey bars) and SAVE37 (VE14089 *zntA$_{Ef}$::tet*) (white bars) inside macrophages (J774 A1 cells) at the indicated time points after addition of the bacteria to a confluent layer of macrophages (moi of 10). Bacterial survival was measured by plating appropriate dilutions on BHI agar plates. * *p* value < 0.002; ** *p* value < 0.0004. Results represent the mean percent of three independent experiments.

In order to determine whether the decreased survival of the mutants inside macrophages was related to exposure to the referred host defenses, the three strains VE14089, VE14089(*ef0759::tet*) and VE14089(*zntA$_{Ef}$::tet*) were tested for their tolerance to oxidative stress resulting from treatment with 20 mM H_2O_2. The *zntA$_{Ef}$* mutant showed a 34-fold decrease in survival just 15 min after exposure and a 70-fold decrease in survival after 30 min of exposure to 20 mM H_2O_2 relative to the wild type (Fig. 5).

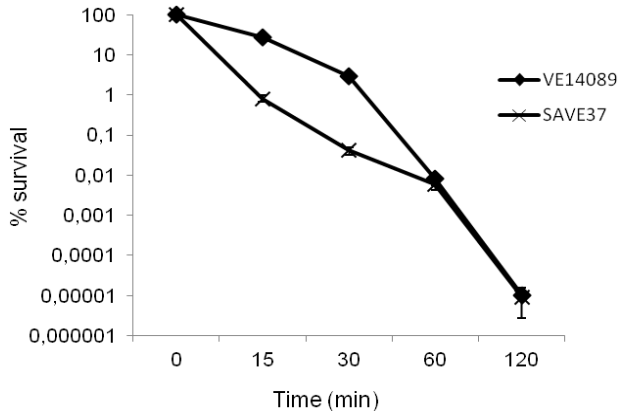

Figure 5 – *E. faecalis* resistance to oxidative stress. Survival of *E. faecalis* VE14089 (diamonds) and its isogenic insertion mutant SAVE37 (VE14089 *zntA$_{Ef}$::tet)* (crosses) at 15, 30, 60 and 120 min after challenge with 20 mM H_2O_2. A value of 100% corresponds to the number of CFUs immediately prior to the submission to 20% H_2O_2 (0 min). The values are the averages of three independent experiments.

E. faecalis is known for its resistance to lysozyme [28]. Resistance to lysozyme was also tested for the same three strains. Only the *zntA$_{Ef}$* mutant showed a marked increased susceptibility to the presence of this enzyme (Fig. 6), suggesting that the decreased survival of *E. faecalis zntA$_{Ef}$* mutant in macrophages may also be linked to increased lysozyme susceptibility.

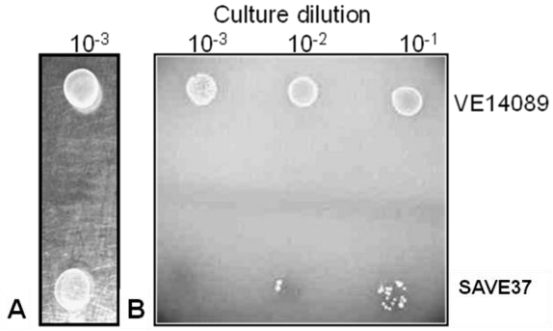

Figure 6 - *E. faecalis* resistance to lysozyme. *E. faecalis* VE14089 and SAVE37 (VE14089 *zntA$_{Ef}$:tet)* strains were grown overnight, adjusted to OD of 1 and diluted up to a thousand fold. Drops of 5 µl were spotted on LB plates with 0 (A) and 10 mg/ml (B) lysozyme and incubated for 24 h at 37ºC.

The results obtained with the *ef0759* mutant suggest that the EF0759 protein may help *E. faecalis* to survive inside macrophages in other ways than by protecting against oxidative stress or lysozyme exposure. The *ef0759* gene encodes a putative SapB protein of unknown function in *E. faecalis*. This protein contains an MgtC superfamily domain. MgtC family proteins are, in some bacterial species, involved in survival inside macrophages. Although the mechanism remains unknown, it has been reported that the protein may activate Na^+-K^+ P-type ATPases, either their own or those from the host [35-37]. Up-regulation of the *ef0759* gene in *E. faecalis* V583 in blood experiments [38] is also an indication that the corresponding protein might indeed have a role in processes related with infection, such as the survival inside macrophages. In *Salmonella typhimurium*, the MgtC protein gene is in an operon with that of a P-type ATPase, MgtB that is induced under magnesium starvation. Despite constituting a magnesium transport system, expression of the MgtCB operon can be modulated by zinc ions [39]. At this stage, any attribution to the role of EF0758-EF0759 would be mere speculation, but it is likely that the proteins have a relevant role in the maintenance of zinc homeostasis, since they were the highest overexpressed genes in the presence of zinc [13].

