Enterococci are members of the gastrointestinal tract microbial consortium of humans and other animals but the genus has proven adept at causing opportunistic infections in immunocompromised patients. Enterococci were the first clinically relevant etiological agents acquiring vancomycin resistance. The appearance of vancomycin resistance in enterococci and its successful spread within the genus and to bacteria from other genus directly implies less therapeutic options for critically ill patients infected with these opportunistic pathogens. Moreover, when antimicrobial therapy is prescribed to treat infection, both the bacteria which are the target of antibiotic treatment and the others are exposed to a range of subinhibitory concentrations in the host, during and after treatment with the therapeutic dose.

In the first part of this thesis we focused on molecular and microbiological approaches to characterize the dissemination of vancomycin resistance determinants among enterococcal strains, contributing to draw a bigger picture of the scenario in Portugal. On the second part we carried out a more extensive study on the extent of the effects of vancomycin exposure and how metabolically *E. faecalis* cope with this exposure. This work contributes to the knowledge of the transcription events that *E. faecalis* undergoes under stress, and hopefully could be used in future to elucidate the biology of this bacterium.

Tânia Ribeiro

Studies on resistance and response to vancomycin in *Enterococcus faecalis*: a last resort antibiotic

Oeiras | January 2011

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Dissertation presented to obtain a Ph.D. degree in Biochemistry by

Instituto de Tecnologia Química e Biológica

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“Fazer qualquer coisa completa, inteira, seja boa ou má - e, se nunca é inteiramente boa, muitas vezes não é inteiramente má -, sim, fazer uma coisa completa causa-me, talvez, mais inveja do que outro qualquer sentimento. É como um filho: é imperfeita como todo o ente humano, mas é nossa como os nossos filhos são.”

Bernardo Soares, *Livro do Desassossego*, 1982

“Do something complete, whole, good or bad – and, if is never entirely good, most of the times it is not entirely bad -, yes, make a complete thing causes me, perhaps, more envy than any other feeling. It's like a child: it is imperfect like any human being, but it is ours like our children are.”

Bernardo Soares, *Book of Disquiet*, 1982

To my Family, to Luísa, Jaime, Arne, Mónica and Inês

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Thank you ALL for standing by over the years.
Tânia

Abstract

Enterococci are part of the normal human and animal gut microbiota and hardly cause infections in healthy individuals. In the last 20 years enterococci have emerged as common causes of hospital-acquired infections. One of the major reasons why these microorganisms easily survive in the hospital environment is their intrinsic resistance to several commonly used antibiotics, and more importantly, their ability to acquire resistance to many currently used antibiotics, including glycopeptides.

Development of resistance to the glycopeptide vancomycin in the *Enterococcus* genus presents a worldwide major problem. Infections with vancomycin resistant enterococci are not only difficult to treat but the organisms show a strong propensity to disseminate and spread from patient to patient in the hospital setting. Accurate knowledge of the real scenario of vancomycin resistance is essential to design national and global strategies and prevent community and nosocomial transmission of vancomycin resistant organisms.

Vancomycin binds with high affinity to the D-Ala C-terminus of the peptidoglycan pentapeptide, thus blocking the addition of late precursors by transglycosylation to the nascent peptidoglycan chain and preventing subsequent cross-linking by transpeptidation. In enterococci, glycopeptide resistance is conferred by the presence of operons that encode enzymes for the synthesis of low-affinity peptidoglycan precursors to the glycopeptides. Eight types of glycopeptide resistance have been characterized on both phenotypic and a genotypic basis in enterococci. The classification of glycopeptide resistance is currently based on the primary sequence of the resistance ligases (*vanA*, *vanB*, *vanC*, *vanD*, *vanE*, *vanG*, *vanL*, *vanM*). The most frequently detected operons are *vanA* and *vanB* operons and both harbor genes with regulatory functions (VanRS, $VanR_BS_B$), resistance (VanHAXY, $VanY_BH_BBX_B$) and accessory proteins (VanZ,

vanW). Their transcription is regulated by the VanRS and $VanR_BS_B$ two-component systems.

In the first part of this thesis we screened phenotypically susceptible strains, from different origins in Portugal, for the presence of vancomycin resistance genes. *vanA* and *vanB* genes were both found in dairy food isolates and in clinical human and veterinary isolates. *vanA* gene was found to be more disseminated in the clinical setting and *vanB* gene was only detected in a restricted number of clinical isolates. However, none of the isolates carrying *vanA* and *vanB* genes had complete vancomycin resistance operons. This finding suggests that the studied strains were, at some point in time, in contact with vancomycin resistance determinants, and probably due to absence of selective pressure, allowed genetic events (deletions or insertions) ultimately leading to *vanA* and *vanB* operons incomplete or corrupted, respectively. However, the presence of some of the vancomycin resistance determinants in susceptible strains may suggest that those genes could play some other biological role in those strains as if they could confer any selective advantage in the presence of vancomycin.

Furthermore, we characterized a set of human clinical enterococcal strains including three enterococcal vancomycin resistant *Enterococcus faecium* (VREfm) strains carrying the insertion sequence IS*Ef1* and two *Enterococcus hirae* strains responsible for nosocomial infection in two different patients. To our knowledge this is the first report of *E. hirae* strains as etiological agents of nosocomial infections in a portuguese hospital. We also point out to the vulnerability of VITEK system alone in providing a correct assessment of vancomycin resistant enterococci (VRE).

Following findings from the first part of this work, namely the hypothesis that *vanA* and *vanB* incomplete operons still play some role in vancomycin resistance,

and recent evidences of involvement of two-component systems (other than VanRS) in response to vancomycin; in the second part of this thesis we aimed identifying complementary and/or alternative mechanisms that may be involved in response to vancomycin.

During antimicrobial therapeutics the concentration of antibiotics in circulation and in the different tissues reach frequently doses that are lower than the therapeutic dose administered. Thus, we found important to study the transcriptional response of *E. faecalis* to different vancomycin doses, thus response to a subinhibitory and to a therapeutic dose of vancomycin was studied in *E. faecalis*. The subinhibitory dose strictly and specifically induced the vancomycin resistance genes. However, the therapeutic dose had a major effect on the overall expression profile, as 14% of the genes were differentially transcribed. These results demonstrate that *E. faecalis* responds globally to a therapeutic vancomycin dose, suggesting that survival/resistance to vancomycin may involve other genes and pathways.

We observed that after vancomycin exposure one ORF presented the same level of transcription than the *van* genes. Our work led to the identification of a new gene belonging to the *vanB* operon of *E. faecalis* (*vanV*). We provide evidence of co-transcription of *vanV* together with $vanY_BWH_BBX_B$ and demonstrate that its expression is under the control of $vanR_BS_B$. However, *vanV* was not found to be required for vancomycin resistance and likely codes for an accessory protein.

Taking together the transcriptomic results we obtained under vancomycin exposure and the available literature we suspect that other enterococcal two-component systems might be involved in sensing and responding to vancomycin. We describe the involvement of CroRS and TCS06 in vancomycin resistance and

found that CroRS is needed for VanRS induction and for TCS06 induction by vancomycin.

To investigate the possibility of *vanB* operon genes influencing the expression of other genes in *E. faecalis,* we used a genome-wide transcriptomic approach to evaluate the differential expression of *E. faecalis* genes in *vanB* and *vanR* independent mutants. In *vanB* mutant 205 and 309 of the V583 *E. faecalis* genes showed differential expression, depending on the growth phase sampled. For the *vanR* mutant 157 and 147 of the genes were differentially expressed. Moreover, both mutants showed a very high number of genes located on mobile genetic elements had their expression levels affected. These results suggest that the *vanB* system may play a wider role in the cell.

In conclusion, the main findings presented in this thesis are that *van*-resistance genes are spread among enterococcal strains from different origins. Although these strains may keep a susceptible background in the presence of vancomycin selective pressure, resistance may arise, suggesting that other alternative mechanisms exist and may develop, by which enterococci might survive in the presence of vancomycin. We also provide evidence that vancomycin resistance is not strictly a product of *vanB* operon fully expression and other two-component systems are involved and contribute to the high-level resistance to the antibiotic. Furthermore, *vanB* operon is likely to have a broader role on enterococcal metabolism.

Resumo

Os *Enterococcus* fazem parte da microbiota normal do tracto gastrointestinal de humanos e animais e raramente causam infecções em indivíduos saudáveis. Nos últimos 20 anos os enterococos emergiram como uma das causa mais comuns de infecções adquiridas em ambiente hospitalar. Uma das principais razões pela qual estes microrganimos sobrevivem com sucesso em ambiente hospitalar deve-se à sua resistência intrínseca a vários antibióticos e, ainda mais relevante, à sua capacidade para adquirir resistência a muitos dos antibióticos frequentemente utilizados em terapêutica, incluindo os glicopéptidos. No género *Enterococcus* o desenvolvimento de resistência ao glicopéptido vancomicina constitui um sério problema a nível global. Infecções provocadas por enterococos resistentes à vancomicina são, não apenas difíceis de tratar, como estas bactérias também apresentam uma marcada propensão para se disseminarem de doente para doente em ambiente hospitalar.

O conhecimento do cenário real do estado da disseminação da resistência à vancomicina é essencial para poderem ser planeadas e adoptadas estratégias globais de forma a prevenir a transmissão nosocomial de bactérias resistentes à vancomicina, bem como a sua transmissão na comunidade.

A vancomicina liga-se com elevada afinidade ao D-Ala C-terminal do pentapéptido do peptidoglicano, bloqueando a adição de mais precursores à cadeia nascente do peptidoglicano e prevenindo o estabelecimento subsequente das ligações cruzadas por transpeptidação. Em enterococos a resistência a glicopéptidos é conferida pela presença de operões que codificam enzimas para a síntese de precursores do peptidoglicano com reduzida afinidade para estes antibióticos. Em enterococos, oito tipos de resistência a glicopéptidos já foram caracterizados aos níveis fenotípico e genotípico. A classificação dos tipos de resistência a glicopéptidos baseai-se na sequência primária das ligases

codificados nos diferentes operões (*vanA*, *vanB*, *vanC*, *vanD*, *vanE*, *vanG*, *vanL*, *vanM*). Os operões mais frequentemente detectados são os *vanA* e *vanB* que possuem genes com funções reguladoras (VanRS, $VanR_BS_B$), de resistência (VanHAXY, $VanY_BH_BBX_B$) e proteínas acessórias (VanZ, vanW). A sua transcrição é regulada pelos sistemas de dois componentes VanRS e $VanR_BS_B$, respectivamente

Na primeira parte desta tese pesquisámos a presença de genes de resistência à vancomicina em estirpes de enterococos fenotipicamente susceptíveis ao antibiótico, de diferentes origens. Os genes *vanA* e *vanB* foram detectados em isolados alimentares provenientes de alimentos lácteos, bem como em isolados clínicos humanos e veterinários. O gene *vanA* está mais disseminado no ambiente clínico e o gene *vanB* foi apenas detectado num número reduzido de isolados clínicos. No entanto, nenhum dos isolados que possuem os genes *vanA* e *vanB* apresentam operões completos de resistência à vancomicina. Estas observações sugerem que as estirpes estudadas estiveram, nalgum momento, em contacto com genes de resistência à vancomicina e, provavelmente devido à ausência de pressão selectiva, ocorreram eventos genéticos (delecções ou inserções) que, em última instância, levaram a operões *vanA* e *vanB* incompletos ou corrompidos, respectivamente. No entanto, a presença de alguns genes de resistência à vancomicina em estirpes susceptíveis pode sugerir que estes genes desempenham outros papéis biológicos nestas estirpes, como se constituíssem alguma vantagem selectiva em presença de vancomicina. Além disso, caracterizámos um conjunto de estirpes clínicas humanas, incluindo três estirpes de *Enterococcus faecium* vancomicina resistentes (VREfm) que têm a sequência de inserção IS*Ef1* e duas estirpes de *Enterococcus hirae* responsáveis por infecções nosocomais em dois doentes

diferentes. Esta é a primeira descrição de estirpes de *E. hirae* enquanto agentes etiológicos de infecção nosocomial em hospitais Portugueses. Também salientámos a vulnerabilidade do sistema VITEK, por si só, em identificar correctamente enterococos resistentes à vancomicina (VRE).

Seguindo os resultados da primeira parte deste trabalho, nomeadamente a hipótese de que operões *vanA* e *vanB* incompletos continuam a ter um papel na célula, e as recentes evidências do envolvimento de outros sistemas de dois componentes (que não o VanRS) na resposta à vancomicina, a segunda parte desta tese teve por objectivo identificar mecanismos alternativos e/ou complementares que estejam envolvidos na resistência à vancomicina.

Durante o processo terapêutico com um agente antimicrobiano a concentração do antibiótico em circulação em nos diferentes tecidos atinge frequentemente doses inferiores à dose terapêutica administrada Por este motivo, considerámos importante estudar as respostas transcricionais de *E. faecalis* a diferentes doses de vancomicina. Assim, a resposta a uma dose sub-inibitória e a uma dose terapêutica de vancomicina foi estudada em *E. faecalis*. A dose sub-inibitória induz estrita e especificamente os genes de resistência à vancomicina. No entanto, a dose terapêutica tem um efeito muito significativo no perfil geral de transcrição, com 14% dos genes a serem transcritos diferencialmente. Estes resultados demonstram que *E. faecalis* responde globalmente a uma dose terapêutica de vancomicina, sugerindo que a sobrevivência/resistência à vancomicina pode envolver outros genes e vias.

Observámos que após exposição à vancomicina uma ORF apresentava o mesmo nível de transcrição que os genes *van*. O nosso trabalho permitiu identificar um novo gene pertencente ao operão *vanB* de *E. faecalis* (*vanV*). Fornecemos evidências da co-transcrição do *vanV* juntamente com

vanY$_B$WH$_B$BX$_B$ e demonstramos que a sua expressão está sob o controlo de *vanR$_B$S$_B$*. No entanto, o *vanV* não é necessário para a resistência à vancomicina e provavelmente codifica para uma proteína acessória.

Considerando os resultados obtidos no estudo de transcriptómica sob exposição à vancomicina e a literatura disponível fomos levados a suspeitar do envolvimento de outros sistemas de dois componentes na detecção do sinal e resposta à vancomicina. Assim, analisámos e descrevemos o envolvimento de CroRS e TCS06 na resistência à vancomicina e percebemos que o CroRS é necessário para a indução do VanRS e para a indução do TCS06 pela vancomicina.

Para investigar a possibilidade dos genes do operão *vanB* influenciarem a expressão de outros genes em *E. faecalis*, usámos uma abordagem transcriptómica para avaliar a expressão diferencial dos genes de *E. faecalis* em dois mutantes independentes, *vanB* e *vanR*. No mutante *vanB*, 205 e 309 genes de *E. faecalis* V583 mostraram-se diferencialmente expressos, dependendo da fase de crescimento. No mutante *vanR*, 157 e 147 genes foram diferencialmente expressos. Nos dois mutantes, um elevado número de genes localizados em elementos genéticos viram os seus níveis de expressão afectados. Estes resultados sugerem que o sistema *vanB* poderá ter um papel muito mais alargado na célula.

As principais conclusões apresentadas nesta tese são, portanto, que os genes de resistência *van* estão disseminados entre estirpes de enterococos de diferentes origens. No entanto estas estirpes mantêm um *background* susceptível mas, em presença de pressão selectiva por vancomicina, a resistência pode surgir, sugerindo que existem mecanismos alternativos que se podem desenvolver e permitir aos enterococos sobreviver em presença de vancomicina.

Fornecemos ainda evidências de que a resistência à vancomicina não é estritamente produto de uma expressão plena do operão *vanB* e que outros sistemas de dois componentes estão envolvidos e contribuem para a elevada resistência ao antibiótico. É provável que o operão *vanB* tenha uma papel mais alargado no metabolismo dos enterococos.

List of Publications

Scientific Articles Peer Reviewed in International Journals:

Tânia Ribeiro, Axel Hartke, Michael S. Gilmore, Fátima Lopes. Transcriptomic profile of *E. faecalis* V583 in response to vancomycin reveals bacterial support for full expression of high-level vancomycin resistance (in preparation).

Tânia Ribeiro, Fátima Lopes. CroRS integrity is essential for VanRS induction and high-level vancomycin resistance phenotype (in preparation).

Tânia Ribeiro, Sofia Santos, Fátima Lopes. Identification of a new gene in the *vanB* operon of *E. faecalis* (submitted for publication).

Tânia C Ribeiro, Vera Pinto, Frèdèric Gaspar, Maria FS Lopes (2008) *Enterococcus hirae* causing wound infections in a hospital. Journal of Chinese Clinical Medicine 3:150-152.

Tânia Ribeiro, Marta Abrantes, Maria de Fátima Silva Lopes, Maria Teresa Barreto Crespo (2007) Vancomycin-susceptible dairy and clinical enterococcal isolates carry *van*A and *van*B genes. International Journal of Food Microbiology 113:289-295.

Benoît Zuber, Marisa Haenni, **Tânia Ribeiro**, Fátima Lopes, Philippe Moreillon, Jacques Dubochet (2006) Granular layer in the periplasmic space of Gram-positive bacteria and fine structure of *Enterococcus gallinarum* and *Streptococcus gordonii* septum revealed by cryo-electron microscopy of vitreous sections. Journal of Bacteriology 188:6652-6660.

Dissertation Outline

The objectives proposed for this dissertation were all focused on vancomycin resistance determinants dissemination and vancomycin response in enterococci. In particular we intended to learn more about the current real amplitude of vancomycin determinants dissemination in different environments and what are the mechanisms that mediate vancomycin response in enterococci.

The present Dissertation is divided in two main parts and into nine chapters. On the first part we present data related to the screening and detection of vancomycin resistance determinants in strains from different environmental sources. On the second part we focus in genes and pathways that might be involved in vancomycin resistance and response

Chapter 1 consists of a general introduction to contextualize the subject. A brief overview of vancomycin resistance in enterococci and its importance as a last resort antibiotic, to treat multidrug resistant infections caused by Gram-positive bacteria, and as stress agent is made. The results of this Doctoral work are presented in the following chapters. Each chapter has its own Introduction, Results and Discussion of the respective work.