The results obtained with the *zntA*$_{Ef}$ mutant indicate that ZntA$_{Ef}$ plays a crucial role in *E. faecalis* survival inside macrophages, helping the cell to deal with the attack by host defenses. Macrophages use at least three defense mechanisms to fight engulfed bacteria, namely oxidative burst, zinc overload and lysozyme [34, 40]. As ZntA$_{Ef}$ was shown to be involved in defending *E. faecalis* against those three host attacks, we propose that this efflux system is one of the main constituents that enable *E. faecalis* cells to endure life inside the host. A BLAST search reveals that ZntA$_{Ef}$ is

ubiquitous among all sequenced enterococcal genomes, strengthening its importance. This work is the first description of a Zn^{2+}- responsive P-type ATPase, $ZntA_{Ef}$, involved in such biologic processes in *E. faecalis*. As had been observed for *M. tuberculosis* and *E.coli*, also *E. faecalis* seems to use a P-type ATPase, $ZntA_{Ef}$, to fight zinc accumulation inside macrophages as a host defense. Saliva, which constitutes one of the first host barriers, is characterized by high zinc and lysozyme concentrations. $ZntA_{Ef}$ is now suggested to be involved in defending *E. faecalis* against both host defense mechanisms and oxidative stress. This could explain the success of *E. faecalis* as an orthodontia pathogen, and also the possible link between the actions of zinc overload and lysozyme against pathogens. Unraveling the mechanisms of action of these defense elements is likely to provide insights into better approaches to fight bacteria such as *E. faecalis*.

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Supplementary Information

Table S1: Primers used in this study.

Primers	Nucleotide sequence 5'- 3'
OEF102	GGCGATCGGACTAAACAATTGAACACGGC
OEF106	TGATGAAACGGCACGGATAG
EF0421fw	TACCTACCTGCAGGAGGGGACATTGCTGAAGTTTCGCCACG
EF0421rev	TACTACCCTAGGGTGGGCAGATACTCACTGACTTCTGTGG
0759mutfw	TACCTACGAATTCGAGGGGACTCGGTATCAGTAGTAATGCC
0759mutrev	TACTACATGCATGTGGGCAGCTGTCACTGCCTCTTGTTCG
EF0966fw	TACCTACCTGCAGGAGGGGACCGTCATTATGAAAAAGCAGG
EF0966rev	TACTACCCTAGGGTGGGCAGCTTCTTGACAACCGTTTTCC
1400mutfw	TACCTACGAATTCGAGGGGACAATGGTTCCAAAGTGCTGCG
1400mutrev	TACTACATGCATGTGGGCAGGCTTCCTTCACTTCTGGTGC
EF1699fw	TACCTACCTGCAGGAGGGGACGAAATTGGTCTTCTTCAACC
EF1699rev	TACTACCCTAGGGTGGGCAGTGACCAAGAGTACATTAACC
EF2225fw	TACCTACCTGCAGGAGGGGACACGATGCCTGTTTCAAATCC
EF2225rev	TACTACCCTAGGGTGGGCAGACACGCAATGAAGAAAACGG
pILORI4-1	CCATTCGCCATTCAGGCT
pILORI4-2	CCGCTACGGATCACATCT
EF1400-1	GTTATGAATTCGTTATCCATTCCACCTACAGC
EF1400-2	AATTTGGATCCCACAACTTGCACAATCAAGTCC

Table S2: *E. faecalis* V583 genes with *zim* in their promoter regions.

Gene*	*zim* motif	Description**
ef0086	ATAAGGACGTGTTTTCATAA	hypothetical protein
ef0089	ATAAGGACGTGTTTTCATAA	hypothetical protein
ef0104	CTATGAAAGCGCATTCTTTT	arginine deiminase
ef0292	AAATGTATCCGTTTACATAT	PTS system transporter subunit IIC
ef0533	ATCTATTTGTATTTTCATTT	hypothetical protein
ef0567	TTTTGAATGTAGAGTCATTT	potassium-transporting ATPase subunit A
ef0585	ATATGAGTTTTATTTCATGT	30S ribosomal protein S14
ef0758	ATATGAATATATATTCATAT	cadmium-translocating P-type ATPase
ef0778	ATATGAATTTGTTTTCATTT	hypothetical protein
ef1098	ATAAGTATTTTTATTCATTA	hypothetical protein
ef1099	ATAAGTATTTTTATTCATTA	collagen adhesin protein
ef1124	ATATAAAAGGGTTTTCATTG	DeoR family transcriptional regulator
ef1125	ATATAAAAGGGTTTTCATTG	L-ascorbate 6-phosphate lactonase
ef1158	ATAAAATTTCATTTTCAGTA	N4-(beta-N-acetylglucosaminyl)-L-asparaginase
ef1172	ATATGTAAGCTTTTTCTTTT	teichoic acid biosynthesis protein B
ef1314	TTATTATTGCTTTTTCATTA	aminotransferase AlaT
ef1329	ATAGAAATATCTCGTCATTT	HesA/MoeB/ThiF family protein
ef1378	ATATTAAAGGAAATTCATTT	transcriptional regulator
ef1380	GTAAGTTTTCATCTTCAATT	hypothetical protein
ef1400	ATATGATTGCATCTTCATTT	cadmium-translocating P-type ATPase
ef1418	ATAAGAACGTATGTTCTTTT	hypothetical protein
ef1487	ATATGATTATATCTGCTAAA	hypothetical protein
ef1741	ATATGAATTTGTTCTCATTT	catabolite control protein A
ef1743	ATATGAATTTGTTCTCATTT	proline dipeptidase
ef1809	AAATGATAACGTTTACATTT	GntR family transcriptional regulator
ef1824	TAATGAATGCGTTTTCATTT	glycosyl hydrolase family protein
ef1869	AACTGAATCTTTATTCATTT	permease
ef2223	ATATAATAGCGTTTTCATAA	ABC transporter permease
ef2311	AAATGCGTTCATGTTCTTTT	hypothetical protein
ef2322	GAATGATTTCATATTGTTTC	hypothetical protein
ef2365	ATATGAACTCCTTTACTTAT	xanthine phosphoribosyltransferase

Gene*	*zim* motif	Description**
ef2404	ATATGATTTTAGTGGCATTA	hypothetical protein
ef2605	ATGAGAATAGGTATTCCTAT	UDP-N-acetylglucosamine 1-carboxyvinyltransferase 1
ef2615	AAAAGAATAAATTTACATTT	hypothetical protein
ef2680	ATCAGAATATTTTTTCATTT	ABC transporter ATP-binding protein/ permease
ef2681	ATCAGAATATTTTTTCATTT	HAD superfamily hydrolase
ef2831	ATATCAATATATTTTCATTC	hypothetical protein
ef3069	ATATGAATTTTTCATCTAAT	formate/nitrite transporter family protein
ef3070	ATATGAATTTTTCATCTAAT	30S ribosomal protein S4
ef3160	ATATGTTCACTTATACATAT	hypothetical protein
ef3207	TTCTGATTTTTTCTTTATTT	dihydrouridine synthase

* Genes with score > 11, according to MOODS software (pfm cutoff = 10^{-4}) [19]. A more stringent selection (score > 15) would include only the top seven genes, which are more likely to be the zinc responsive genes; the first three genes, *ef1400, ef0778* and *ef0758*, responded to zinc in our previous transcriptome experiments [13].
** According to the National Center for Biotechnology Information (NCBI).