In chapter 2 we describe the dissemination and prevalence of vancomycin resistance determinants (*vanA* and *vanB*) in a considerably large group of dairy isolates and human and veterinary clinical isolates and this way study the dissemination of these determinants in different ecological niches. The arrangement of the prototype resistance element Tn*1546* is determined and high heterogeneity of this mobile element was observed.

Chapter 3 consists on the characterization of enterococcal strains in a Portuguese Hospital. We report the detection of *E. faecium* strains belonging to CC17 and the presence of IS*Ef1* in Leiria Hospital. We also report the first two

cases of *E. hirae* as etiological agent of nosocomial infections in Portugal associated with wound infections.

In chapter 4 we describe and discuss microarray data on how *E. faecalis* V583 responds to a therapeutic dose of vancomycin.

In chapter 5 we demonstrate that *vanB* operon has one more gene on its constitution than previously acknowledged.

Chapter 6 characterizes the involvement of two two-component systems in vancomycin sensing and potential induction of vancomycin resistance.

Chapter 7 describes the behavior of vancomycin susceptible strains when exposed to serial passages in presence of antibiotic and discuss the possible mechanims behind the observed adaptation.

Chapter 8 is centered on unraveling the role of *vanB* operon genes in enterococci in absence of vancomycin induction. We present and discuss transcriptomic results on *vanB* and *vanR* mutants.

Chapter 9 is an integrated Discussion of the global results of the previous chapters and proposes the Future Perspectives regarding the vancomycin response mechanisms and alternative regulation pathways. There is an Appendix section where the pdfs of the published papers are presented.

Abbreviations

A adenine

AI after induction

Ala alanine

AMP ampicillin

APS antimicrobial peptide sensor

AS aggregation substance

Asp aspartate

ATCC american type culture collection

ATP adenosine triphosphate

B bacitracin

BC before Christ

BHI brain hearth infusion

BI before induction

bp base pair

°C degree Celsius

CA catalytic and ATP-binding domain

CAT chloramphenicol acetyltransferase

CC clonal complex

cDNA complementary desoxyribonucleic acid

CDP cytidine diphosphate

CECT colección española de cultivos tipo

CESR cell envelope stress response

CFP cefoperazone

CHEF clamped homogeneous electric fields

CIP ciprofloxacin

CLSI clinical and laboratory standards institute

CN gentamicin

CoNS coagulase-negative staphylococci

CS coefficient of similarity

CTP cytidine triphosphate

CWAP cell-wall anchored proteins

D aspartate

DA clindamycin

DHF dihydropholate

DHp dimerization and histidine-containing phosphotransfer domain

DNA desoxiribonucleic acid

dNTP deoxynucleotide triphosphate

DSMZ deutsche sammlung von mikroorganismen und zellkulturen

E erythromycin

EARSS european antimicrobial resistance surveillance system

ECF extracytoplasmic function sigma factor

EDTA ethylenediaminetetraacetic acid

Ehk enterococcal histidine kinase

Err enterococcal response regulator

Ery erythromycin

Esp enterococcal surface protein

F nitrofurantoin

G guanine

GI gastrointestinal

GISA glycopeptide intermediate *Staphylococcus aureus*

GlcN glucosamine

GlcNAc N-acetyl glucosamine

GRE glycopeptide resistant enterococci

H histidine

HiRCC high risk clonal complex

His histidine

HK histidine kinase

HLGR high-level gentamicin resistance

HLVR high-level vancomycin resistance

HPT histidine phosphotransferase

HSP heat shock protein

HTH helix-turn-helix

IE infectious endocarditis

IPN imipenem

IPP isopentenyl pyrophosphate

IR intergenic region

IS insertion sequence

K kanamycin

kb kilobase

Lac lactate

LMG Laboratorium voor Microbiologie

LTA lipoteichoic acid

µg microgram

µl microliter

µM micromolar

MDR multi-drug resistance

MGE mobile genetic element

MIC minimum inhibitory concentration

min minute

ml milliliter

MLST multilocus sequence typing

mM milliMolar

MOPS 3-(N-morpholino)propanesulfonic acid

mRNA messenger ribonucleic acid

MRSA methicillin resistant *Staphylococcus aureus*

MurNAc N-acetyl muramic acid

NA nicotinic acid

NAD nicotinamide adenine dinucleotide

Nam nicotinamide

NamND nicotinamide mononucleotide

NCCLS national committee on clinical laboratpry standards

ng nanogram

nm nanometer

NMR nuclear magnetic resonance

NR nicotinamide riboside

OA oxaloacetate

ORF open reading frame

P penicillin B

PABA para-aminobenzoic acid

PAI pathogenicity island

PBP penicillin binding protein

PCR polymerase chain reaction

PFGE pulsed field gel electrophoresis

PG peptidoglycan

pH hydrogen potential

pmol picomol

ppGpp, pppGpp guanosine 3',5'-bispyrophosphate, guanosine 3'-diphosphate 5'-triphosphate, collectivelly ppGpp and pppGpp are termed (p)ppGpp

PRPP phosphoribosyl pyrophosphate

PTS phosphoenolpyruvate phosphotransferase system

RD rifampicin

RDO registered designation of origin

RNA ribonucleic acid

RNase ribonuclease

rpm rotations per minute

RR response regulator

RT-PCR reverse transcriptase PCR

S streptomycin

s second

SAM S-adenosylmethionine

SDS sodium dodecyl sulphate

Ser serine

SI subinhibitory

SLR signal log ratio

SNP single nucleotide polymorphism

Spp. species

sRNA small non-coding RNA

SSC saline-sodium citrate buffer

ST sequence type

T thymine

TAE tris acetate EDTA buffer

TAIL-PCR thermal asymmetric interlaced-PCR

TBE tris borate EDTA buffer

TCS two-component system

TE/Tet tetracycline

THF trihydropholate

ThMP hydroxymethyl pyrimidine

Tn tranposon

TPP thiamine pyrophosphate

TRAP tripartite ATP- independent periplasmic

Tris trishydroxymethylaminomethane (2-Amino-2-(hydroxymethyl)propane-1,3-diol**)**

U unit

UDP uridine diphosphate

UMP uridine monophosphate

UPGMA unweighted pair group method with arithmetic mean

UPP undecaprenyl pyrophosphate

US United States

UTI urinary tract infections

UV ultraviolet radiation

V volt

Van vancomycin

VISA vancomycin-intermediate *Staphylococcus aureus*

VRE vancomycin resistant enterococci

VREfm vancomycin resistant *E. faecium*

VREfs vancomycin resistant *E. faecalis*

VRSA vancomycin-resistant *Staphlococcus aureus*

VSE vancomycin susceptible enterococci

VSEfm vancomycin susceptible *Enterococcus faecium*

wt wild type

w/v weight/volume

Chapter 1

General Introduction

CONTENTS

I. VANCOMYCIN

HISTORICAL INTRODUCTION TO VANCOMYCIN DISCOVERY

The use of substances with antibacterial activity has been documented throughout history. Over 50,000 years ago the Neanderthals used certain herbs and natural substances to kill bacteria in the wounds and stop their propagation. Human remains have been found with various herbs that are said to be used for therapeutic purposes by the prehistoric man. There is evidence in the Ebers papyrus (1550 BC) that the Egyptians have used beer yeast on wounds and numerous other ancient civilizations used molds in one form or another for cures and rituals. A chemical analysis of the bones of ancient Nubian mummies, shows that they were regularly consuming tetracycline, most likely in their beer since the grain used to make the fermented gruel contained the soil bacteria streptomyces, which produces tetracycline (Levy, 2002). The ancient Chinese also used molds to treat boils, carbuncles and other skin infections.

Nevertheless, it is only in the end of the 19th century that the first scientific report of mold use with the specific aim of killing bacteria appeared. In 1897 Ernest Duchesne reported that certain penicillium molds could indeed destroy *Salmonella typhi* and *Escherichia coli* in the test tube. He further showed that inoculating intraperitoneally a guinea pig with a solution containing *Penicillium glaucum* and *E. coli*, would protect the guinea pig against the lethal dose of *E. coli*. He had shown that certain molds could kill bacteria – he had discovered penicillin (reviewed in Duckett, 1999).

However, it is generally accepted that the discovery of penicillin dates from 1928 (reviewed in Podolsky, 1997) when the young bacteriologist Merlin Pryce noticed that one of the petri dishes floating in the sink and ready to be discarded was contaminated with a mold and, more important, that there was a clear ring of

lysed bacteria. Fleming, who was interested in the lysis phenomena, got intrigued by this observation and decided to pursue it further. After several experiments he showed that an active substance, produced by this mold, could kill bacteria such as staphylococci, streptococci, gonococci but not others such as those responsible for typhoid fever or cholera. Later he and his young assistant, Stuart Craddock, proved that injection of the mold filtrate could not only save rabbits and mice from lethal infections of staphylococci but it could also cure his assistant´s sinus and eye infections.

Despite the successful experiments done by Fleming, the application of penicillin to treat infections would have remained only an academic curiosity, if it were not for the work of two other future Nobel Prize laureates. Fleming was discouraged, with no research funds to continue the experiments and with a very unstable compound of which only very small amounts could be purified. He was further disappointed by his own unpublished observation that staphylococci became resistant to penicillin very quickly (Podolsky, 1997). Fleming discretely described his preliminary findings and then turned his back on penicillin. Later Florey and Chain rescued penicillin and succeeded in purifying large amounts of the compound, generating enough interest so that when World War II started there was a bigger production of this antibiotic in order to decrease the fatalities in Europe and Asia. By this time penicillin had been used in fewer than 100 people in the United States of America.

On Sunday morning, November 29, 1942, the Boston Herald front page announced the most devastating fire in the history of Boston, the Cocoanut Grove Nightclub disaster. There were more than 450 victims and the physicians were caring for the 200 survivors who resisted for the first 24 hours. The fire also occasioned a medical event now regarded as historically significant as the fire

itself – the trial of a new, unique drug available only through government sources. Limited amounts of penicillin were released to quell many of the infections contracted by survivors of the fire. Actually, the Cocoanut Grove victims became one of penicillin´s most important clinical trials and the success of skin grafting and treating the severely burned patients was largely attributed to the action of penicillin (Levy, 2002). Soon after, the use of antibiotics was widely spread and penicillin was considered as a miraculous drug. Penicillin discovery marked the beginning of the “antibiotic era”.

The success of penicillin encouraged scientists to search for and discover new antibiotics that could treat other bacteria, including penicillin-resistant strains. As more antibiotics were discovered, from the late 1940s into the 1970s, the problem of resistance seemed little more than an annoyance.

By the 1950s, it was apparent that penicillin was not going to remain effective against staphylococci. The pharmaceutical Eli Lilly and Company was soon operating a large-scale screening program aimed solely at the isolation of antibiotics with high level of specificity and activity against staphylococci. Soil samples were obtained from all parts of the world and were subjected to the screening procedures. An organic chemist at Lilly, Dr. E. C. Kornfeld, had a missionary friend in Borneo who sent him dirt from the deep interior of the jungle. In it, he found an organism that later was named *Streptomyces orientalis*. Fermentation broths of the organism were highly active and bactericidal against virtually all staphylococci tested. Studies in animals showed the level of toxicity to be relatively low. The active compound in *S. orientalis* fermentation was labeled compound 05865 (Levine, 2006).

The difficulty with which staphylococci would become resistant to the compound 05865 was obviously a matter that required immediate appraisal.

Consequently, the earliest *in vitro* studies consisted on experiments on the induction of resistance to compound 05865 by daily serial passage. The results obtained lead to consider that the laboratory-induced level of staphylococcal resistance to compound 05865 was negligible (Levine, 2006).

Since the impurities from the preparation and fermentation broth were linked to ototoxicity and nephrotoxicity and due to its brownish color, compound 05865 earned the nickname "Mississippi mud". Soon, however, "Mississippi mud" was given the generic name vancomycin, derived from the word vanquish, and efforts were directed towards its purification (Levine, 2006).

Although the early clinical trials of vancomycin were modest in scope and involved relatively few researchers, information concerning the efficacy of the antibiotic in difficult staphylococcal infections quickly spread. With the increased demand for vancomycin to be used in clinical studies and in emergency treatment of serious staphylococcal infection, supplies were often inadequate. On various occasions it was necessary for a representative of Eli Lilly and Company to locate some vancomycin in one hospital and deliver it to another where it was more urgently needed. The large volume of requests for vancomycin for emergency use was followed by numerous reports of satisfactory therapeutic responses. Because there was no other alternative in the treatment of serious infections caused by staphylococci resistant to the then available antibiotics, the U.S. Food and Drug Administration strongly urged Eli Lilly and Company to make vancomycin generally available as soon as possible.

Early concerns regarding safety, which were based on results of studies in animals, may have been responsible for the limited use of vancomycin for 20 years. It was reformulated and retested in animal models for toxicity in the 1970s. It was then found to have no otoxocity and little nephrotoxicity. In the late 1970s

vancomycin re-emerged as a key component in treating methicillin-resistant *Staphylococcus aureus* (MRSA). The clinical need for an agent to fight MRSA was apparent and vancomycin quickly became the drug of choice.

VANCOMYCIN MODE OF ACTION

The simultaneous worldwide emergence, first of methicillin-resistant *S. aureus*, and then, the penicillin-resistant *Streptococcus pneumonia*, together with the reported success on the treatment of pseudomembranous enterocolitis led to the clinical re-introduction of vancomycin (Levine, 2006). In the 1980s vancomycin was being widely used worldwide and its use increased specially in the United States. In contrast, the rise of vancomycin use has been less pronounced in Europe (Kirst *et al.*, 1998).

Vancomycin is the most relevant member of the glycopeptide class of antibiotics, which include also teicoplanin, telavancin and ramoplanin. The chemical structure shared by the glycopeptide antibiotic family has been determined by different methods as mass spectrometry, NMR and X-ray crystallography (Barna & Williams, 1984), elucidating its mode of action. Vancomycin, and other glycopeptide antibiotics, do not interact with the cell wall biosynthetic enzymes but form complexes with the carboxy-terminal D-alanine (D-Ala) residues of peptidoglycan precursors thus preventing their incorporation into the cell wall, leading to cell wall synthesis inhibition (Reynolds, 1989).

The synthesis of peptidoglycan involves several steps (Figure 1). In the cytoplasm, a racemase converts L-alanine to D-alanine (D-Ala), and then two molecules of D-Ala are joined by a ligase, creating the dipeptide D-Ala-D-Ala, which is then added to uracil diphosphate-N-acetylmuramyl-tripeptide to form uracil diphosphate-N-acetylmuramyl-pentapeptide. Uracil diphosphate-N-

acetylmuramyl-pentapeptide is bound to the undecaprenol lipid carrier, which, after the addition of N-acetyl glucosamine (GlcNAc) from uracil diphosphate-GlcNAc, allows translocation of the precursors to the outer surface of the cytoplasmic membrane. N-acetylmuramyl-pentapeptide is then incorporated into nascent peptidoglycan by transglycosylation and allows the formation of cross-bridges by transpeptidation (Reynolds, 1989).

Vancomycin binds with high affinity to the D-Ala-D-Ala C-terminus of the pentapeptide (Figure 1A), therefore blocking the addition of late precursors by transglycosylation to the nascent peptidoglycan chain and preventing subsequent cross-linking by transpeptidation (Reynolds, 1989). As a secondary and less relevant mechanism, glycopeptides inhibit the transglycosilation reaction of the peptidoglycan unit into the growing polymer, presumably by steric hindrance from the antibiotic-peptidoglycan precursor complex (Ge *et al.*, 1999; Walsh, 1999).

At present, glycopeptides are used for the treatment of infections caused by methicillin-resistant *S. aureus* (MRSA), coagulase-negative staphylococci (CoNS) or by ampicillin-resistant enterococci. Furthermore, these drugs are indicated in patients with serious β-lactam allergies (Levine, 2006). Glycopeptides are virtually not active against Gram-negative bacteria which are protected by an outer membrane.

Resistance to vancomycin in a clinical enterococcal isolate was first reported in Europe in 1986 (Leclercq *et al.*, 1988; Uttley *et al.*, 1988) and in 1987 in the United States (Sahm *et al.*, 1989). Enterococci were the first clinically relevant bacterial genus to acquire a resistance mechanism that specifically confers high-level vancomycin resistance.