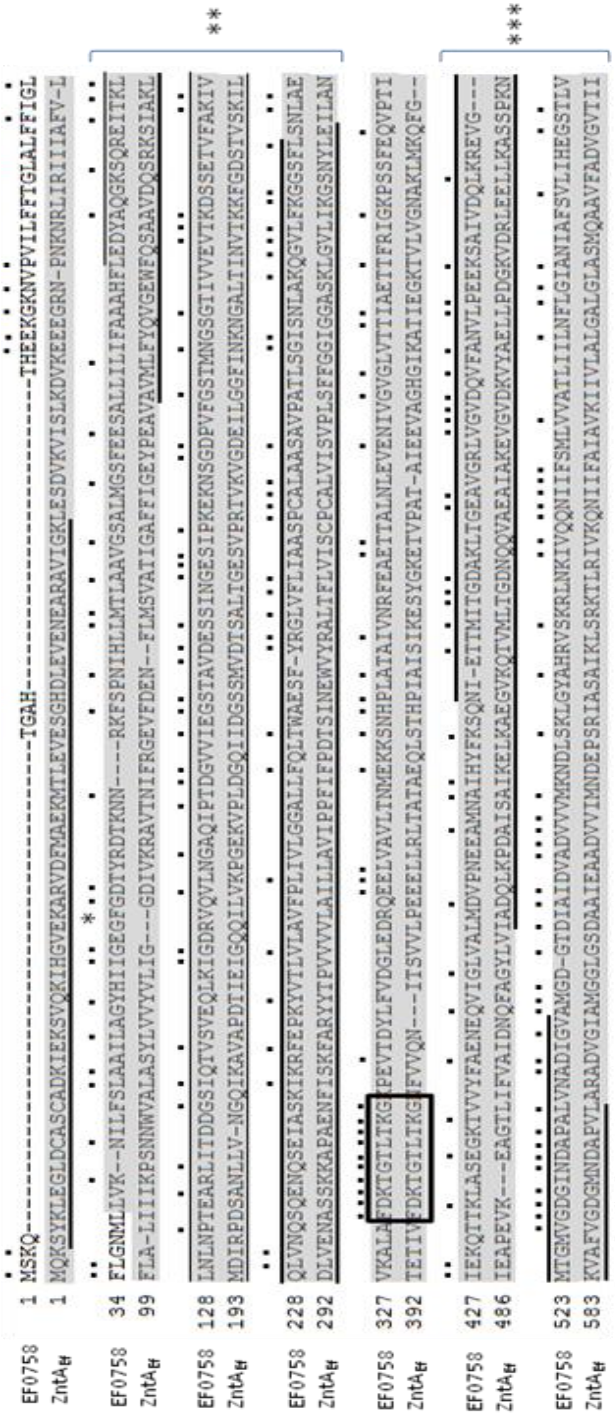

Figure S1 – Alignment of EF0758 and ZntA$_{Ef}$ protein sequences (Clone Manager Suite 7). The ZntA domains are shown in grey; the phosphorylation site is shown within the black box; black lines mark the different domains: * Heavy-metal-associated domain, ** E1-E2 ATPase domains, *** Haloacid dehalogenase-like hydrolase domain; ▪ Consensus amino acid residues.

Chapter 5

General Discussion

Transition metals are key elements in *E. faecalis* adaptation to different host sites

Enterococci are commensal, ubiquitous bacteria present in the oral and gut flora of the animal host. They maintain a dynamic homeostasis with the host, without becoming virulent or causing infections. However, when an opportunity emerges for penetrating cells at more internal sites, including the bloodstream, enterococci may seize it. The environment that bacteria meet when invading internal tissues is very different from that found at colonization sites, as it generally has higher redox potentials, limited essential nutrients and is protected by numerous host defenses. These new conditions are much less favorable for bacterial growth and enterococci will have to adapt to the new environment in order to survive. This adaptation requires a rearrangement of their transcriptional profile in order to express the proper proteins and virulence factors that enable them to colonize new tissues and consequently cause infection. Many enterococcal virulence factors have been described and studied, as referred in Table 1 in Chapter 1, which contributed to our partial understanding of mechanisms these bacteria employ to facilitate infection. The stresses that enterococci encounter when colonizing different sites in the host, and when causing infection, have been mimicked in recent studies in order to unravel the mechanisms and "tools" that support enterococcal adaptation to these harsh host environments (Table 2, Chapter 1).

One of the key elements for bacterial survival that changes when bacteria move into internal sites of the host is metal availability. Several metals are essential for numerous processes in all kingdoms of life. They are crucial components of many proteins, which would not function without these elements. Dysfunction of metal-associated proteins and unbalance of metal homeostasis are often correlated with several chromosomal and neurodegenerative diseases in man, as mentioned in Chapter 1. In

bacteria, metals are involved in processes ranging from metabolism to infection and virulence [1]. Metal homeostasis has been linked to pathogenicity in several important Gram positive pathogens. Little is known on the relevance of metals in *Enterococcus* survival, colonization and virulence. This lack of knowledge propelled the work presented in this thesis. It is intended to contribute to the study of the systems and regulators involved in metal homeostasis and, in particular, of the role of zinc, manganese and copper ions in important enterococcal life processes related to colonization, adaptation and infection.

Transport systems have the major role in *E. faecalis* metal stress responses

Maintenance of metal homeostasis in bacteria is achieved mainly by metal influx- and efflux systems and metalloregulatory proteins. These systems are still largely unknown in *Enterococcus faecalis*. The work presented in Chapter 2 contributes to our knowledge on the mechanisms these organisms have to modulate metal ion homeostasis, namely for zinc, manganese and copper ions. The work suggests that mostly transport systems are responsible for metal homeostasis (Figure 1). Our results agree with most annotations of genes (putatively) involved in metal transport.

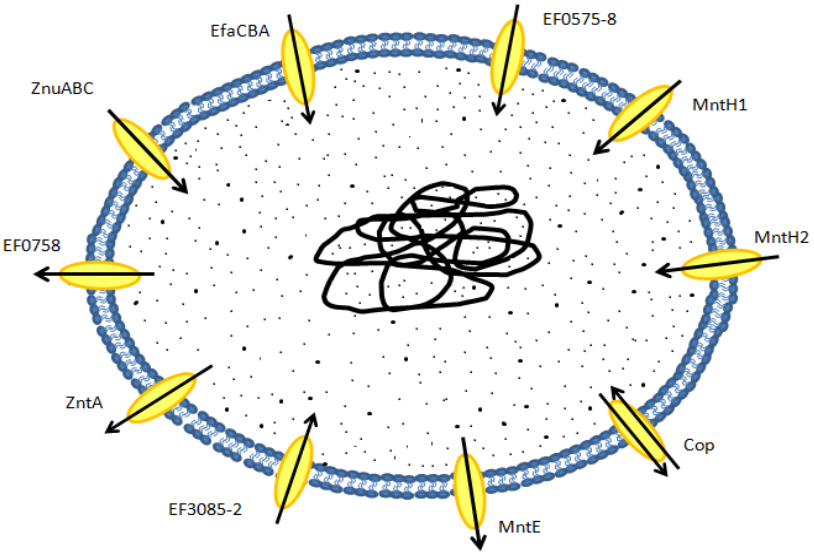

Figure 1 – *E. faecalis* annotated metal transport systems suggested to be involved in zinc, manganese or copper transport. Arrows indicate the way of the metal ion flux.

Several genes that are a part of other transport systems and two-component systems are suggested by our results to have a role in the homeostasis of zinc, manganese or copper ions. This is the case, for example, for *ef0082*, encoding an annotated major facilitator family transporter, *ef0583-4*, specifying two putative ABC transporters and that of a two-component system of unknown function, Err06-Ehk06. Prior to this work there were no clues as to the function of these systems; our results suggest that they may play a part in metal transport and homeostasis. The transporter genes mentioned above (*ef0082*, *ef0583-4*) were also differentially expressed in experiments with blood and urine [2, 3], which may be an indication that they also have a role in host-pathogen interactions. It would be very interesting to continue the study of these genes in order to unravel their primary roles in or connection with metal

homeostasis, and their potential involvement in colonization and infection processes.

In addition, expression of genes coding for proteins involved in cellular processes, in energy and amino acid metabolism and related to the cell wall was also altered in the presence of high concentrations of metals. It seems that *E. faecalis* had adapted its transcriptome to the experimental conditions in order to survive and grow properly. The *E. faecalis* V583 genome carries over 25% of mobile and/or exogenously acquired DNA, including a number of conjugative and composite transposons, a pathogenicity island (PAI), integrated plasmid genes and phage regions, and a high number of insertion sequence elements [4]. Many of these mobile genetic elements were affected in our DNA microarray experiments, particularly in the presence of manganese, when more than half of the differentially expressed genes were located on mobile elements, as described in Chapter 2. The ability of differentially expressing such genes under stressful conditions has likely helped enterococci to adapt to environments by conferring plasticity to the genome and contributing to the organism's adaptability.

Many *E. faecalis* strains have been isolated from bloodstream and urinary tract infections, an observation that has propelled the interest in studying these clinical bacteria under conditions mimicking the environments where they usually function as pathogens. We were able to correlate transcriptomic results from such studies [2, 3] with some of our results. The expression of genes from the arginine deiminase (ADI) operon was affected by the three metals used in our studies and experiments in which the strains were grown in the presence of blood or urine [2, 3]. The ADI pathway has been suggested to play a role in host-pathogen relationships in *Giardia lamblia* [5]. Hence, it would be interesting to confirm such a role of ADI in *E. faecalis* and study the putative influence of metals therein.

The same correlation with the mentioned urine and blood transcriptomic results [2, 3] was observed in the case of manganese transport systems. The results concerning these systems agree with the idea that bacteria sense their surrounding environment in terms of, among others, its metal composition and quickly adjust their transcriptional profile to variations, which can then lead to changes in the expression of proteins involved in *E. faecalis* colonization and virulence. Adaptation to changing metal concentrations is part of the overall process of adaptation to a new environment. In particular, when entering more internal sites in the host, where metals are scarce, metal scavenging systems are crucial to bacterial survival. In addition, as metals are so important for both the host and the bacteria, the (human) host tries to fight bacterial invaders by sequestering these elements [6].

The bacterial cell wall has an important role in response to copper stress

Copper is essential as a cofactor of numerous proteins; it can also be particularly dangerous as a catalyst in the production of reactive oxygen species (ROS) by the Fenton reaction, as described in Chapter 1. In agreement with this, we observed that cell wall-related genes, namely the *dltABCD* operon, were particularly responsive to high concentrations of copper ions (Chapter 2). This operon encodes proteins with the function to incorporate D-alanine residues into cell wall-associated teichoic acids and lipoteichoic acids (LTA). The D-alanylation of LTA contributes to the regulation of processes such as cation regulation [7]. LTA and peptidoglycans provide anionic sites for binding of metal cations. The overexpression of the referred genes suggests an effort of *E. faecalis* in sequestering the excess Cu^{2+} ions to prevent an increase in redox reactions that would contribute to cell damage.