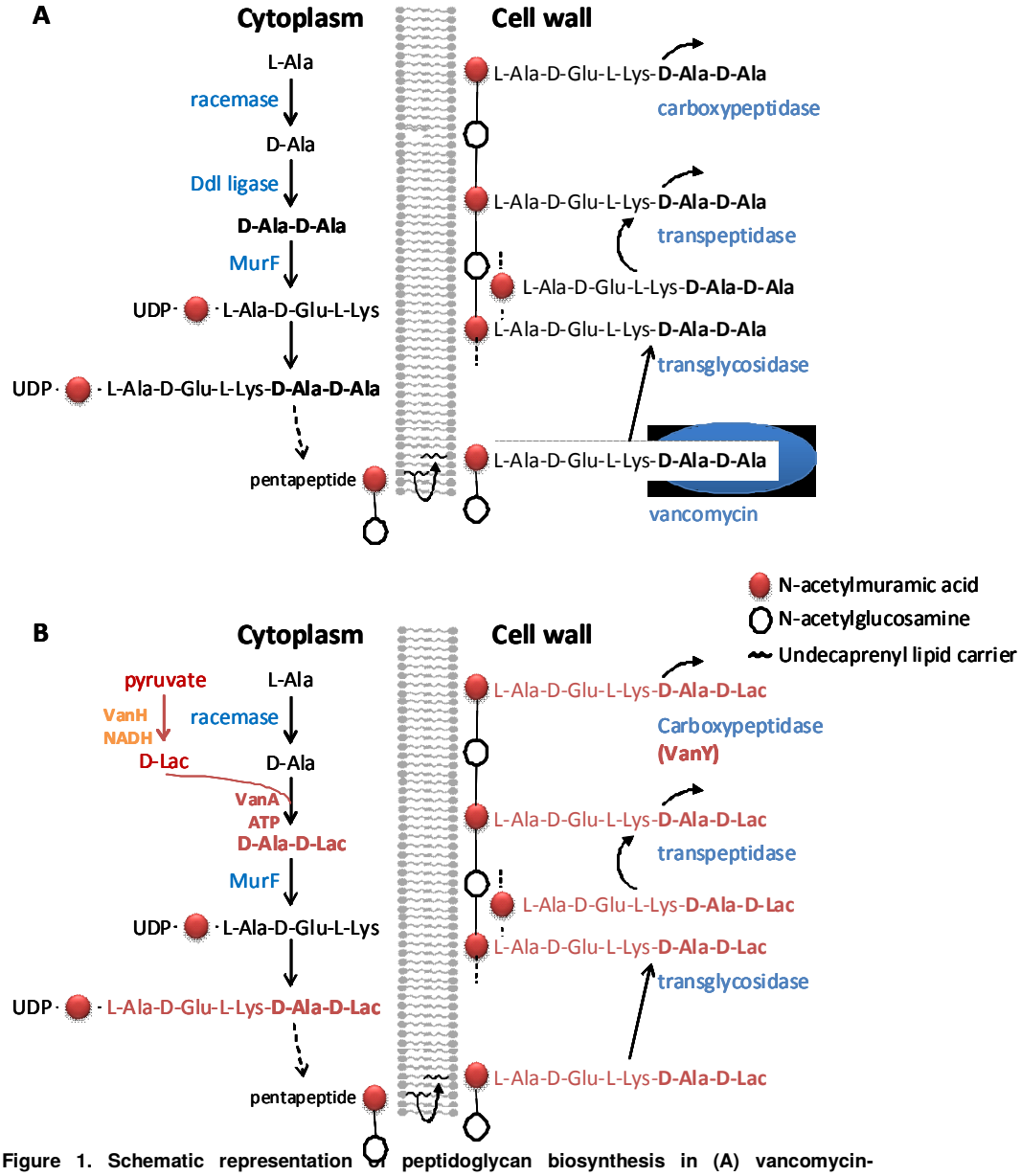

Figure 1. Schematic representation of peptidoglycan biosynthesis in (A) vancomycin-susceptible and (B) vancomycin resistant bacteria. Binding of vancomycin to the C-terminal D-Ala-D-Ala of the late peptidoglycan precursors prevents the reactions catalyzed by transglycosylases, transpeptidases and D,D-carboxypeptidases. In vancomycin resistant strains there is still production of native peptidoglycan precursors ending in D-Ala-D-Ala resulting from the endogenous chromosomal pathway. Is on the native pathway that VanX and VanY act sequencing hydrolyzing the dipeptide D-Ala-D-Ala. Ddl, D-Ala-D-Ala ligase; MurF, a synthetase protein; UDP, uracil diphosphate. Adapted from (Courvalin, 2006).

During the first several decades of use, staphylococci did not mount a resistance to vancomycin. It has only been within the last years that vancomycin-intermediate *Staphylococcus aureus* (VISA) and vancomycin-resistant *Staphylococcus aureus* (VRSA) have surfaced.

II. *ENTEROCOCCUS*

GENERAL DESCRIPTION OF THE GENUS

In 1899, Thiercelin described Gram-positive coccoid-shaped bacteria from the human intestine and used the name “entérocoque” to emphasize their intestinal origin (Thiercelin, 1899). However, the term *Streptococcus* kept being more commonly used. In 1937, Sherman developed a scheme classifying the genus *Streptococcus* into four main groups: pyogenic, viridians, lactic and enterococci (Sherman, 1937). Enterococci and streptococci were proven to be different based on DNA hybridization experiments and subsequently the genus *Enterococcus* was introduced in 1984 (Schleifer & Kilpper-Balz, 1984). Enterococci are also separated from other *Streptococcus* species as they grow between 10 and 45ºC, in 6.5% NaCl and at pH 9.6, survive heating at 60ºC for 30 min., hydrolyze esculine into esculitine (Devriese *et al.*, 1991) and react with the Lancefield group D antisera (Murray, 1990). Enterococci produce L(+)-lactic acid homofermentatively from glucose.

Although about 40 *Enterococcus* species are now recognized (http://www.dsmz.de/microorganisms/html/bacteria.genus/enterococcus.html), *E. faecalis* and *E. faecium* are the two most common species found in the human microbiota (Devriese *et al.*, 1991). Enterococci are members of the gastrointestinal (GI) tract microbial consortium of humans and other animals

(Tannock, 2002). In humans enterococci also colonize the genito-urinary tract and the oral and vaginal cavities (Murray, 1990). They are also capable of surviving in many other environmental niches like soil, sand, water (usually as faecal pollutants), food products and in plants (Mundt, 1986).

E. faecalis is currently the most intensively studied enterococcal specie due to its prominence in the nosocomial setting. Until recently the only publically sequenced genome available belonged to *E. faecalis* V583 (Paulsen *et al.*, 2003), which was the first reported vancomycin resistant clinical isolate in the United States (Sahm & Olsen, 1990). In 2008 another *E. faecalis* strain, OG1RF, was sequenced but the genome was not made publically available (Bourgogne *et al.*, 2008). Very recently the genome of other 28 enterococcal strains (including *E. faecalis*, *E. faecium*, *E. casseliflavus* and *E. gallinarum* species) became available (Palmer *et al.*, 2010).

ENTEROCOCCI AS PATHOGENS

The staphylococci and the streptococci were the most prevalent hospital pathogens in the 1960s and 1970s. Then, over a period of time, the distribution of organisms involved in nosocomial infections shifted from Gram-positive to Gram-negative in the 1970s and 1980s and since then Gram-positive organism have re-emerged as major hospital pathogens (Levy, 1998). In large part this trend may be attributed to the selection pressure created by antibiotics used in the hospital setting. Although enterococci have been recognized as an important cause of infectious endocarditis (IE) for more than a century (Singh *et al.*, 2010), just in the last two decades they were recognized as one of the leading causes of nosocomial infections (Murray, 1990). The most prevalent infections caused by enterococci are urinary tract infections (UTI), bacteremia, abdominal wound

infections and endocarditis (Moellering, 1992; Murray, 1998). Most of these infections are caused by *E. faecalis* and only a small number are caused by *E. faecium* (Witte *et al.*, 1999). However, in recent years a progressive increase of infections caused by *E. faecium* has been reported (Mundy *et al.*, 2000; Treitman *et al.*, 2005). With exception of *E. faecalis* and *E. faecium*, other enterococcal species are rarely associated to human pathogenesis and their clinical relevance is often neglected, although there are reports of infections caused *E. durans* (Stepanovic *et al.*, 2004), *E. hirae* (Gilad *et al.*, 1998; Poyart *et al.*, 2002; Ribeiro *et al.*, 2008), *E. raffinosus* (Wilke *et al.*, 1997; Sandoe *et al.*, 2001, Savini *et al.*, 2008, Mastroiani, 2009), *E. gallinarum* (Reid *et al.*, 2001; Takayama *et al.*, 2003), *E. avium* (Swaminathan & Ritter, 1999), *E. casseliflavus* (Reid *et al*., 2001; Pappas *et al.*, 2004; Iaria *et al.*, 2005), *E. cecorum* (Hsueh *et al.*, 2000) and *E. mundtii* (Higashide *et al.*, 2005). Enterococci are considered opportunistic pathogens considering that they are generally harmless for healthy people but able to cause serious infection in immunocompromised patients that are more susceptible to infections.

Two main features contribute to enterococcal pathogenic aptitude: a wide range of virulence factors, and the accumulation of different antimicrobial resistance mechanisms.

E. faecalis possess a plethora of virulence factors designed to help establishing infection and persist in the presence of the host immune response (Shankar *et al.*, 1999). There are four main stages in the pathogenesis of enterococcal infections. The first is persistence in inanimate objects due to intrinsic properties of bacteria, namely resistance to desiccation, heat and other conditions that otherwise impair bacterial endurance outside of the host. This

persistence contributes largely to enterococci prevalence as a nosocomial pathogen by providing a means of survival until an opportunity for infection presents itself. Next, entry into the bloodstream or other non-native areas of the host is a critical step in the transformation of enterococci from commensals to damaging pathogens. This phase is usually facilitated by damage to host tissues and presence of bacterial factors such as bile acid hydrolases, adhesins like aggregation substance (AS) and enterococcal surface protein (Esp), and antibiotic resistance that give enterococci an edge in surviving these niches. Once inside the host, enterococci have been shown to utilize several classified virulence factors for adherence and colonization in the establishment of an infection site. In this step, adhesins play a vital role in attachment to host tissues, as well as in the formation of cell aggregates as biofilms. Also, factors that possess immune invasion qualities are vital to cell survival, such as cytolysin, AS, Esp, and lipoteicoic acid (LTA). The final stage is the clinical manifestation of infection resulting in damage of vital tissues. This results from the activity of proteins against host tissues and includes cytolysin, gelatinase and serine protease.

Despite the accepted and demonstrated relevance of virulence factors in enterococci pathogenesis, those factors are present both in environmental and commensal strains. In fact it is still not clear yet what triggers the transition from commensal to pathogenic. It would be reasonable to admit that it would happen by induction of the molecules that are essential for the virulence. However, not all factors confer a selective advantage at the same infection stage or at the same infection target, which complicates the study and comprehension of enterococci-host interactions.

Antibiotics interrupt vital cellular functions through different modes of action. It is usually achieved by attacking the cell-wall and cell membrane integrity, by interfering with DNA, RNA or protein synthesis (Figure 2). They can be either bactericidal or bacteriostatic depending on their mechanism of action. Bactericidals effectively kill the target bacteria and bacteriostatics halt cell growth and replication.

From a clinical perspective, multidrug-resistant enterococci present a major problem. Infections caused by multidrug resistant enterococci are difficult to treat due to limited therapeutic options. The treatment of such infections varies widely from patient to patient, but it usually involves a combination of broad spectrum antibiotics. Drug regimens mainly utilize a cell-wall acting antibiotic coupled with an antibiotic that affects protein synthesis. It is conceivable that using broad spectrum antibiotics fails to specifically target the infectious strain and will likely remove beneficial commensal microorganisms. Furthermore, by exposing the entire bacterial community of the host to these antibiotics promotes the creation of an environment of extreme selective pressure with therapeutic and sub-therapeutic levels of ongoing antimicrobial exposure (Mundy *et al.*, 2000).

Enterococci have been shown to possess a broad range of intrinsic antibiotic resistances (Moellering, 1990; Murray, 1990, Leclercq, 1997) and are able to acquire high-level drug resistance to certain antibiotics (Figure 2). The resistance may arise by point mutations in the drug binding site, like in quinolones (Onodera *et al.*, 2002) and ampicillin, or by acquisition of resistance genes, as in the case of aminoglycosides, macrolides, chloramphenicol, tetracycline and glycopeptides, of which vancomycin resistance is the most relevant clinically (Moellering, 1990; Murray, 1990, Landman & Quale, 1997; Leclercq, 1997; Onodera *et al*., 2002).

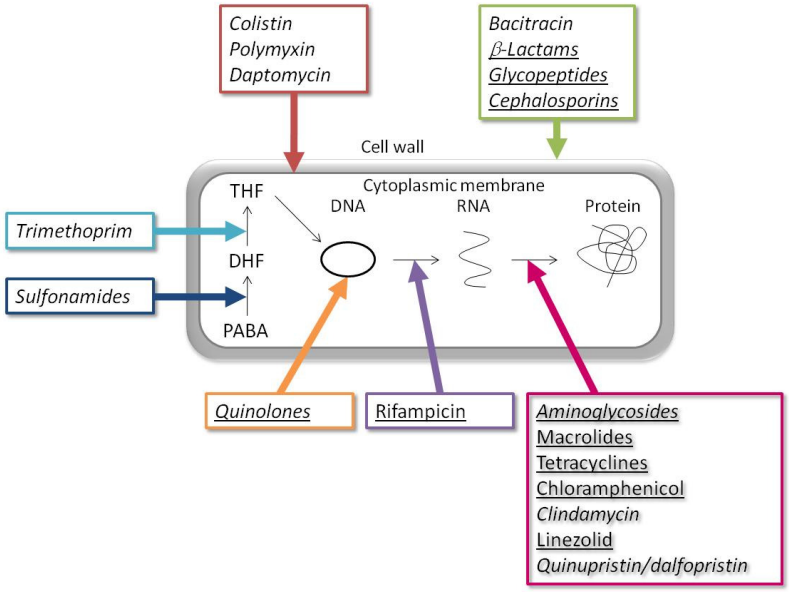

Figure 2. Mechanism of action for common antimicrobials. Antimicrobials mainly target the cell wall and cytoplasmic membrane, or affect DNA structure or protein synthesis. Colistin, polymyxin and daptomycin target the cytoplasmic membrane, while bacitracin, β-lactams, glycopeptides and cephalosporins disrupt the cell wall. Folate synthesis, which is required for construction of DNA base pairs, is a process that includes the conversion of para-aminobenzoic acid (PABA) into dihydropholate (DHF) and then finally into trihydropholate (THF). Trimethoprim and sulfonamides block these conversions and effectively arrest DNA synthesis. Quinolones function by interrupting DNA replication, while rifampicin blocks RNA synthesis. A wide group of antibiotics act by affecting protein synthesis. Antimicrobial resistance in enterococci can be divided in two classes, *intrinsic resistance* and acquired resistance. Bacteria are intrinsically resistant to antimicrobial agents because they either lack the target site for that drug, or the drug is unable to transfer through the organism´s cell wall or membrane to reach its site of action. In contrast, acquired resistance is usually transposon or plasmid encoded (Witte, 1999).

III. VANCOMYCIN RESISTANCE

MECHANISMS OF VANCOMYCIN RESISTANCE IN ENTEROCOCCI

Resistance to vancomycin is due to the synthesis of modified cell wall precursors (Figure 1B) that show decreased affinity to vancomycin or to vancomycin and teicoplanin. Vancomycin resistance is conferred by the presence

of operons that encode enzymes for synthesis of low-affinity precursors, in which the C-terminal D-Ala residue is replaced by D-lactate (D-Lac) or D-serine (D-Ser), thus modifying the vancomycin-binding target. The operons encode enzymes for elimination of the high-affinity precursors that are normally produced by the host, thus removing the vancomycin-binding target (Arthur *et al.*, 1996b). The genetics of vancomycin resistance has been studied in detail over the past decade and various molecular mechanisms have been elucidated (Arthur *et al.*, 1993; Baptista *et al.*, 1996; Leclercq & Courvalin, 1997; Rao *et al.*, 1998).

Replacement of the D-Ala C-terminal residue by a D-Lac suppresses a hydrogen bond crucial for antibiotic binding and considerably lowers the affinity for glycopeptides whereas substitution by a D-Ser does not alter the hydrogen bonds but it is responsible for a conformational change which slightly reduces the affinity for vancomycin (Gholizadeh & Courvalin, 2000). The D-Ala by D-Lac substitution results in a more-than-1000-fold lowering of the affinity to vancomycin while D-Ala by D-Ser substitution results in a six fold-lower affinity to vancomycin (Billot-Klein *et al.*, 1994).

In enterococci, resistance to glycopeptides is phenotypically and genotypically heterogeneous. Eight types of vancomycin resistance have been characterized so far. They can be distinguished on the basis of the range of glycopeptides to which the strains are resistant, resistance level and transferability (Table 1). Although the 8 types of resistance involve related enzymatic functions, they can be distinguished by the organization of the corresponding genes and by the mode of regulation of gene expression.

The *vanA* cluster was originally detected in the non-conjugative Tn*1546* transposon (Arthur *et al.*, 1993). VanA-type resistance is mediated by genetic

elements, identical or closely related to Tn*1546*, that are generally carried by self-transferable plasmids (Leclercq *et al.*, 1988) and, occasionally, by the host chromosome as part of larger conjugative elements (Handwerger & Skoble, 1995).

Gene clusters related to *vanB* are generally carried by large elements (90-250 kb) that are transferable by conjugation from chromosome to chromosome (Quintiliani & Courvalin, 1994). Generally as part of Tn*5382* (27 kb in size) (Carias *et al.*, 1998), Tn*1549* (34 kb in size) (Garnier *et al.*, 2000) and related elements. Plasmid-borne *vanB* clusters have also been detected in clinical isolates of enterococci (Rice *et al.*, 1998). By contrast to *vanA* gene cluster, *vanB* gene cluster shows greater sequence divergence. Three alleles have been reported to date (*vanB1*, *vanB2*, *vanB3*), of which *vanB2* appears to be the most widespread (Dahl *et al.*, 1999).

Table 1. Types of vancomycin resistance found in *Enterococcus* spp.

Type† (ligase gene)	Expression	Terminus of peptidoglycan precursor	MIC (µg/ml)	
			Vancomycin	Teicoplanin
VanA (*vanA*)	Inducible	D-Ala-D-Lac	64-1000	16-512
VanB (*vanB*)	Inducible	D-Ala-D-Lac	4-1000	0.5-1‡
VanC (*vanC*)	Both inducible and constitutive	D-Ala-D-Ser	2-32	0.5-1
VanD (*vanD*)	Constitutive	D-Ala-D-Lac	64-128	4-64
VanE (*vanE*)	Inducible	D-Ala-D-Ser	16	0.5
VanG (*vanG*)	Inducible	D-Ala-D-Ser	8-16	0.5
VanL (*vanL*)	Inducible	D-Ala-D-Ser	8	0.5
VanM (*vanM*)	Inducible	D-Ala-D-Lac	>256	96

†All glycopeptide resistance types are acquired, except for VanC which is an intrinsic property of *E. gallinarum* (Leclercq *et al.*, 1992) and *E. casseliflavus/E. flavescens* (Navarro & Courvalin, 1994). *E. casseliflavus* and *E. flavescens* were recently considered to be the same species and *E. flavescens* was reclassified as a later synonym of *E. casseliflavus* (Naser *et al.*, 2006). ‡ Typical *vanB* isolates are susceptible to teicoplanin but resistance to this antibiotic can develop both *in vitro* and *in vivo* (Gutmann *et al.*, 1992; Hayden *et al.*, 1993).