Copper regulation, performed mainly by the *cop* operon, is very well described in *E. hirae* [8]. From our results and previous studies [9], it is clear that the same operon seems to have the preponderant role in copper homeostasis in *E. faecalis.* However, our results suggest that there may be other genes involved in copper regulation in *E. faecalis*, namely those in an operon (*ef1673-76*) encoding two ABC transporters, a hypothetical protein and a GntR family transcriptional regulator, all of unknown function. These genes significantly responded to high concentrations of copper. It would be interesting to explore the possibility of this operon being directly involved in dealing with this metal and if it has a link with the *cop* operon.

We advanced our studies by examining some of the results presented in Chapter 2 in more depth, aiming to find a link between metal transport, metal homeostasis and important biological processes. In particular, we were interested in studying Mn^{2+}-transport systems and their putative regulator EfaR.

Transition metals are involved in pathogenesis-related processes

Recent studies in Gram positive bacteria have shown that mutations in metal-dependent regulators of virulence factors affect biofilm formation (SloR, [10]), intramacrophage survival (IdeR, [11]) and resistance to oxidative stress (ScaR, [12]), confirming the importance of metal regulation in these processes, which also have been shown to influence the pathogenicity of those bacteria. Prior to this thesis, such a link had only been suggested for the *E. faecalis* endocarditis-specific antigen EfaA, an adhesion lipoprotein that is under manganese-dependent regulation by EfaR [13]. In Chapter 3 we show that, in fact, EfaR is a key player in manganese homeostasis in *E. faecalis* and regulates the manganese

transport systems EfaCBA, EF0575-8, MntH1 and MntH2; it uses Mn^{2+} as a cofactor. We show that the genes of these systems carry a common DNA binding motif in their promoter regions, named EfaR binding motif (*ebm*), which is suggested to be a key element in their regulation by EfaR. As EF0577 is similar to EfaA, it might also be a virulence factor involved in sensing and binding to host cells. Future studies could help establish the role of this protein in virulence, namely through experiments using animal models of infection (endocarditis) and colonization, thus contributing to new strategies of prevention of enterococcal infections.

The *ef0575-8* genes, encoding a second ABC Mn^{2+} transport system, are positioned in the vicinity of virulence factor genes and genes encoding stress proteins on the *E. faecalis* V583 PAI. This PAI is believed to increase virulence ability [4]. The fact that two Mn^{2+} ABC transport systems and two MntH transporters are encoded in the *E. faecalis* V583 genome highlights the importance of this metal in the life of these bacteria. The awareness of the relevance of manganese in biologic processes has grown in the last decade, together with the finding that many bacteria that used to require iron in numerous processes have evolved in order to require manganese instead [14]. This is due to the fact that Fe^{2+} ions are particularly noxious as they catalyze Fenton-type reactions that lead to the production of damaging hydroxyl radicals [15]. On the contrary, Mn^{2+} does not catalyze hydroxyl radical formation [16]; it is essential for the detoxification of ROS in most bacteria, principally as a cofactor for superoxide dismutase (MnSOD). As ROS play an important role in the host defense against bacteria [17], Mn^{2+} sequestration by infecting bacteria is essential for activation of defense systems against ROS. Hence it is important for bacteria such as *E. faecalis* to have Mn^{2+} scavenging systems that, together, could provide a fine-tuned and quick response to changes in manganese concentration.

The work presented in Chapter 3 also suggests that Mn^{2+}, and not Zn^{2+} or Cu^{2+}, is the only true cofactor of EfaR. Nevertheless, evidence points to

Zn^{2+} and Cu^{2+} as being able to compete with Mn^{2+} for EfaR binding, impairing manganese co-repressor activity and potentially affecting Mn^{2+} scavenging. Destabilization of manganese homeostasis, in particular by the inability to scavenge this metal ion, could seriously affect a number of cellular processes and jeopardize the ability of *E. faecalis* to survive and colonize its host. Our observations also suggest that there might be another regulator involved in the maintenance of manganese homeostasis. In future work, it would be important to explore this possibility and in particular to study the putative role of EF0578 in the regulation of this essential metal, as hypothesized in Chapter 3. Such studies would be required to fully understand the mechanisms that *E. faecalis* employs to maintain Mn^{2+} homeostasis.

Deletion of the *efaR* gene showed that EfaR plays a major role in *E. faecalis* pathogenicity, interfering with biological processes such as intramacrophage survival and biofilm formation and contributing to *E. faecalis* resistance to lysozyme and to oxidative stress. Mutation of the genes of the Mn^{2+} transport systems that are under control of EfaR did not affect the biologic processes tested, which suggests that these Mn^{2+} scavenging systems may compensate for each other when one is not functional or that as yet unidentified genes regulated by EfaR may be involved. Nevertheless, we observed that when EfaR is not functional, Mn^{2+} requirements are likely to be compromised, affecting the mentioned biologic processes. These observations suggest that manganese does play a crucial role in the pathogenesis of these bacteria and without a functional EfaR, coordinating the processes of manganese ion scavenging, *E. faecalis* is probably not able to obtain enough of this essential metal. Manganese has antioxidant properties, as discussed above, which explains the sensitivity of an EfaR mutant to this stress and its decreased intramacrophage survival. The important role of EfaR in biofilm formation and resistance to oxidative stress, which also correlates with

intramacrophage survival, may derive from a limit in manganese availability or may indicate that EfaR is a more global regulator which, in addition to metal transport systems, modulates other systems affecting these biologic processes. This hypothesis demands further scrutiny.

The work described in this thesis provides the first description of a DtxR/MntR regulator, EfaR, of which the absence of function has such severe implications in different cellular processes crucial for survival and host colonization. Further evidence, namely obtained by *in vivo* studies, may support the development of drugs or small peptides that could block this regulator and interfere with Mn^{2+} scavenging and therefore with the biological processes already discussed. This might ultimately help to prevent enterococcal infections.

E. faecalis relies on proteins involved in zinc homeostasis to stand host defenses

Host cells defend themselves from bacterial attack through, among others, the immune system. Some of the immune system cells, such as macrophages, engulf bacteria and subject them to a plethora of antimicrobial factors, such as lysozyme [18], ROS and metal stress [19]. In Chapter 4 we present one zinc P-type ATPase, $ZntA_{Ef}$, which seems to have a fundamental role in the fight of *E. faecalis* against these host defenses. In *E. faecalis*, nothing was yet described on zinc homeostasis, and a putative role for this metal in biological processes has never been determined. As zinc is a very important metal and component of many essential proteins, this lack of knowledge encouraged us to contribute to filling this gap.

The study focused on two proteins, EF0759 and $ZntA_{Ef}$, of which the corresponding genes were by far the most highly up-regulated when *E.*

faecalis was grown in the presence of a high concentration of zinc. These genes were found to have a Zn^{2+}-responsive DNA binding motif, *zim,* in their promoter regions. Our work revealed that EF0759 contributes to *E. faecalis* intramacrophage survival. Nevertheless, it does not seem to interfere with resistance to host defenses such as exposure to lysozyme, oxidative stress or zinc overload. Hence, its mode of action as an *E. faecalis* defense must involve other mechanisms. The gene *ef0759* is annotated as encoding a putative SapB protein of unknown function that contains an MgtC superfamily domain. MgtC family proteins have been described in some bacterial species to be involved in survival inside macrophages. Furthermore, they have also been suggested to be able to activate P-type ATPases [20, 21]. Our results suggest that the function of EF0759 is probably more related to the MgtC domain due to the observed influence on intramacrophage survival and the fact that its gene is in operon with the gene of the P-type ATPase EF0758, which was also highly up-regulated in the presence of zinc. Further studies are required to understand the function of EF0759, to know the mechanisms by which it contributes to the survival inside macrophages and to clarify if such a contribution has any relation with activation of P-type ATPases. In the future, it would also be important to study the role of EF0758 in biologic processes and examine if it has functions similar to those of $ZntA_{Ef}$. If that would be the case this would add to the toolbox that *E. faecalis* employs to fight the defense of the host. It would also be relevant to understand the regulation of the genes of these proteins, possibly through interaction of the, as yet unknown, regulator with *zim*, and to test the hypothesis of this being an ArsR/SmtB family regulator, as is the case for *S. aureus* [22].

Macrophages use zinc overload to kill engulfed bacteria and prevent bacterial proliferation [23]. It was reported that some bacteria, namely *M. tuberculosis* and *E. coli*, use P-type ATPases to get rid of the excess zinc inside macrophages [23]. Interestingly, our results suggest that, similar to

M. tuberculosis and *E. coli*, *E. faecalis* also uses P-type ATPases, namely $ZntA_{Ef}$, to fight zinc accumulation and other host defenses inside macrophages. We show that mutation of $ZntA_{Ef}$ decreased bacterial intramacrophage survival and resistance to oxidative stress, lysozyme and zinc excess. $ZntA_{Ef}$ inactivation will thus probably affect the subsequent steps of infection. Another possibility that should be considered is that impairment of the maintenance of zinc homeostasis through $ZntA_{Ef}$ inactivation may interfere with the proper functioning of other zinc-requiring proteins, which could ultimately lead to a poor bacterial defense against host attack. More studies would be necessary, for example in metal depleted environments, to fully understand the role of $ZntA_{Ef}$.