Transfer of VanG-type resistance is associated to movement, from chromosome to chromosome, of genetic elements of about 240 kb that also carry *ermB*-encoded erythromycin resistance (Depardieu *et al.*, 2003). VanM transfer was achieved and is associated with its putative location on a plasmid (Levine, 2006). The other *van* clusters, apart from *vanA* and *vanB*, will not be considered further here.

VanA is the most frequently encountered type of vancomycin in enterococci and the first to be described (Brisson-Noel *et al.*, 1990, Dutka-Malen *et al.*, 1990). The prototype Tn*1546* VanA-type resistance element, which was originally detected on a plasmid in an *Enterococcus faecium* clinical isolate, is an 11 kb transposon. It encodes 9 polypeptides that can be assigned to various functional groups: transposition (ORF1 and ORF2), regulation of resistance gene expression (VanR and VanS), synthesis of the D-Ala-D-Lac depsipeptide (VanH and VanA), and hydrolysis of peptidoglycan precursors (VanX and VanY). There is an additional peptide, VanZ, which function remains unknown (Figure 3).

The VanH dehydrogenase reduces pyruvate to D-Lac (Arthur *et al.*, 1992a), the VanA ligase synthesizes the depsipeptide D-Ala-D-Lac which replaces the dipeptide D-Ala-D-Ala in the pathway of synthesis of the late peptidoglycan precursors, and the VanX D,D-dipeptidase hydrolyses the dipeptide D-Ala-D-Ala resulting from the endogenous chromosomal pathway (Reynolds *et al.*, 1994). VanY encodes a D,D-carboxypeptidase (Arthur *et al.*, 1996b) and hydrolyses the pentapeptide synthesized from the dipeptide D-Ala-D-Ala which as escaped VanX hydrolysis. VanZ confers low-level resistance to teicoplanin by an unknown mechanism (Arthur *et al.*, 1995). VanY and VanZ are not required for glycopeptide resistance.

The VanR and VanS proteins are part of a two-component regulatory system (TCS) that modulates transcription of the resistance gene cluster (Arthur *et al.*, 1992b).

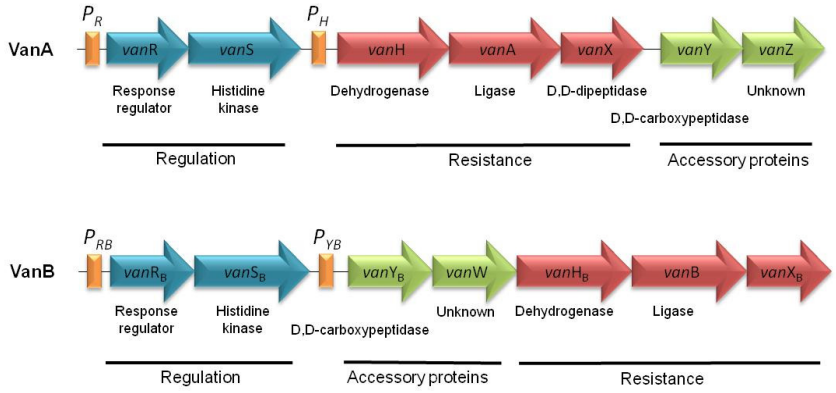

Figure 3. Genetic organization of *vanA* and *vanB* operons. Regulatory genes are represented in blue, essential genes for resistance are represented in red and accessory proteins in green. The genes required for transposition, ORF 1 (transposase) and ORF 2 (resolvase), are not represented. The genes are not scale represented. Adapted from (Courvalin, 2006).

As in VanA-type strains, acquired VanB-type resistance is due to synthesis of peptidoglycan precursors ending in the depsipeptide D-Ala-D-Lac instead of the dipeptide D-Ala-D-Ala (Arthur *et al.*, 1996a). The organization and functionality of the v*anB* cluster is similar to that of *vanA* but differs in its regulation because vancomycin, but not teicoplanin, is an inducer of the *vanB* cluster. The *vanB* operon contains genes encoding a dehydrogenase ($vanH_B$), a ligase (*vanB*), and a dipeptidase ($vanX_B$) (Figure 3), all of which have a high level of sequence identity (67-76% identity) with the corresponding deduced proteins for the *vanA* operon and the $vanR_BS_B$ regulatory genes that encode a two-component system only distantly related to *vanRS* (34% and 24% identity) (Evers & Courvalin, 1996).

The function of the additional VanW protein found only in the *vanB* cluster is unknown, and there is no gene related to *vanZ*.

Some VanA and VanB strains require vancomycin for growth (Dever *et al.*, 1995; Green *et al.*, 1995). This phenomenon is clinically important, since these strains require special growth conditions, their presence and prevalence will probably be underestimated since they are not detected on routine laboratory testing. These strains lack a functional ligase (Ddl) due to mutations in the *ddl* gene. Thus, they require the presence of vancomycin to grow because the *vanA*- or *vanB*-encoded D-Ala-D-Lac ligase is induced overcoming the defect in the synthesis of the regular peptidoglycan precursors (Van Bambeke *et al.*, 1999). Dependent strains can revert to a non-dependent, highly resistant phenotype. It can occur as a result of a mutation in either *vanS* or $vanS_B$ sensor, which lead to constitutive production of D-Ala-D-Lac, or in the *ddl* gene, which restores synthesis of D-Ala-D-Ala (Cetinkaya *et al.*, 2000).

The widespread of vancomycin-resistant enterococci (VRE) and the report of dependence on vancomycin for growth highlights the need to discover new therapeutic targets to treat infection.

VANCOMYCIN RESISTANCE IN PORTUGAL

The highest rates of VRE associated with nosocomial infections in Europe were reported in some countries of southern Europe. In recent years, the levels of detected VRE are up to 45% in Portugal (EARSS, 2008). As observed in other geographical regions, *vanA E. faecium* isolates are mainly responsible for the high rates of infections caused by VRE in Portugal (Novais *et al.*, 2005b; EARSS, 2008).

The first large VRE surveillance study in Portugal which included data from ten participating hospitals was performed in 1994 and revealed rates of 1% of VREfs and 9% of VREfm among isolates causing urinary tract and invasive infections (Melo-Cristino *et al.*, 1996). A remarkable increase in VREfm was documented in subsequent years with rates rising from 20% in 1996 (for the same 10 hospitals screened in 1994) to 47% in 2003 (Figure 4) (EARSS, 2008, Melo-Cristino, 1998). In 2008 EARSS (European Resistance Surveillance System) reported a decrease in VREfm rates to 24% (Figure 4). Whether the observed decrease is a result of the implementation of specific successful infection control measures or not, is not known.

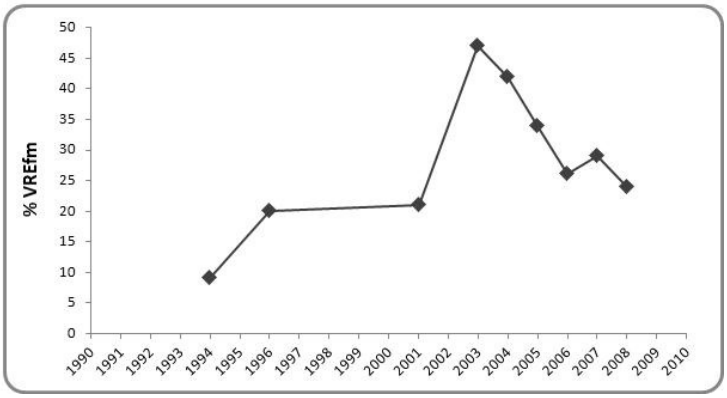

Figure 4. Reported percentage of VREfm in Portugal. Based on results from the European Antimicrobial Resistance Surveillance System. Available from http://www.rivm.nl/earss/result/.

According to EARSS, the number of Portuguese participating laboratories has increased, providing nowadays coverage of almost 90% of the total population.

Although polyclonality was frequently observed among VREfm, intra- and inter-hospital dissemination of persisting *E. faecium* and *E. faecalis* clones and specific *vanA* transposon (Tn*1546*) types seemed to have contributed to the rapid

and extensive spread of VRE in Portuguese hospitals (Novais *et al.*, 2004; Novais *et al*., 2005b, Novais *et al.*, 2008, Mato *et al.*, 2009).

Aware of the potential risk that VRE constitute outside of the clinical environment an effort has also been done in recent years to characterize vancomycin-resistant enterococci from non-clinical sources. VRE have been detected and isolated from healthy human volunteers (Novais *et al.*, 2008), poultry food samples (Novais *et al.*, 2005a; Novais *et al*., 2008), pets and wild animals (Poeta *et al.*, 2005; Poeta *et al.*, 2007; Radhouani *et al.*, 2010) and environmental sources (Novais *et al.*, 2008). Despite the valuable contribution of these studies for the characterization of vancomycin resistance in enterococci from different sources and geographic regions, characterization of the dissemination of vancomycin resistance determinants is still poor, as the existing studies do not cover all country geographical areas and only look for VRE strains and not for the presence of *van*-genes in vancomycin-susceptible enterococci (VSE).

VANCOMYCIN RESISTANCE IN OTHER BACTERIA

Vancomycin resistance emerged in enterococci in the middle 1980s, and it took more than a decade until significant resistance to vancomycin was also discovered in staphylococci. In staphylococci, vancomycin reduced susceptibility is not related with the presence of vancomycin resistance genes, as in enterococci. Vancomycin-intermediate and –resistant coagulase negative staphylococci have been found in large numbers (Sieradzki *et al.*, 1998; van den Braak *et al.*, 1998). Vancomycin intermediate *S. aureus* (VISA) and glycopeptide-intermediate *S. aureus* (GISA) were first reported in Japan in 1996 (Hiramatsu *et al.*, 1997), almost immediately followed by two additional cases in the United States (CDC, 1997). The mechanisms by which *S. aureus* isolates become

intermediately resistant to vancomycin are poorly understood, but several common characteristics are shared by clinical and laboratory-derived strains. Those include slower growth rate, decreased lysostaphin susceptibility and reduced autolytic activity (Sieradzki & Tomasz, 1997; Boyle-Vavra *et al.*, 2000, Pfeltz *et al.*, 2000). These intermediary strains produce an excess amount of non-cross-linked D-alanyl-D-alanine targets which sequester vancomycin molecules. A detailed description of the mechanism of heterogeneous resistance in *Staphylococcus aureus* can be found elsewhere (Liu & Chambers, 2003). Although the genetic basis behind this phenotype is not yet well defined, it is expected to involve chromosomal mutations (Wootton *et al.*, 2004) and/or the disruption of the *agr* accessory gene regulator (Sakoulas *et al.*, 2002), as well as mutations on the two-component regulatory systems *vraSR* and *graSR* (Cui *et al.*, 2009).

The description of VISA/GISA strains was anticipated as a prelude for the emergence of vancomycin-resistant *S. aureus* and jeopardizes vancomycin clinical relevance. In fact, transfer of resistance genes from VRE to *S. aureus* was demonstrated *in vitro* several years ago (Noble *et al.*, 1992), and a first clinical case of infection with vancomycin-resistant *S. aureus* strain carrying *vanA*, originated in a VRE isolate, was described in 2002 (CDC, 2002) from a diabetic foot ulcer. There are already 11 VRSA reported in the United States (Sievert *et al.*, 2008; Tenover, 2008; Zhu *et al.*, 2008).

The description of *S. aureus* harbouring vancomycin resistance determinants transferred from enterococci is the irrefutable proof of the threat that VRE constitute (Tenover *et al.*, 2008; Zhu *et al*., 2008). Now that VRSA are a reality is probably just a matter of time until vancomycin resistance determinants are found in other clinically relevant microorganisms. This possibility is of utmost concern

since nowadays vancomycin is the last resort antibiotic for the treatment of infections caused by multidrug resistant Gram-positive bacteria.

Although *vanA* and *vanB* gene clusters have been detected in several enterococcal species, *E. faecium* and *E. faecalis* are the most prevalent and clinically important (Woodford, 1998). Enterococcal glycopeptide resistance genes have occasionally been reported in non-enterococcal species. Homologues of the *vanA* gene have been detected in vancomycin resistant clinical isolates of *Oerskovia turbata*, *Arcanobacterium haemolyticum* isolated from stools of two patients during an outbreak of glycopeptide-resistant enterococci (GRE) infection in England (Power *et al.*, 1995) and from a *Bacillus circulans* isolate in a case of a catheter-related infection (Fontana *et al.*, 1997). A transferable v*anB* gene homologue was found in *Streptococcus bovis* isolated from a stool swab collected on admission from a patient as surveillance for GRE (Poyart *et al.*, 1997) and the occurrence of *Streptococcus gallolyticus* strains containing *vanA* and/or *vanB* in faecal samples from avoparcin-fed veal calves was also reported (Mevius *et al.*, 1998). Experimentally, *vanA* resistance has been transferred from enterococci to *Streptococcus sanguis*, *Lactococcus lactis*, *Streptococcus pyogenes* as well as *Listeria monocytogenes* (Leclercq *et al.*, 1989; Power *et al*., 1995; Biavasco *et al.*, 1996, Poyart *et al*., 1997).

Vancomycin resistant *S. pneumoniae* has not been isolated yet, however, clinical strains of vancomycin tolerant *S. pneumonia* have been reported (Novak *et al.*, 1999, McCullers *et al.*, 2000; Rodriguez *et al.*, 2004). Antibiotic tolerance is particularly insidious because it cannot be detected using conventional *in vitro* tests – tolerant strains seem to be sensitive to antibiotics (Gilmore & Hoch, 1999) and tolerance is thought to be a precursor to resistance development.

REGULATION OF VANCOMYCIN RESISTANCE

The transduction signal systems are involved on the perception of the different extracellular signals and on the internal communication. They allow a rapid response to environmental fluctuations and make a fundamental link between the external stimuli and a specific response by inducing bacteria to synthesize the molecules that are, at each situation, essential. The general two-component signal transduction mechanism by which bacteria sense and respond to different environmental conditions has been illustrated on Figure 5.

The TCSs use energy in form of ATP to induce the response to a stress condition. They are composed of a sensor histidine kinase (HK) protein, located in the membrane, and a cytoplasmic response regulator (RR) that will induce the cellular response. In response to a specific extracellular signal, the HK undergoes *trans*-autophosphorylation on a specific histidine residue on a reaction that is ATP dependent. The phosphate group is then transferred to an aspartate residue of the cognate response regulator within its receiver domain and typically activates an attached output domain (Figure 5a).

Regulation of VanA and VanB vancomycin resistance clusters occurs by a similar mechanism. On Figure 6 the regulation of VanA cluster is represented. Expression of glycopeptide resistance is regulated by a VanS/VanR-type TCS composed of a membrane-bound histidine kinase and a cytoplasmic response regulator that acts as a transcriptional activator (Arthur *et al*., 1997).

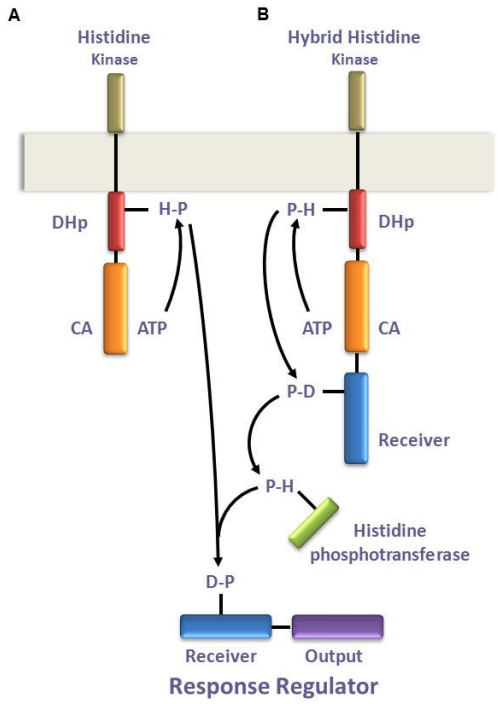

Figure 5. Schematic overview of a two-component signal transduction systems (A) and phosphorelays (B). A) On the prototypical two-component system a histidine kinase autophosphorylates on a conserved histidine residue (H) with subsequent transfer of the phosphoryl group to a conserved aspartate residue (D) on a cognate response regulator. Input domain on histidine kinases vary widely and typically do not share substantial homology to one another. The catalytic and TPase (CA) domain of the histidine kinase is responsible for binding ATP and catalyzing autophosphorylation of a conserved histidine found within the dimerization and histidine phosphotransferase (DHp) domain. The DHp domain mediates homodimerization and serves as the phosphodonor for a cognate response regulator. Many histidine kinases are bifunctional and also dephosphorylate their cognate response regulator. Response regulators typically contain two domains, a receiver domain and an output domain. Receiver domains contain the phosphoacceptor aspartate residue (D) and several other highly conserved amino acids and catalize phosphotransfer from a histidine kinase. Output domains, which are activated by phosphorylation of the receiver domain, are varied, but are often involved in binding DNA; B) On phosphorelays a stimulus activates autophosphorylation of a hybrid histidine kinase. The sensor kinase first transfers the phosphoryl group to a response regulator possessing the domain with the conserved aspartate but no output domain. The response regulator subsequently transfers the phosphoryl group to a histidine-containing phosphotransfer protein, and it is the latter protein that serves as a phosphodonor to the terminal response regulator, which possesses an output domain mediating a cellular response. Adapted from (Laub & Goulian, 2007).

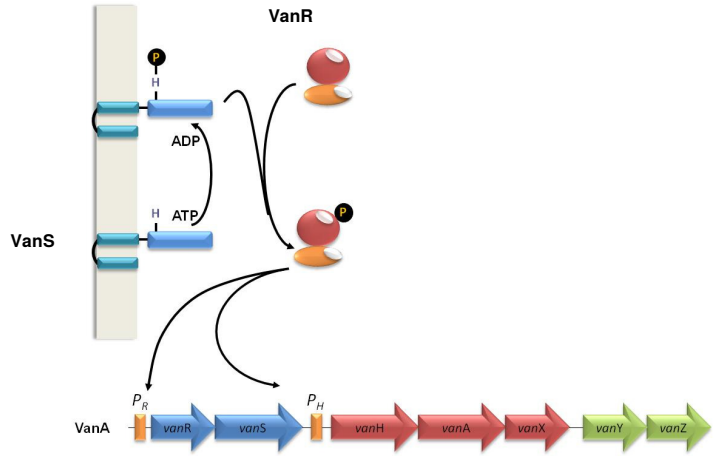

Figure 6. Regulation of VanA cluster. Phosphorylation of the VanR by the VanS sensor. The colored arrows represent coding sequences and indicate the direction of transcription, The regulatory and resistance genes are co-transcribed from promoters P_R and P_H, respectively. The organization and functionality of the *vanB* cluster is similar to *vanA* but differs in its regulation, because vancomycin, but not teicoplanin, is an inducer of the *vanB* cluster. However, the phosphorylation events are similar in both clusters. Adapted from (Courvalin, 2006).