The concentration of zinc is high in saliva, in which lysozyme is also present. These two components seem to be a part of the first line of host defenses against opportunistic pathogens. The existence of P-type ATPases in *E. faecalis* would allow the organism to deal with these components and helps explain the success of *E. faecalis* as an orthodontia pathogen. This work is the first report of an *E. faecalis* zinc ATPase being an important determinant in processes involved in infection and virulence. Furthermore, it provides the first link between zinc homeostasis and pathogenesis in this organism. After *in vivo* confirmation of the relevance of this ATPase in enterococcal infection processes, development of blockers for such ATPases could be a good future strategy to fight enterococcal infections.

Knowledge on *E. faecalis* metal-related mechanisms may help minimize enterococcal infections in the future

The work presented in this thesis establishes the relevance of transition metals, and of the mechanisms involved in metal homeostasis in *E. faecalis* survival, growth, colonization and infection of the host (Figure 2).

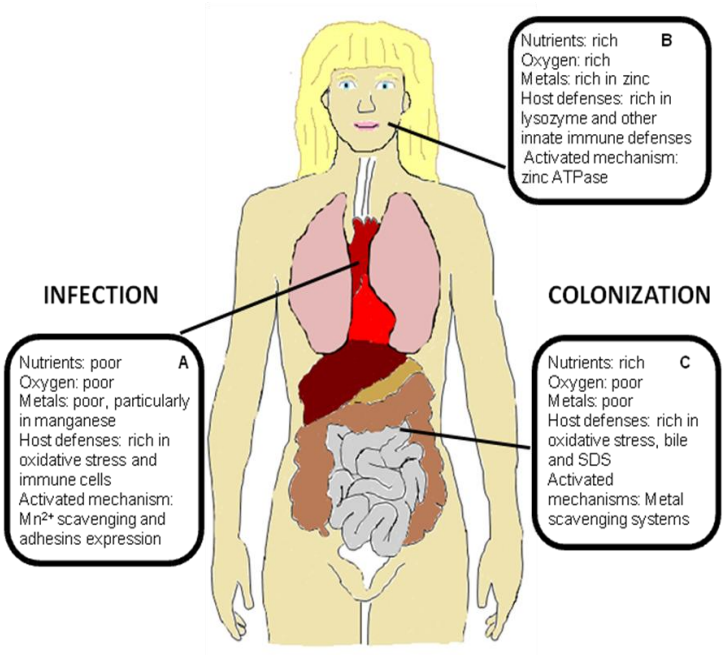

Figure 2 – Enterococcal infection and colonization sites in the human host. A. Bloodstream [3]; **B.** Oral cavity [24]; **C.** Gastrointestinal tract [25]. Boxed: characteristics of each site that influence bacterial colonization and infection processes.

Figure 2 gives an overview of relevant conditions encountered by invading *E. faecalis* at different sites of the host that precondition its colonization and infection processes, as well as the metal-related mechanisms they may use to facilitate adaptation. These are complex fluctuating environments in which metals are crucial elements.

It was shown how *E. faecalis* uses mechanisms involved in metal homeostasis to their benefit. On one hand, when these bacteria are present in environments poor in Mn^{2+} they use Mn^{2+} transport systems to obtain this metal and express virulence factors, namely adhesins, which facilitate bacterial adhesion to host cells and thus infection of new internal sites; it is fascinating how *E. faecalis* reacts to an adverse condition, metal limitation, by activating a scavenging system for that metal and a virulence factor that will help their infection process. On the other hand, when *E. faecalis* is being attacked by host defenses such as zinc overload, lysozyme and oxidative stress, it has the possibility to use a metal P-type ATPase, $ZntA_{Ef}$, which enables the bacterium to survive inside macrophages. This shows how *E. faecalis* is resourceful, incredibly well-adapted and suited to quickly deal with new challenges.

The work presented in this thesis acknowledges the relevant role of metal homeostasis in *E. faecalis* pathogenicity. The obtained know-how on metal transport and regulation in this organism adds to our understanding on the switch of Enterococcus from commensal to pathogen. Hopefully this knowledge may in the future help in dealing with enterococcal infections in nosocomial and other environments.

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