VanS-type sensors comprise a N-terminal glycopeptide sensor domain with two membrane-spanning segments and a C-terminal cytoplasmic kinase domain. After a signal associated with the presence of vancomycin in the culture medium, the cytoplasmic domain of VanS/VanS$_B$ catalyzes ATP-dependent autophosphorylation on a conserved histidine residue (H164) and transfers the phosphate group to a conserved aspartate residue (D53) of VanR/VanR$_B$ present in the effector domain (Arthur *et al.*, 1992b; Haldimann *et al.*, 1997). VanS/VanS$_B$ also stimulates dephosphorylation of VanR/VanR$_B$ in absence of vancomycin (Arthur *et al.*, 1997). The phosphorylated form of VanR/VanR$_B$ activates co-transcription of *vanHAXY*/*vanY$_B$WH$_B$BX$_B$* genes by binding to the P_H/P_{YB} promoter (Arthur *et al.*, 1992b), and of *vanRS*/*vanR$_B$S$_B$* genes by binding to the P_R/P_{RB} promoter. The phosphorylated forms of VanR and VanR$_B$ bind with increased

affinity to the P_R/P_H and P_R/P_{YB} promoter regions. The VanS/$VanS_B$ sensor therefore modulates the phosphorylation level of the VanR/$VanR_B$ regulator: it acts as a phosphatase under non-inducing conditions and as a kinase in the presence of glycopeptides, leading to phosphorylation of the response regulator and activation of the resistance genes (Arthur *et al.*, 1997). Moreover, VanR/$VanR_B$ can catalyze its own phosphorylation in the absence of VanS/$VanS_B$ by using acetyl-phosphate as a substrate or by a histidine kinase protein chromosomally encoded (Holman *et al.*, 1994). Although, in this case the expression of the vancomycin resistance genes becomes constitutive due to the constitutive activation of the P_R/P_{RB} and P_H/P_{YB} promoters by VanR/$VanR_B$ in the absence of VanS/$VanS_B$.

Despite general common features on the regulation mechanism there are differences in the regulation of gene expression. The two VanR-VanS and $VanR_B$-$VanS_B$ systems display only limited homology. Furthermore, the putative membrane-associated sensor domains of VanS and $VanS_B$ are unrelated (Evers & Courvalin, 1996). It is therefore possible that VanS and $VanS_B$ sense the presence of glycopeptides by different mechanisms, which could account for the difference in induction specificity. These differences may also explain the large range of vancomycin MICs observed for the VanB phenotype (Quintiliani *et al.*, 1993).

The general aspects of *vanA* and *vanB* gene clusters regulation, including the phosphotransfer reactions and promoter activation, are well characterized. In contrast, there are some aspects that have not yet been fully elucidated. The mechanism by which VanS and $VanS_B$ detect the presence of vancomycin is still unknown and the available work on this matter provides contradictable results. In

fact, it has never been established if signal recognition involves a direct interaction between the sensors and a specific ligand such as glycopeptides or peptidoglycan precursors (Arthur & Quintiliani, 2001). It is not likely that the accumulation of cytoplasmic peptidoglycan precursors could be the signal that triggers induction (Baptista *et al*., 1996; Ma *et al.*, 2008). The heterologous expression of enterococcal HKs in *E. coli* inner membranes was evaluated and it was attempted to identify the activating signal for some of these HKs. In the case of VanS, there was no detectable increase in activity levels upon addition of a 10-fold molar excess of vancomycin, indicating that vancomycin is not an activating signal for this specific kinase under this condition (Ma *et al.*, 2008). Conversely Koteva and co-workers, using a synthesized vancomycin photoaffinity probe, proved that vancomycin directly binds *Streptomyces coelicolor* VanS (VanSsc) (Koteva *et al.*, 2010). More studies are needed before a general assumption regarding the VanS ligand can be made since VanS proteins from different bacteria share little homology, raising the possibility that there is not only a single and common ligand to all VanS proteins.

Although vancomycin resistance mechanism is very well described in enterococci, there is the suspicion that enterococci might have other cellular mechanisms contributing to vancomycin resistance and/or vancomycin response. In a study by Hancock & Perego (2004), the authors systematically inactivated 17 response regulators genes belonging to the TCSs described for *E. faecalis* V583. A phenotypic analysis of mutants revealed that a mutant, other than *vanR*, showed an increased sensitivity to vancomycin (*croR*) (Hancock & Perego, 2004b).

ENTEROCOCCAL TWO-COMPONENT SYSTEMS

Bacteria have different mechanisms available for the regulation of stress response (sigma factors, small RNAs and two-component systems). Alternative σ factors have been shown to be important for the survival of various organisms under extreme conditions and to be involved with virulence and pathogenicity (Kazmierczak *et al.*, 2005). Findings obtained in the past few decades have identified a new class of gene expression regulators, the small non-coding RNAs (sRNAs). sRNA candidate genes have been identified in different bacteria (Gottesman, 2005; Pichon & Felden, 2005).

One of the differences between *Enterococcus* species, *B. subtilis* and *S. aureus*, is the absence of a σ^B hortologue in enterococci. In *E. faecalis* the general stress response involves other regulation mechanisms, although there are six described σ factors in V583 genome namely, RpoD or σ^D (Frere *et al.*, 1996), σ^N implicated in the nitrogen limitation response (Dalet *et al.*, 2000) and other four putative extracytoplamic function sigma factors (ECF) namely, σ^V (Benachour *et al.*, 2005), σ^X, σ^G and σ^H (Benachour, personal communication).

However, in enterococci the best characterized stress response mechanisms are the two-component systems.

The two-component systems were first reported in 1986 (Ninfa & Magasanik, 1986; Nixon *et al.*, 1986) and with the genomic advances it is known that the chromosome of most bacteria encodes several two-component systems. For instance, *Synechocystis* sp. has 80 TCS, *E. coli* has 62 proteins associated to TCS (Mizuno, 1998) and 36 TCS are identified in *B.subtilis*. There are other bacteria with a reduced number, as is the case of *Haemophilus influenza* with 6 TCS and *Mycoplasma genitalium* with no described TCS.

On *E. faecalis* V583 genome there are 17 TCS Ehk-Err (enterococcal histidine kinase and enterococcal response regulator) composed of a HK and a RR, and an orphan RR (i. e. an RR without a partner HK) (Paulsen *et al*., 2003). However, the content in TCSs varies among other *E. faecalis* strains, e. g. OG1RF has 15 TCS and JH2-2 has 14 TCS (Teng *et al*., 2002; Le Breton *et al*., 2003; Mcbride *et al*., 2007; Bourgogne *et al*., 2008). By comparative genomic hybridizations it was proposed that 12 out of the 17 enterococcal TCSs known, as well as the orphaned response regulator, are part of *E. faecalis* core genome (McBride *et al*., 2007). The TCS that belong to *E. faecalis* core genome include Ehk-Err01, Ehk-Err02, Ehk-Err03, Ehk-Err04, Ehk-Err05, Ehk-Err06, Ehk-Err07, Ehk-Err09, Ehk-Err10, Ehk-Err13, Ehk-Err14, Ehk-Err17 and Err18 (McBride *et al*., 2007). The functions of most of enterococcal TCSs are largely unknown.

These systems were grouped according to the classification of Fabret *et al*. (1999) (Table 2), based on the analysis of *B. subtilis* genome. This classification is based on the structure similarities and on the sequence of each of the TCS components. Therefore, the TCSs are clustered according to the structure of the ATP binding site and the motif containing the phosphorylable histidine residue on the HK, as well as the structure of the receptor domain containing the aspartate residue on the response regulator. Based on this classification 6 classes were defined (I, II, IIIA, IIIB, IV and V) (Fabret *et al*., 1999), each one corresponding to the classes defined by Mizuno *et al.* (1997) in *E. coli* (Others-B, NarL, OmpR, NtrB, Others-A and CheY, respectively) (Mizuno, 1997).

E. faecalis TCSs have been studied in more detail in three strains, namely V583 (Hancock & Perego, 2004b), JH2-2 (Teng *et al*., 2002) and OG1RF (Le Breton *et al*., 2003). The results of these studies, however, do not allow a definition of the precise role of each TCS, as the role of each TCS is strain

dependent. However, taken together, these studies can emphasize the role of some two-component systems on virulence, biofilm formation, antibiotic resistance and stress response.

The number of TCSs varies among the studied strains which can be, in part, explained by the fact that both TCS Err11-Ehk11 (VanRS) and Ehk12-Err12 are located on mobile elements or on a pathogenicity island (PAI). Err07-Ehk07 (VicRS) is considered to be an essential TCS to the cellular viability since a viable mutant has not been obtained. Err07-Ehk07 could be an equivalent to the essential system YycFG from *B.subtilis* (Fabret & Hoch, 1998).

Err05 mutant presents some growth deficiencies and abnormal cellular morphology in JH2-2. s*alB* expression, encoding for a putative stress protein, is altered on the mutant (Le Breton *et al*., 2003). Err05-Ehk05 has been implicated in resistance to antimicrobial agents that target the cell wall, like β-lactams (Comenge *et al*., 2003), and was named CroR-CroS, standing for ceftriaxone resistance. An *err05* mutant also presents reduced susceptibility to vancomycin (Hancock & Perego, 2004b), revealing a potential role of this TCS in response to cell-wall active antibiotics.

As mentioned above, TCS Err11-Ehk11 ($VanR_BS_B$) is the most widely studied enterococcal TCS and it is responsible for vancomycin resistance expression and regulation (Arthur *et al*., 1992b; Arthur & Courvalin, 1993; Evers *et al.*, 1996, Arthur & Quintiliani, 2001). The systems Ehk01-Err01 and Ehk03-Err03 are involved in bacitracin resistance (Hancock & Perego, 2004b) but their exact role remains unclear.

Table 2. Classification of *E. faecalis* TCSs, according to Hancock and Perego (2002). The homologies with known proteins are indicated, with *Bli* : *B. licheniformis*, *Bce* : *B. cereus*, *Bsu* : *B. subtilis*, *Efa* : *E. faecalis*, *Lmo* : *L. monocytogenes*, *Sag* : *S. agalactiae*, *Sau* : *S. aureus*, *Spy* : *S. pyogenes.*

TCS	Group*	Histidine kinase		Response Regulator		
		Locus	Homologies (% identic residues /% conserved residues)	Locus	Family†	Homologies (% identic residues /% conserved residues)
Ehk01-Err01	I	EF2219	YesM (37/58) *Spy*	EF2218	AraC	YesN (43/64) *Spy*
Ehk02-Err02		EF3197	LytS (55/74) *Sau*	EF3196	Lyt	LytR (39/64) *Sau*
Ehk03-Err03	II	EF2912	VraS (48/69) *Sau*	EF2911	NarL	VraR (62/79) *Sau*
Err04-Ehk04	IIIA	EF1704	PhoR (31/50) *Bsu*	EF1703		PhoP (55/76) *Bsu*
Err05-Ehk05		EF3290	VanS (38/61) *Bce*	EF3289		VanR (57/77) *Bce* ; BacR (44/64) *Bli*
Err06-Ehk06		EF1261	YclK (44/62) *Bsu*	EF1260		YclJ (60/72) *Bsu*
Err07-Ehk07		EF1194	VicK(100) *Efa* ; YycG (45/69) *Bsu*	EF1193		VicR (100) *Efa* ; YycF (77/88) *Bsu*
Err08-Ehk08		EF1863	VncS (77/89) *Sag*	EF1864		VncR (95/98) *Sag*
Err09-Ehk09		EF0927	YxdK (41/65) *Bsu*	EF0926	OmpR	YtsA (47/70) *Bli* ; YdxJ (42/66) *Bsu*
Err10-Ehk10		EF1051	EtaS (100) *Efa* ; LisK (50/69) *Lmo*	EF1050		EtaR (100) *Efa* ; LisR (75/85) *Lmo*
Err11-Ehk11		EF2298	VanS (100) *Efa*	EF2299		VanR (100) *Efa*
Ehk12-Err12		EF0570	KdpD (41/63) *Lmo*	EF0571		KdpE (49/71) *Lmo*
Ehk13-Err13		EF0373	CovS (35/62) *Spy*	EF0372		SrrA (37/62) *Sau*
Err18			-	EF3329		?
Ehk14-Err14	IV	EF1209	CitA (32/55) *Bce* ; DpiB (32/56) *Spy*	EF1210	Other-A	DpiA (44/64) *Spy* ; CitT (32/52) *Bce*
Err15-Ehk15		EF1820	FsrC (100) *Efa*	EF1822	Agr	FsrA (100) *Efa*
Err16-Ehk16		EF1335	?	EF1336	Agr	?
Err17-Ehk17		EF1632	?	EF1633		?

*TCS are grouped according to Fabret *et al.* (1999) classification, based on *B. subtilis* ; †TCS were classified in families according to Mizuno *et al.* (1997) classification, based on *E. coli*.

Err10 mutant has an increased survival rate in a mouse model by peritoneal injection compared with the wild type OG1RF, suggesting that the system Err10-Ehk10 (EtaRS) is involved in virulence. Ehk13-Err13 (EtbRS) might also be involved in virulence although the evidences are not so compelling (Teng *et al*., 2002).

It was demonstrated that TCS Err15-Ehk15 (FsrCA) is activated by quorum sensing mechanisms (Nakayama *et al*., 2001) and has a role in biofilm formation (Hancock & Perego, 2004a) and virulence (Qin *et al*., 2000; Mylonakis *et al*., 2002; Sifri *et al*., 2002) through transcriptional regulation of *gelE*, encoding a metalloprotease, and *sprE*, encoding a serine protease (Qin *et al*., 2001).

Although some of the TCSs are associated to stress response, antimicrobial resistance and virulence, all together contributing to the opportunist character of *E. faecalis*, little is known about the target genes of these systems.

TWO-COMPONENT SYSTEMS AND CROSS-REGULATION

Histidine kinases and response regulators each comprise paralog gene families and members of each family share significant homology at both the primary sequence level and at the structural level. The similarity of these signaling proteins raises the possibility of cross-talk between different two-component pathways (Chang & Stewart, 1998). Although the existence of such cross-talk has been widely suggested, it is unclear to what extent it occurs *in vivo*. Cross-talk is defined as the communication between two pathways that, if eliminated, would leave intact two distinct, functioning pathways. In general cross-talk between distinct pathways should be kept to a minimum, otherwise an organism would not be able to evoke the necessary response to a specific input stimulus. However, under certain conditions, it may be advantageous to an organism to allow or use

cross-talk as a means of either integrating multiple signals or of diversifying the response to a single input. When cross-talk benefits the organism it is called "cross-regulation" (Wanner, 1992) (Figure 7).

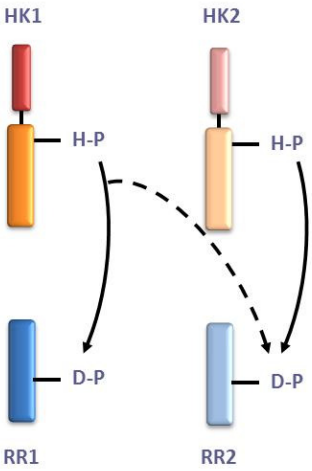

Figure 7. Cross-talk schematic representation. Cross-talk is defined as the communication between pathways that, if eliminated, would leave intact two distinct pathways. Histidine kinase (HK), response regulator (RR), histidine residue (H), aspartate residue (D). Adapted from (Laub & Goulian, 2007).

The available data on cross-talk in *E. faecalis* are limited. Studies of the PhoR-PhoB and the VanS-VanR two-component signalling systems have been particularly useful in highlighting the mechanisms used by cells to prevent cross-talk (Figure 8) (Fisher *et al.*, 1995; Fisher *et al.*, 1996; Haldimann *et al.*, 1996; Haldimann *et al.*, 1997; Silva *et al.*, 1998). The PhoR-PhoB two-component system is endogenous to *E. coli* and allows the organism to sense and respond to changes in phosphate availability. The enterococci VanS-VanR two-component system can be expressed in *E. coli*. Cross-talk from the kinase VanS to the response regulator PhoB can occur in *E. coli* but only in the absense of PhoR (Fisher *et al*., 1995; Silva *et al*., 1998). Similarly, the kinase PhoR can cross-talk to the response regulator VanR but only in the absence of VanS (Haldimann *et al*., 1997; Silva *et al*., 1998).

In a study using flow cytometry, Baptista *et al.* (1999) examined the induction of *van* gene expression in single cells of an enterococcal VanB strain. In enterococcal VanB strains, null mutations in $vanS_B$ lead to a phenotype termed "heterogeneous", in which, in the absence of antibiotic, only a minority of the bacteria expresses the *van* genes (Baptista *et al.*, 1999). Furthermore, addition of the antibiotic leads to uniform induction of the whole population, rather than selection of the subpopulation initially expressing resistance under non-inducing conditions. They concluded that a heterologous kinase activates $VanR_B$ in the absence of $VanS_B$. Interestingly, this putative kinase was stimulated by vancomycin, teicoplanin and the non-glycopeptide cell wall inhibitor moenomycin.

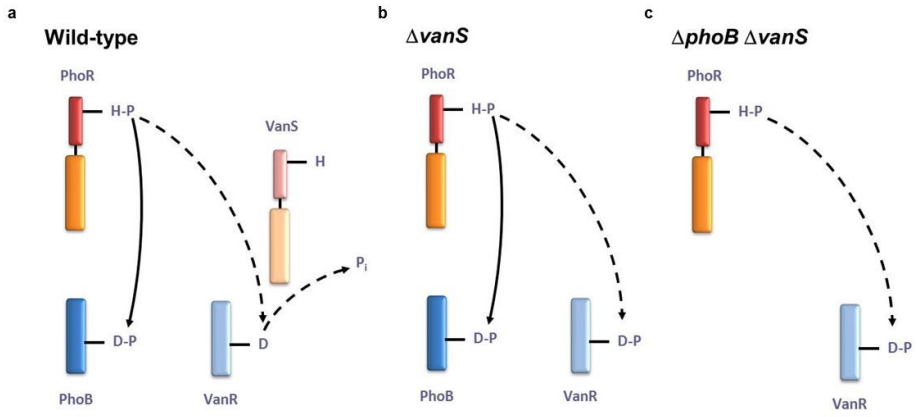

Figure 8. Cellular mechanims for preventing cross-talk. Studies of the PhoR-PhoB and VanS-VanR two-component pathways in *E. coli* demonstrate two major mechanisms by which cells prevent unwanted cross-talk between different pathways. (a) In wild-type cells any inadvertent cross-phosphorylation of VanR is eliminated by the phosphatase activity of VanS, thereby preventing the accumulation of VanR-P. (b) Eliminating VanS can thus lead to increased cross-talk, represented by thicker arrows, from PhoR-P (or acetyl phosphate). (c) Competition between response regulators for the phosphodonor PhoR-P also helps prevent cross-talk. PhoR-P has a higher affinity for PhoB relative to VanR, and so preferentially phosphorylates PhoB. Eliminating PhoB therefore leads to even more cross-phosphorylation of VanR. Adapted from (Laub & Goulian, 2007).

Presumably, in the wild-type enterococcal VanB strains, the phosphatase activity of $VanS_B$ keeps $VanR_B$ in the unphosphorylated state in the presence of teicoplanin and moenomycin, preventing the putative heterologous kinase from activating *van* gene expression. The putative heterologous kinase has not yet been identified but a possible candidate is CroS, since it is known to be induced by vancomycin, teicoplanin and moenomycin A (Comenge *et al.*, 2003).

BACTERIAL RESPONSE TO VANCOMYCIN AND OTHER CELL WALL-ACTIVE ANTIBIOTICS

The regulatory network of the cell wall response is well studied in *B. subtilis* and includes several overlapping regulons: in the presence of cell wall active antibiotics, the inducible defensive response is coordinated by, at least, two alternative extracytoplasmic sigma factors (σ), σ^W and σ^M and four two-component systems BceRS, LiaRS, YvcPQ and YxdJK (Cao *et al.*, 2002; Mascher *et al.*, 2003; Mascher *et al.*, 2004). It was proposed that σ^W is involved in defending the cell against antimicrobial agents and that σ^M modulates the surface properties and maintains the envelope integrity. It was also speculated that the σ^B general stress response is activated by cell wall antibiotics as a secondary response (Mascher *et al.*, 2003).

In *S. aureus*, the mechanisms of antibiotic induction are poorly characterized and the available knowledge is based on wide genomic studies that have identified the regulation of several genetic determinants or proteins. A first indication that antibiotic challenge in *S. aureus* is linked to a physiologically wide-range response came from the identification of the auxiliary genes and from the implication of σ^B regulatory element in the cell wall antibiotics resistance mechanism (Wu *et al.*, 1996; De Lencastre *et al.*, 1999). In this context, a cell wall

stimulon was defined as being the specific cell response to inhibition of peptidoglycan biosynthesis (Jablonski & Mychajlonka, 1988). However, the regulatory pathways associated to such stimulon are still unknown.

Proteomic studies where a *S. aureus* strain was challenged with cell wall targeting antibiotics revealed a characteristic protein pattern which was not induced by non-cell wall targeting antibiotics (Singh *et al.*, 2001). Later, microarray studies were performed in different *S. aureus* genetic backgrounds. The unique effects of oxacillin, bacitracin and D-cycloserine were studied (Utaida *et al.*, 2003). Vancomycin effect was determined and a specific set of genes belonging to the regulon of the VraSR two-component system (standing for Vancomycin-resistance associated sensor/regulator) was described (Kuroda *et al.*, 2003). The vancomycin effect was later determined in other *S. aureus* strains (McAleese *et al.*, 2006; McCallum *et al.*, 2006). The transcriptional profiles of all these studies showed subsets of 15 genes, proposed to belong to a "cell wall stimulon", and the majority of the diversity observed is likely due to strain genetic diversity (McCallum *et al*., 2006). Whether any of the core genes specifically contribute to *S. aureus* response to antibiotic stress is not known, but is generally speculated that they act as part of a concerted action that would be essential for the bacteria to express physiological resistance.

In *E. faecalis*, the mechanisms of antibiotic induction and antibiotic stress response are almost completely unknown with just a limited number of studies, using microarray transcriptomic approach, to understand the overall response of *E. faecalis* to antibiotics (Aakra *et al.*, 2005; Aakra *et al.*, 2010). Abranches *et al.*, (2009) provide evidence that (p)ppGpp pools modulate vancomycin tolerance (Abranches *et al.*, 2009). However, and opposite to *B. subtilis* and *S. aureus*,

nothing is yet documented on antibiotic cell wall stress response. Considering that enterococci were the first clinically relevant etiological agents acquiring vancomycin resistance and that they constitute primary carriers and a potential dissemination source of vancomycin resistance genes to other clinically relevant bacterial species, it is of utmost importance to study the enterococcal response to vancomycin. Moreover, the suspicion about the potential involvement of other TCSs suggests that vancomycin response can be more intricated than previously suspected.

In summary, the response to vancomycin, as the majority of the biological processes cannot, be well understood if we do not have a detailed knowledge of the general and specific stress response mechanisms. There are several factors contributing to different response mechanisms to vancomycin exposure in bacteria. Vancomycin response is a multifactorial mechanism and works through the concerted action of different stress response mechanisms. In enterococci is fundamental the establishment of the molecular role of the two-component systems that will for sure allow the definition of stress response regulons in general. A deep knowledge of these regulons, in particular the antibiotic response regulons, is crucial for the determination of new targets for the development of alternative antibiotic therapies.

With the work presented in this thesis we intend to contribute to identify and define other molecular players involved in the vancomycin resistance mechanims. Moreover, we intend to contribute to the future definition of the vancomycin response regulon in *E. faecalis*, through the identification of putative genes and metabolic pathways involved in this response.

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PART 1:

SPREAD OF VANCOMYCIN RESISTANCE DETERMINANTS AMONGST ISOLATES FROM DIFFERENT ENVIRONMENTS

Chapter 2

Vancomycin-Susceptible Dairy and Clinical Enterococcal Isolates carry *vanA* and *vanB* genes

This chapter contains data published in:

Ribeiro, T., Abrantes, M., Lopes, M. De F. S., Crespo, M. T. B. (2007) "Vancomycin-susceptible dairy and clinical enterococcal isolates carry *vanA* and *vanB* genes" *International Journal of Food Microbiology* **113**:289-295.

The author had a major contribution in the performance of the experimental work. The *vanA* gene screening and part of Tn*1546* mapping was performed together with M. Abrantes.

CONTENTS

ABSTRACT

A total of 113 enterococcal isolates from dairy food products and from human and dog infections, isolated in Portugal, and 26 type and reference strains of the genus *Enterococcus* were screened for vancomycin resistance. MIC values, for both vancomycin and teicoplanin, were determined. The genetic relatedness of isolates carrying *vanA* and/or *vanB* was determined using Pulsed Field Gel Electrophoresis. For *vanA* carrying isolates, transposon Tn*1546* was partially mapped using PCR. None of the 63 dairy isolates was resistant to vancomycin and only one (out of 50 clinical isolates) was resistant, with a MIC value of 256 µg/ml. The type and reference strains used were susceptible to both vancomycin and teicoplanin. *vanA* was found in 35% of the dairy isolates and 40% of the clinical isolates. *vanB* was detected in 18% of the clinical isolates, both human and dog isolates, and only in 6% of the dairy isolates. PCR partial mapping of Tn*1546* on *vanA*-containing strains revealed 23 different patterns among 42 isolates. Some patterns were shared between dairy and clinical isolates. Mapping of *vanB* operon on *vanB* carrying strains also revealed that these strains carry an incomplete *vanB* resistance operon.

Using Pulsed Field Gel Electrophoresis for *vanA*-carrying strains, six groups of isolates were found to be genetically undistinguishable and grouping was found to be geographically and location specific/related. No genetic relatedness was found between isolates from dairy, human and veterinary sources. An incomplete and/or unfunctional Tn*1546* element explains the absence of resistant behaviour in the studied isolates, even when *vanA* gene is present. As well, an incomplete *vanB* operon explains the absence of a resistant phenotype. Moreover, the work reported shows that both clinical (human and animal) and dairy isolates have been in contact with *vanA* and *vanB* genotypes of resistance and suggest that

dissemination of *vanA* gene has been through transposable elements, like Tn*1546*, and not by clonal dissemination of a resistant strain. Therefore, a national strategy should be implemented to survey both vancomycin resistance and its genetic dissemination.

INTRODUCTION

Enterococci constitute part of the natural gut microflora in mammals (Devriese *et al.*, 1992). They are also found in a variety of food products, namely milk and cheese produced in the south of Europe (Flahaut *et al.*, 1997; Barros, 1998; Lopes *et al.*, 1999). In some of these cheeses enterococci play a key role in the maturation and final organoleptic characteristics of the food product and constitute the main microbial population in the final product (Franz *et al.*, 1999). For many years enterococci have been considered harmless to man. However, in the last decades, they have become the second most frequently reported cause of surgical wound infections and nosocomial urinary tract infections and the third most frequently reported cause of bacteraemia (Klein *et al.*, 1998). Moreover, treatment of these infections has become more and more difficult due to the increasing number of antibiotic resistant enterococci.

The optimal therapy for endocarditis, one of the most serious enterococcal infections, involves a combination of a cell-wall active agent and an aminoglycoside, usually gentamicin (Malani *et al.*, 2002). With the emergence of high-level gentamicin resistance the only antibiotic available for a successful treatment of enterococcal infections is vancomycin, a glycopeptide. However, over the past decade, there has been a rapid increase in vancomycin resistant enterococci (VRE). Colonization and infection by VRE have been reported in hospitalized patients and in the community, and has also been associated with environmental sources and meat products. Glycopeptide resistance in enterococci is generally acquired and is encoded in genetic mobile elements, like transposons. This is the case of VanA, VanB, VanD, VanE, VanG and VanM phenotypes (Xu *et al.*, 2010). It can also be encoded in a non-transferable chromosomal determinant, as in VanC phenotype and most likely in VanL

(Tendolkar *et al.*, 2003; Boyd *et al.*, 2008). VanA and VanB are the most globally widespread and prevalent phenotypes. The prototype VanA element is Tn*1546*, a 10.8-kb transposon (GenBank accession number M97297) that carries a cluster of nine genes (Arthur *et al.*, 1993), encoding nine different proteins, which mediates high-level inducible resistance to both vancomycin and teicoplanin. The nine polypeptides can be divided in four functional groups: transposition (ORF 1 and 2), regulation (VanR and VanS), resistance to glycopeptides (VanH, VanA and VanX) and synthesis of peptidoglycan (VanY and VanZ). The *vanB* cluster encodes inducible resistance to vancomycin, but not to teicoplanin and is disseminated by large transposons such as Tn*1547* and Tn*5382* (Tendolkar *et al.*, 2003). Tn*1547* is homologous to Tn*1546* and most of the *vanB* genes share a large percentage of homology to the *vanA* cluster genes. In Europe, *vanA* has been found in VRE isolated in the community, from sewage, animal faeces and raw meat, which suggests that these environments can act as reservoirs of these microorganisms (Simjee *et al.*, 2002). In fact, the emergence of VRE has been related to the use of antimicrobial agents both in human and veterinary medicine and as growth promoters in animal feed. Although enterococci isolated from traditional portuguese cheeses have not been subjected to the selective pressure of the use of glycopeptides, it would be of the utmost importance to ascertain the dissemination of vancomycin resistance in this environment. Therefore, the aim of this study was to survey vancomycin resistance among a group of dairy and clinical isolates and to ascertain the possible relatedness of the VRE isolates. *vanA* and *vanB* genes were chosen since they are the most widely found among VRE. Type and reference strains of the genus *Enterococcus* were included in this study since they are the representatives of the genus.

MATERIALS AND METHODS

BACTERIAL STRAINS AND GROWTH CONDITIONS

All strains were grown at 37°C without shaking, in Brain Heart Infusion medium (BHI) (Oxoid). A total of 139 enterococci (113 wild type strains plus 26 control and reference) were used in this study. Sixty three strains were isolated from Portuguese dairy products (ewe's cheese and milk) collected from four different Registered Designation of Origin (RDO) designated as A, B, C and D, as described by Lopes *et al.* (1999) (Lopes *et al.*, 1999). Thirty-nine isolates were obtained from human infections in several Portuguese hospitals. Eleven dog-infecting isolates were obtained from Lisbon Veterinary Faculty. The dairy isolates were identified by phenotypic and molecular methods as 28 *Enterococcus faecalis*, 6 *E. faecium*, 24 *E. durans*, 1 *E. raffinosus*, 6 *E. hirae*, 1 *E. dispar* and 34 *Enterococcus* spp. (Alves *et al.*, 2004). The same methods applied to the clinical isolates identified 31 as *E. faecalis*, 8 as *E. faecium*, 1 as *E. solitarius*, 1 as *E. durans* and 9 as *Enterococcus* spp.. Twenty-six type and reference strains of the genus *Enterococcus* were also studied and are described in Table 3. The more recently described species *E. pallens*, *E. gilvus* (Tyrrell *et al.*, 2002), *E. phoeniculicola* (Law-Brown & Meyers, 2003), *E. italicus* (Fortina *et al.*, 2004), *E. hermanniensis* (Koort *et al.*, 2004), *E. devriesei* (Svec *et al.*, 2005b), *E. aquimarinus* (Svec *et al.*, 2005a), *E. caninstestini* (Naser *et al.*, 2005), *E. caccae* (Carvalho Mda *et al.*, 2006) and *E. silesiacus*, *E. termitis* (Svec *et al.*, 2006) and *E. thailandicus* (Tanasupawat *et al.*, 2008) were not included in this study. *E. faecalis* DSMZ 2570 (equivalent to ATCC 29212) was used as a control strain in the E-test, for MIC determination (Minimum Inhibitory Concentration). Vancomycin MIC values for this control strain coincided always with the expected ones. *E. faecium* BM 4147 and *E. faecalis* DSMZ 12956 were used as positive

control strains for *vanA* and *vanB* PCR reactions, respectively.

MIC DETERMINATION

Minimum inhibitory concentrations for vancomycin and teicoplanin were determined using the E-test (AB Biodisk), according to manufacturer's instructions. Each plate was inoculated with a suspension of the microorganism equivalent to 2 in the McFarland turbidity standard. Vancomycin and teicoplanin were purchased from Sigma. *E. faecalis* DSMZ 2570 was used as a control strain. Strains were classified as resistant (MIC≥ 32 μg/ml), intermediate (MIC≥ 8 μg/ml and MIC≤ 16 μg/ml) and susceptible (MIC≤ 4 μg/ml).

PREPARATION OF DNA

Total DNA was extracted from cells according to the method of Pitcher *et al*. (1989).

PCR AND SEQUENCING

Except for the type and reference strains, all dairy and clinical isolates were searched for the presence of the genes *vanA* and *vanB* using PCR. Primers used were as presented in Table 1. For the isolates for which *vanA* was detected by PCR eight primer pairs (Table 1) were used to partially map the Tn*1546* transposon. The primers used to map the *vanB* operon on dairy *vanB* carrying strains are also listed on Table 1.

PCR was performed with T-personal Combi thermocycler (Biometra). Each 50 μl PCR reaction mixture contained 250 ng of DNA, 0.5 μM of each primer, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates (dATP, dCTP, dGTP and dTTP), 1X PCR buffer, 0.005% W1 and 2.5 U of *Taq* DNA polymerase. All

reagents were purchased from GibcoBRL, except the primers that were purchased from MWG-Biotech.

Table 1. Sequence of primers used to amplify genes and intergenic regions (IR) from *vanA* (Tn*1546*) and the *vanB* operons.

Gene / IR	Sequence	Amplicon size (bp)
VanA OPERON MAPPING – STRUCTURAL GENES and INTERGENIC REGIONS[1]		
vanA	f 5′-GGGAAAACGACAATTGC-3′ r 5′-GTACAATGCGGCCGTTA-3′	732
vanR	f 5′-AGCGATAAAATACTTATTGTGGA-3′ r 5′-TCGGAGCTAACCACATTC-3′	533
vanS	f 5′-AACGACTATTCCAAACTAGAAC-3′ r 5′-GCTGGAAGCTCTACCCTAAA-3′	1049
SH	f 5′-ACGTTTAGGGTAGAGCTTCC-3′ r 5′-CTCATCCTGCTCACATCCATAAACA-3′	313
vanH	f 5′-ATCGGCATTACTGTTTATGGAT-3′ r 5′-TTACGTCATGCTCCTCTGAG-3′	1016
vanX	f 5′-ACTTGGGATAATTTCACCGG-3′ r 5′-TGCGATTTTGCGCTTCATTG-3′	404
XY	f 5′-GCATATAGCCTCGAATGG-3′ r 5′-TCCTGGAAAACAGTGCTTCATTAA-3′	594
vanY	f 5′-ACTTAGGTTATGACTACGTTAAT-3′ r 5′-TCGTCAAGCTTGATCCTA-3′	486
YZ	f 5′-GTTTCCCGGATCAACACATACTA-3′ r 5′-CCCAGTAGCAGTAAATGGAGTCA-3′	336
VanB OPERON MAPPING – STRUCTURAL GENES[2]		
vanB	f 5′-ATGGGAAGCCGATAGTC-3′ r 5′-GATTTCGTTCCTCGACC-3′	635
$vanR_B$	f 5′-GTAAGGGCGTTTTTGGCTGA-3′ r 5′-TCCTCCAATCGGTAACCTACAC-3′	602
$vanS_B$	f 5′-CGATCATTGTCCTGTTACTGCT-3′ r 5′-TAGTGTTGATGTGGGCGGTA-3′	1301
$vanY_B$	f 5′- GAATCATCACAAACGGCATA-3′ r 5′-GCAAAGCCCTTGGTGGTAT-3′	749
vanW	f 5′-ATGAACAGAAAAAGATTGACACAGC-3′ r 5′-TCATTGGTTCGCCTCCTG-3′	828
$vanH_B$	f 5′-ACTGGGGGATATGACTGTGG-3′ r 5′-GCGTAGGCCATAAGTCCGAG-3′	453
$vanX_B$	f 5′-GGGATGCCAAGTACGCTACA-3′ r 5′-GGGGTATGGCTCATCAATCA-3′	530

[1] Sequences published by (Miele *et al.*, 1995) except for *vanX* (Manson *et al.*, 2003). [2] The primers used to amplify the *vanB* operon genes were designed for this study (Chapter 5). f) forward primer, r) reverse primer.

The thermocycler was programmed with the following conditions: 5 min at 95ºC; 30 s at 94ºC, 30 s at 47ºC (*vanA*-related primers) or 49ºC (*vanB*-related primers) and 30 s at 72ºC for 30 cycles; 10 min at 72ºC; and 4ºC until analysis. Visualisation of amplicons was done with ethidium bromide (Sigma) under UV irradiation, after electrophoresis on 2% agarose (w/v) (GibcoBRL) gels. Image analysis was performed with Kodak Digital Science™. Amplification of the above mentioned genes was confirmed with sequencing of the PCR products, after their purification either with Concert™ Rapid PCR Purification System or with Concert™ Matrix Gel Extraction System, both purchased from GibcoBRL. Sequencing was performed by MWG.

INVERSE PCR

Inverse PCR was performed for four *vanA*$^+$ strains for which the presence and sequence of *vanA* gene was confirmed by sequencing. Strains from different origins and PCR patterns were chosen: one dairy strain (pattern T), one human clinical strain (pattern C) and two veterinary clinical strains (patterns J and G). As a control for the PCR reaction and for PCR products gel analysis comparison the strain BM4147, which carries the complete Tn*1546,* was used (Ochman *et al.*, 1988).

In order to optimize this procedure different conditions were tested. Restriction digests were performed using 5 µg of source DNA treated with 20 units of three different restriction endonucleases (*Hind*III, *Eco*RI, *Nsi*I) according to the supplier´s specifications. Digested DNA samples were checked on a 1% (w/v) agarose gel in 1×TAE buffer (40 mM Tris-acetate, 1 mM EDTA) to verify the efficacy of digestion. Digested DNA samples were purified by phenol-chlorophorm extraction followed by an ethanol precipitation. Circularization was performed under conditions that favor intramolecular ligation. 1 µg of the appropriated

restriction fragment was diluted to a concentration of 0.002 µg/µl. The ligation was initiated by the addition of T4 DNA ligase to a concentration of 1 unit/µl and the reaction was allowed to proceed at 16ºC overnight. The ligated sample was purified by a phenol-chlorophorm extraction followed by an ethanol precipitation. The circularized fragment were linearized using either *Aeg*I or *Eag*I and the PCR reactions were performed using the primers InVanA_1 (5'-GCAAGGTTTTTCGCACATTTTCC-3') and InVanA_2 (5'-CGTATGTAACTGGTT CTTAGGG-3') purchased from STAB Vida. The PCR reactions were performed using either 100 ng or 250 ng of digested DNA, 10 pmol of each primer, 0.2 mM of dNTPs, and 2.5 U of Expand long template High Fidelity Taq DNA polymerase (Roche Diagnostics). PCR reactions were performed according to the manufacturer´s indications. The DNA products were fractionated in a 0.8% (w/v) agarose gel and bands of interest were further purified and sequenced. All the restriction enzymes and the T4 DNA ligase were purchased from New England Biolabs.

PFGE

PFGE was performed with dairy and clinical isolates carrying *vanA* gene or *vanB* gene. Genomic DNA agarose discs and DNA digestion with *Sma*I were performed according to the method of Chung *et al.* (2000) except for lysis solution, which contained only RNase A and lysozyme (Chung *et al.*, 2000),. Agarose discs were electrophoresed on a 1% (w/v) Seakem GTG agarose gel in 0.5× TBE buffer low EDTA (50 mM Tris, 50 mM boric acid, 0.2 mM EDTA, pH 8.0) in a CHEF apparatus (Clamped Homogeneous Electric Fields) (BioRad) at 8ºC at 220V with pulse times of 5 s rising to 35 s during 18 hours. Gels were stained by immersion in ethidium bromide solution (1 µg/ml) for 30 minutes and photographed with a UV light source.

RESULTS

In this work 113 enterococcal isolates from two different environments, dairy products and clinical settings (both human and pets), and identified as different species, and 26 type and reference strains of the genus *Enterococcus*, were firstly investigated on their resistance to vancomycin using the E-test for MIC determination. Teicoplanin MICs were also determined and the study was completed with a survey of the presence of *vanA* and *vanB* genes using PCR. *vanA* positive isolates were subjected to PFGE to ascertain their relatedness and other regions of the Tn*1546* transposon were also searched in these isolates, using eight primer pairs. *vanB* operon was mapped by PCR on *vanB* carrying isolates.

MIC DETERMINATION

According to results shown in Table 2, no resistant isolates were found among dairy isolates, although nearly 16% (10 isolates) had MIC values above 6 μg/ml, and can be considered intermediate.

Table 2. Distribution of dairy and clinical isolates, carrying *vanA*/*B* genes of resistance, and of type and reference strains, between different MIC values for vancomycin.

Strains[a]		MIC (μg/ml)											
		0.38	0.5	0.75	1	1.5	2	3	4	6	8	12	256
Dairy	*vanA*$^+$	1	0	0	3	0	0	7	7	4	0	0	0
	vanA$^-$/*vanB*$^-$	1	0	1	3	9	7	11	2	3	0	0	0
	vanB$^+$	0	0	0	0	0	0	0	1	0	1	2	0
	vanA$^+$*vanB*$^+$	0	0	0	0	0	0	0	0	0	0	0	0
	Total	**2**	**0**	**1**	**6**	**9**	**7**	**18**	**10**	**7**	**1**	**2**	**0**
Clinical	*vanA*$^+$	0	0	0	0	0	3	3	6	4	0	0	0
	vanA$^-$/*vanB*$^-$	0	0	0	0	2	3	11	5	3	0	0	0
	vanB$^+$	0	0	0	0	0	1	0	1	1	1	0	1
	vanA$^+$*vanB*$^+$	0	0	0	0	0	1	0	0	1	1	1	0
	Total	**0**	**0**	**0**	**0**	**2**	**8**	**14**	**12**	**9**	**2**	**1**	**1**
Reference Strains	Total	**1**	**3**	**5**	**9**	**1**	**0**	**7**	**0**	**0**	**0**	**0**	**0**

[a] Total number of isolates/strains in each column are in bold; *vanA*$^{+/-}$, presence/absence of *vanA* gene only; *vanB*$^+$, presence of *vanB* gene only; *vanA*$^+$*B*$^+$, presence of both *vanA* and *vanB* genes; *vanA*$^-$*B*$^-$, absence of both *vanA* and *vanB* gene.

In contrast, among the clinical isolates, 72% behaved as susceptible, 22% were intermediate and 6% were found to be resistant. All type and reference strains behaved as vancomycin susceptible, as they showed MIC values below 4 μg/ml, and 70% of these strains had MIC values below 1 μg/ml (Table 3).

Table 3. Vancomycin and teicoplanin MIC values for the type and reference strains of the genus *Enterococcus*.

Strains[a]	Taxon	MIC (μg/ml)	
		Vancomycin	Teicoplanin
DSMZ 11492^T	*E.asini*	0.5	0.25
DSMZ 20679^T	*E. avium*	0.75	0.75
DSMZ 20680^T	*E. casseliflavus*	0.5	0.094
DSMZ 20682^T	*E. cecorum*	3	0.75
DSMZ 7374^T	*E. columbae*	0.5	0.5
DSMZ 6630^T	*E. dispar*	3	0.5
DSMZ 20633^T	*E. durans*	1	0.094
DSMZ 20478^T	*E. faecalis*	0.75	0.75
CECT 187	*E. faecalis*	3	0.25
CECT 184	*E. faecalis*	3	0.25
DSMZ 20477^T	*E. faecium*	1	0.75
DSMZ 7370^T	*E. flavescens*	0.75	0.094
DSMZ 20628^T	*E. gallinarum*	0.75	0.75
LMG 19487	*E. haemoperoxidus*	1	0.38
DSMZ 20160^T	*E. hirae*	1	0.75
DSMZ 20681^T	*E. malodoratus*	1	0.38
LMG 19486	*E. moraviensis*	1	0.25
DSMZ 4838^T	*E. mundtii*	1	0.38
ATCC 700913	*E. porcinus*	3	0.38
DSMZ 5632^T	*E.pseudoavium*	0.38	0.032
DSMZ 5633^T	*E. raffinosus*	3	0.38
ATCC 700914	*E. ratti*	1	0.25
DSMZ 20726^T	*E. saccharolyticus*	1	1
DSMZ 5634^T	*E. solitarius*	1.5	0.38
DSMZ 6905^T	*E. sulfureus*	0.75	0.38
LMG 12287	*E. villorum*	3	0.38

[a] DSMZ, Deutsch Sammlung von Mikroorganismen and Zellkulturen (Braunschweig, Germany); CECT, Colección Española de Cultivos Tipo (Valencia, Spain); LMG, Laboratorium voor Microbiologie (Gent, Belgium); ATCC, American Type Culture Collection (Rockville, USA).

Overall, reference strains had lower MIC values than those of the other isolates studied. The clinical isolates showed higher MIC values than dairy isolates, with 28% of the clinical isolates presenting MIC values between 6 and 256 μg/ml, against only 16% of the dairy isolates with MIC values above 6 μg/ml. All MIC values for teicoplanin fell under the susceptible classification, for all the isolates and reference strains studied (results not shown).

SEARCH FOR THE *vanA* AND *vanB* GENES

vanA gene was found in 35% of the dairy isolates and in 40% of the clinical isolates. *vanB* gene was found in only 6.3% (4 isolates) of the dairy isolates and in 18% (seven human isolates and two dog isolates) of the clinical enterococci studied. In four of the *vanB* positive clinical enterococci (two from humans and two from dogs), *vanA* was also detected. Among the dairy isolates, *vanA* was detected in four different species (*E. faecalis, E. faecium, E. durans* and *E. hirae*), both in milk and cheese isolates. In clinical enterococci, *vanA* was detected in *E. faecalis* and *E. faecium*, and in *E. solitarius*. The four isolates that showed both *vanA* and *vanB* were identified as *E. solitarius*, *E. faecium* and two as *Enterococcus* spp.

No correlation was evident between the presence of *vanA* gene and higher MIC values for vancomycin (Table 2) or teicoplanin. In fact, both dairy and clinical isolates that were positive for *vanA* had MIC values under 6 μg/ml. Only two clinical isolates had MIC values of 8 and 12 μg/ml and in both of them *vanB* gene was also detected. In fact, the presence of *vanB* gene seems to increase MIC values for vancomycin since nine, among thirteen isolates positive for *vanB*, had MIC values between 6 and 256 μg/ml. In the *E. faecium* isolate with a MIC value of 256 μg/ml, only *vanB* gene was detected.

MAPPING OF *vanA* AND *vanB* OPERONS

Strains carrying *vanA*, both clinical and dairy, were surveyed for the presence of other elements of Tn*1546*, the prototype mobile element carrying the *vanA* operon. Eight primer pairs were used in this screen assay. Two of these primer pairs were directed to two intergenic regions, XY and YZ, and the other six pairs of primers were directed to more conserved regions of this transposon, namely genes *vanR*, *vanS*, *vanH, vanX* and *vanY*. Among 42 isolates, 23 different PCR patterns were found (Table 4). All the genes and intergenic regions were detected, at least in one isolate, and with the exact size as predicted from the Tn*1546* sequence. The absence of *vanX* was the only common feature among all the 42 isolates. The only two isolates for which all (except *vanX*) the searched elements were detected (pattern B), come from the clinical environment, but from different hospitals and with collection dates 10 years apart. They were both identified as *E. faecalis* and their MIC values for vancomycin were 4 and 6 µg/ml. PCR pattern A, corresponding to absence of all the fragments, was found in four isolates, one from dairy origin (*E. durans*, with a MIC value for vancomycin of 4 µg/ml) and three from clinical environment (two *E. faecalis* and one *Enterococcus spp*., with MIC values for vancomycin of 4 and 3 µg/ml), and none of these strains was highly susceptible.

A higher variability in PCR patterns was found among dairy isolates. In fact, seven patterns (from B to H) were only found in clinical enterococci (13 isolates), and 12 different patterns (from L to X) were found in dairy isolates (16 enterococci). Four patterns were common to both environments (A, I, J and K). The only common feature among these four patterns was the absence of the X and S fragment. The S fragment, corresponding to *vanS* gene, was only found in six isolates and is the second most absent after the *vanX* gene. Region H,

corresponding to *vanH* gene, was present in 43% of the isolates and the intergenic region XY in 16 isolates (38%). The intergenic region YZ was found in 55% of the isolates and is, overall, the most conserved region of the transposon.

Despite the variability of PCR patterns found in these 42 enterococcal isolates, results from Table 4 suggest a higher presence of fragments XY, Y and YZ in dairy isolates and of fragments R, S, SH and H in clinical isolates. In fact, among the total of fragments detected in dairy isolates, 60% were XY, Y and YZ, and in clinical isolates 62% included the R, S, SH and H fragments. No correlation was found between the different PCR patterns and MIC values for vancomycin. The six veterinary isolates carrying *vanA* gene had PCR patterns similar to both human and dairy isolates (Table 4). No association was found between species and PCR pattern. *E. faecalis*, which was the most common species in both samples, were found in 11 of the 23 PCR patterns and shared patterns with either *E. faecium* or *E. durans*. Among patterns where dairy isolates were assigned, M and U are the most similar, sharing four fragments S, XY, Y and YZ. Isolates assigned to these two patterns are both from cheeses from RDO A. With three fragments in common, namely fragment XY, Y and YZ, are patterns J, L, M and U. Isolates assigned to these patterns come from RDO A cheese (three isolates) and RDO B milk. Although distant geographically, enterococci from these two regions are phenotypically related.

Among the nine patterns with only one or two fragments (patterns I and O to X), patterns I, O and V, that share *vanR*, were found only in cheese and milk isolates from RDO B. Fragment S was only detected in RDO A. Fragment SH was not detected in RDO C. However *vanH* was mainly detected in this RDO. These observations point to a possible geographic correlation with the genetic events that led to the observed differences in Tn*1546*.

Table 4. Distribution of *vanA* carrying isolates in the different PCR patterns of the Tn*1546* element found in this work. Patterns are represented as + (presence of the amplicon with the expected size) and - (absence of an amplicon) signs for each of the seven regions searched: genes *vanR*, *vanS*, *vanH*, *vanX* and *vanY* and intergenic regions SH, XY and YZ.

	Region of Tn*1546*								Origin	
Pattern	*vanR*	*vanS*	SH	*vanH*	*vanX*	XY	*vanY*	YZ	Dairy	Clinical
A	-	-	-	-	-	-	-	-	1	3
B	+	+	+	+	-	+	+	+	0	2
C	+	-	+	+	-	-	-	-	0	2
D	+	+	+	+	-	-	-	-	0	1
E	+	+	-	-	-	+	-	+	0	1
F	-	-	+	+	-	-	-	+	0	2 [a]
G	-	-	+	-	-	-	-	+	0	2 [a]
H	-	-	-	-	-	-	-	+	0	3 [b]
I	+	-	-	-	-	-	-	-	2	1 [c]
J	-	-	+	+	-	+	+	+	1	1 [c]
K	+	-	-	+	-	-	-	+	2	2
L	-	-	-	-	-	+	+	+	2	0
M	+	+	-	-	-	+	+	+	1	0
N	-	-	-	+	-	+	-	+	2	0
O	+	-	-	-	-	-	-	+	1	0
P	-	-	+	-	-	+	-	-	1	0
Q	-	-	-	-	-	+	+	-	1	0
R	-	-	-	+	-	+	-	-	1	0
S	-	-	-	-	-	-	+	-	1	0
T	-	-	-	+	-	-	-	-	2	0
U	-	+	-	-	-	+	+	+	1	0
V	+	-	-	-	-	-	+	-	1	0
X	-	-	-	-	-	+	-	-	2	0
SUM[d]	16	6	12	18	0	16	11	23	22	20

[a] one of the isolates is from a dog infection; [b] two of the isolates come from dog infections; [c] it is an enterococci isolated from a dog infection; [d] sum of isolates from each column.

Dairy strains carrying *vanB* were searched for the presence of other *vanB* operon genes. The objective of screening *vanB* strains was to confirm that, as for *vanA* strains, the lack of phenotypic expression was also related with the presence of an incomplete operon structure.

We observed that none of the four mapped strains presented a complete *vanB* operon (Table 5). The common features to all isolates are that $vanR_B$ and $vanX_B$ are present in all strains while $vanH_B$ is absent in all strains.

Table 5. Mapping of the *vanB* operon on *vanB*$^+$ dairy strains. Patterns are represented as + (presence of the amplicon with the expected size) and - (absence of an amplicon) signs for each of the genes investigated.

Strain	$vanR_B$	$vanS_B$	$vanY_B$	$vanW$	$vanH_B$	$vanX_B$
Dairy_*vanB*1	+	-	-	+	-	+
Dairy_*vanB*2	+	+	+	+	-	+
Dairy_*vanB*3	+	+	-	-	-	+
Dairy_*vanB*4	+	+	-	-	-	+

Finally in order to have more information on the *vanA* and *vanB* forms of the studied isolates, for genes which we did not succeed to amplify, we performed PCR using different combinations of the primers, i. e., when a gene was not PCR detected we used the forward primer of the upstream gene/IR paired with the reverse primer of the downstream gene/IR. For example, for strains exhibiting pattern C, we performed PCR using the forward primer for *vanR* and the reverse primer for vanSH IR. However, we did not obtain the expected PCR products.

INVERSE PCR

Since no strains harboring the complete transposon Tn*1546* were detected, inverse PCR was performed in an attempt to characterize the upstream and downstream regions of *vanA*. Inverse PCR was chosen because it allows the amplification of the regions that flank the DNA segment of a known sequence,

both upstream and downstream. The method has general applicability in genetics and can be used to establish the insertion sites of mobile genetic elements, analyze the regions adjacent to a specific sequence, or to proceed along a stretch of uncharacterized DNA.

Despite several attempts to obtain PCR fragments eligible for sequencing, we did not succeed in amplifying any fragment, including for the strain that was used as positive control. Different experimental conditions were tested: three restriction enzymes were tested to digest the chromosomal DNA; two single cut enzymes were used to cut inside the *vanA* gene sequence; and DNA amounts for the self-ligation step and for the PCR reactions. Also different sets of primers were designed to perform the inverse PCR reactions.

Inverse PCR is straightforward, but revealed itself as being very time consuming and with many optimization steps. It is our goal to characterize the different polymorphisms on the Tn*1546*-type element and on the *vanB* operon present in these strains, as well as locate the *vanA* and *vanB* genes on the chromosome and know the surrounding genes. In order to do so we are considering different approaches such as TAIL-PCR (Thermal Asymmetric Interlaced Polymerase Chain Reaction) previously used by Boyd and coworkers to characterize *vanD*, *vanG* and *vanL* operons (Boyd *et al.*, 2006a; Boyd *et al.*, 2006b; Boyd *et al*., 2008).

GENETIC RELATEDNESS OF ISOLATES

Dairy and clinical isolates for which either *vanA* or *vanB* were detected by PCR were subjected to PFGE and genetic relatedness was determined using criteria defined by Tenover *et al.* (1995) (Tenover *et al.*, 1995). No relatedness was found between dairy, human clinical and animal clinical enterococcal isolates. Among the 22 dairy isolates with *vanA* gene, only eight were found to be

related, in pairs (Table 6). These four pairs were from the three RDOs where *vanA* was detected (RDO A, B and C). Each pair of isolates came from the same cheese manufacturing place, from the same product (milk or cheese) and belongs to the same species, but MIC values were different and so were the Tn*1546* patterns.

Table 6. Relevant characteristics of isolates that were grouped according to their genetic relatedness, as determined by their PFGE banding patterns.

Group	Origin	Identification	MIC (μg/ml)	PCR pattern[a]
1:				
QA10	Cheese RDO A	*Enterococcus* spp.	6	M
QA41b	Cheese RDO A	*Enterococcus* spp.	4	A
2:				
LN37	Milk RDO B	*Enterococcus* spp.	0,38	T
LN60	Milk RDO B	*E. hirae*	1	I
3:				
QN1	Cheese RDO B	*E. durans*	3	O
QN8	Cheese RDO B	*E. durans*	6	I
4:				
QCB1	Cheese RDO C	*E. faecalis*	4	X
QCB4	Cheese RDO C	*E. faecalis*	3	R
5:				
HSM 3221	Hospital A	*Enterococcus* spp.	6	C
HSM 3720	Hospital A	*E. faecalis*	6	C
HSM 4182	Hospital A	*E. faecalis*	6	B
6:				
Vet 85r	Dog, otite ext, 2000	*E. faecalis*	2	H
344	Dog, urinary inf, 1999	*E. faecalis*	2	G

[a] see Table 4.

Among the 20 clinical isolates carrying *vanA*, only two PFGE groups were found (Table 6). The three isolates in group 5 were isolated in the same Hospital, were identified as *E. faecalis* and presented the same MIC value for vancomycin. Two of them had the same PCR pattern C, and the other had pattern B. Group 6 isolates both came from dog infections, in two consequent years. They both had the same MIC value for vancomycin and their PCR patterns, H and G, although different, are related.

Among *vanB* carriers no genetic relatedness was found.

DISCUSSION

As mentioned before, vancomycin is still at the moment an antibiotic that can be used to treat infections caused by multiresistant enterococci and by other gram-positive bacteria. It is important to have an accurate knowledge of the dissemination of the genes responsible for vancomycin resistance in other environments than the clinical setting, namely in food enterococci since these are microorganisms that contact both with human commensal microorganisms and with those in soil, water, different solid interfaces and animals.

The present work has demonstrated that *vanA* gene is equally disseminated in dairy enterococci, as it is in clinical (human and pets) environments. However, its presence does not correspond to a resistant phenotype. In order to understand the reason for this discrepancy between the susceptible behaviour and the presence of *vanA* and/or *vanB* genes, some elements of the Tn*1546* transposon and of the *vanB* operon were searched, in *vanA* and *vanB* carrying isolates, respectively. For *vanA* carrying isolates, among 42 isolates, 23 different PCR patterns were found, showing either a high variability and heterogeneity in this element, as also reported by other works (Woodford *et al.*, 1998; Van den Braak, 2001), or that we are in the presence of remnant forms of Tn*1546*. In fact, if this transposable element had been present in these isolates, it had to suffer some independent deletions or insertions that would prevent the primers used from annealing with DNA. This is a very likely possibility in the light of some reports on the instability of enterococci genomes. For example, the strain V586, vancomycin resistant and appearing to have identical pulsed field gel electrophoretic banding patterns to the vancomycin resistant V583 strain, has been shown to derive from this strain as a result of high-frequency spontaneous excisions by an yet unknown mechanism (Tendolkar *et al*., 2003). This genetic instability of the Tn*1546*

transposon is also evident even in genetically undistinguishable isolates. Several authors support the thesis that the heterogeneity of Tn*1546* is mainly explained by the presence of different ISs, which serve as hot spots for the rearrangement of genetic fragments (Heaton & Handwerger, 1995; Borgen *et al.*, 2002; Paulsen *et al.*, 2003).

The lack of complete and functional Tn*1546* may therefore account for the lack of resistance found both in dairy and clinical enterococci studied. In the particular case of pattern B, the fact that no full resistance was observed can be ascribed to the absence of *vanX*, that code for a carboxypeptidase essential for the vancomycin resistance phenotype. Although presenting only *vanA* gene, strains with pattern A were not fully susceptible. This fact suggests that the absence of *vanX* cannot, *per se*, explain the absence of resistant phenotype in the strains carrying pattern B. If the presence of *vanA* alone can account for the absence of full susceptibility in strains with pattern A remains to be established.

No genetic association was found between dairy and clinical (human or animal) isolates, despite the equal presence of *vanA* in both environments. This excludes the hypothesis of clonal dissemination of the VanA-type resistance. This is in accordance with previous reports stating that in Europe there is a high degree of heterogeneity among VRE isolates (Van den Braak, 2001). However, the fact that dairy and clinical isolates share some Tn*1546* PCR patterns is indicative of a possible genetic trading and share of a resistance genetic pool between these two environments. Despite these differences in PCR patterns, reasonable explanations based on deletion/insertions events can be hypothesized. Pair nº1 presents patterns A, characterized by the absence of all the fragments searched for, and M, that present all the genes, except *vanH* and

the intergenic region SH. One molecular event, like a deletion and/or an insertion in the region upstream from *vanH* and downstream from *vanS* would have been sufficient to prevent one of the primers of either primer pair to anneal to DNA. Pair nº2 isolates present patterns T, with only *vanH*, and pattern I, with only *vanR*. Although apparently not related, both these patterns are probably the result of multiple insertion/deletion events. Pair nº3 isolates present patterns O and I, which are, as mentioned above, similar. Pair nº4 isolates present patterns X and R, which are also related.

The scenario is slightly different with *vanB* genes, since they were found in only 6.3% of the dairy enterococcal isolates, suggesting that dairy strains have less contact with *vanB* resistant isolates. In fact, to our knowledge this is the first time *vanB* gene is described in dairy isolates. Other papers where *vanA*/*vanB* genes were screened in cheese isolates report the non-detection of *vanB* gene (Mannu *et al.*, 2003; Canzek Majhenic *et al.*, 2005; Jurkovic *et al.*, 2006; Serio *et al.*, 2007; Javed *et al.*, 2010; Ozmen Togay *et al.*, 2010). However, there are a few reports were *vanB* isolates are present in other food products and from environmental sources (Mammina *et al.*, 2005; Messi *et al.*, 2006; Caplin *et al.*, 2008; Roberts *et al.*, 2009). This is, again, in agreement with other reports showing that *vanA* resistance is more disseminated in the environment than *vanB* resistance. On the other hand, *vanB* carrier isolates, both human and animal, were found not to be genetically related, demonstrating that dissemination of *vanB* resistance is not clonal.

Like all *vanA* isolates possess an incomplete Tn*1546* element, the *vanB* dairy strains mapped also present an incomplete operon, explaining the lack of resistance expression on these strains. Due to the reduced number of strains we can only speculate about hypothesis regarding phenotype *versus* operon

composition. The absence of $vanY_B$ and *vanW* is not supposed to affect the resistance phenotype. However, the fact that $vanH_B$ is absent in all four strains can explain by itself the lack of a resistant phenotype since $vanH_B$ is essential for the expression of resistance. Absence of $vanH_B$ likely allows some level of resistance as three of the isolates have MIC values falling on the intermediate category (one with 8 µg/ml and two with 12 µg/ml). The strain with lower MIC (4 µg/ml), also lacks $vanS_B$. This result is in agreement with the fact that $vanS_B$ is also an essential gene for resistance, since it encodes for the histidine kinase protein responsible for sensing the antibiotic and induce the regulatory gene and thus all the resistance operon.

VRE are often identified as *E. faecalis*, *E. faecium*, *E. gallinarum* and *E. casseliflavus*. The present work reports detection of *vanA* gene in VSE also from other species, like *E. durans* and *E. hirae* and *E. solitarius*, not often associated with infectious processes in clinical settings, and therefore neglected. This again shows that species is not a barrier for trading antibiotic resistance genes among the genus *Enterococcus*.

If some years ago, vancomycin resistance enterococci were not relevant in the clinical environment, nowadays Portugal, together with Greece, has the highest rates of VRE associated with nosocomial infections in Southern Europe with levels up to 47% in 2003, being the *vanA E. faecium* isolates the main responsible (Werner *et al.*, 2008). However, a decrease in the rates of *vanA E. faecium* was observed. And in 2007 a decrease to 27% was reported by EARSS (European Antimicrobial Resistance Surveillance System), suggesting the successful implementation of infection control measures (Werner *et al*., 2008).

In conclusion, this work demonstrated that, although vancomycin resistant enterococci are not relevant in the food environment in Portugal, resistance

genes, namely *vanA* and *vanB*, are disseminated both in clinical and dairy food environments, although *vanB* to a less extend than *vanA*. However, their presence does not imply a resistance phenotype, which is probably due to lack of complete and fully functional Tn*1546* element and *vanB* operon. The heterogeneity of *vanA*-carrying element in the isolates studied, even in genetically undistinguishable isolates, is once again described. But, as opposite to the generally published works, it describes a genetically variable unfunctional Tn*1546*. This is probably due to the lack of selective pressure of the studied isolates. Both the antibiotic and the resistance genes are needed to produce a clinical resistance problem (Levy, 2002). The more controlled use of vancomycin in Portugal, and in Europe in general, as well as the implementation of control measures, has so far protected people from a burden of vancomycin resistance in hospitals. However, from what has been shown with our work, the resistance genes are, or have been, present in both dairy food and in clinical environments. Therefore, if vancomycin use increases in Portuguese hospitals, the resistance gene will become more prevalent and the resistance problem may thus rise. This work also shows that MIC determinations together with the search for *vanA* or *vanB* genes is not enough to categorize and predict the isolate behaviour to vancomycin, and the importance of characterize isolates with vancomycin low-level resistance.

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Chapter 3

Molecular Characterization of Uncommonly found Enterococci in Portuguese Hospitals: VREfm from CC17 Carrying IS*Ef1* and *Enterococcus hirae* Causing Infections

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The author had a major contribution in this manuscript, namely in the planning of the experimental work and in the performance of the experiments. Other contributions for this work: F. Gaspar (design of primers and optimization of PCR conditions for virulence factors screening), M. Ruivo (MLST), V. Pinto (PFGE of *E. hirae* strains).

CONTENTS

ABSTRACT

Portugal has been reported as one of the major contributors to nosocomial vancomycin resistant enterococci in Europe, although very few publications deal with the molecular characterization of these isolates by Portuguese laboratories. During one year our laboratory received 24 enterococcal isolates from a Portuguese Hospital, four of them reported as vancomycin resistant *Enterococcus faecium* (VREfm) and two identified as *Enterococcus hirae* by the Hospital (using VITEK 2 Advanced Expert System).

This study was set up to investigate the genetic relatedness of the collected isolates and to characterize the vancomycin resistance genotype of VREfm. Resistance to fifteen different antibiotics was also screened, revealing *E. faecium* as the multiresistant species, also associated with ampicillin and penicillin resistance. PFGE showed the existence of a high number of clones among *E. faecium* isolates.

Among the four VREfm reported by the Hospital only three had MIC values accordingly, although *vanA* gene was detected in the four isolates. The confirmed three VREfm, collected from different patients over a period of three months, were found to be genetically related, presenting the same PFGE type, and belonging to the global Hospital-adapted clonal lineage CC17. Accordingly, these VREfm strains were found to be ampicillin and ciprofloxacin resistant and carried the genetic information for high level gentamycin resistance. IS*Ef1* was detected in the VREfm strains in the *vanX-vanY* intergenic region, as reported previously by another Portuguese study. Hybridization studies revealed that *vanA* is located in plasmid DNA.

The strain that was found to carry the entire *vanA* operon but had a susceptible MIC (2 µg/ml), was re-tested using VITEK system and presented

divergent vancomycin resistance results. We decided to investigate this strain further in order to understand the reasons for discrepancies between phenotype and genotype of vancomycin resistance and different results in the two VITEK assays. We found that the isolate was a mixture of two *E. faecium* strains, one susceptible and the other vancomycin resistant, the resistant one belonging to CC17. These results indicate that, in the particular case of polymicrobial infection with the same enterococcal species, VITEK alone may not provide a correct assessment of VRE. To overcome the possibility of VRE misdiagnosis we suggest that VITEK should be complemented with growth in the presence of selective media with vancomycin.

Reports of nosocomial infections caused by *E. hirae* are rare. The two *E. hirae* strains were isolated from wound infections in different patients. They were both resistant to methicillin, kanamycin, netilmycin, clindamycin, lincomycin, colistin and polymyxin B, described as associated to the genus *Enterococcus*. The screening of virulence factors associated with enterococcal infectious ability revealed that both *E. hirae* strains carried the *cyl*, *fsr* and *gelE-sprE* operons, all ascribed a role in virulence in *E. faecalis*. Although not clones, the two strains were related, as revealed by PFGE analysis. Overall, our results demonstrate the potential of *E. hirae* strains both to cause hospital outbreaks and as virulence trait carriers. To our knowledge this is the first description of *E. hirae* associated with wound infections in humans in Portugal.

INTRODUCTION

Enterococci are part of the normal intestinal microbiota of humans and other animals. However, they are also prominent nosocomial opportunistic pathogens and rank among the top three most common pathogens in the nosocomial setting (Murray, 2000). Infections caused by enterococci include urinary tract infections, bloodstream infections, endocarditis, bacteremia, catheter-related and wound infections, and intra-abdominal and pelvic infections (Moellering, 1992; Murray, 1998). *Enterococcus faecalis* and *Enterococcus faecium* are the most prevalent species cultured from humans. Although *E. faecalis* is alone responsible for 80%-90% of the infections caused by enterococci, *E. faecium* has increased its contribution to nosocomial enterococcal infections in the last two decades (Wade, 1997; Mundy *et al.*, 2000; Treitman *et al.*, 2005). Frequently the relevance of other enterococcal species is neglected because they are rarely associated with severe infections in humans. However, there are some reports of infections due to *E. gallinarum*, *E. casseliflavus* (Reid *et al.*, 2001; Takayama *et al.*, 2003; Iaria *et al.*, 2005), *E. raffinosus* (Sandoe *et al.*, 2001, Savini *et al.*, 2008), *E. durans* (Stepanovic *et al.*, 2004), *E. avium* (Swaminathan & Ritter, 1999), *E. cecorum* (Hsueh *et al.*, 2000), *E. mundtii* (Higashide *et al.*, 2005) and *E. hirae* (Gilad *et al.*, 1998, Poyart *et al.*, 2002). The *E. hirae* species is widely known to cause infections in animals (Etheridge *et al.*, 1988, Lapointe *et al.*, 2000), but is rarely associated with human infections. In all studies published the isolation of *E. hirae* corresponds, in average, to 1% of all enterococcal isolates. Since this is such a rare species in clinical environment it is comprehensible that is infrequently associated with infections in humans.

In the USA, VREfm is endemic in hospitals, despite lack of carriage among healthy individuals. In Europe, however, hospital outbreaks are rare, but VREfm

carriage among healthy individuals is common. Greater use of glycopeptides such as vancomycin in US hospitals, and use of avoparcin as an animal growth promoter in Europe are assumed to be the cause of these epidemiological differences (Goossens, 1998). However, the predominant role of antibiotic pressure in the epidemiology of VREfm was challenged and VREfm outbreak have been reported (Willems *et al.*, 2001).

One of the characteristics of enterococci is their intrinsic and easily acquired resistance towards many of the antibiotics used in the clinical practice. The acquisition of vancomycin resistance by enterococci strains poses serious problem in terms of treatment and infection control. Vancomycin resistant enterococci (VRE), particularly *E. faecium* strains, are frequently resistant towards all antibiotics, creating serious limitations in treatment options (Murray, 2000). Thus, VRE are a prominent clinical and public health concern, contributing greatly to healthcare-associated infections (Huang *et al.*, 2007). Despite their ubiquity, the population structure of *E. faecium* comprises a diversity of sequence types that seem to have host-specificity. Particular host-specific human clonal lineages are considered high-risk CCs (HiRCCs) since they are recovered mostly from hospitalized patients (Leavis *et al.*, 2006). The most worrisome of these HiRCCs is clonal complex 17 (CC17), which is a hospital-adapted genetic subtype of *E. faecium* associated with epidemics and clinical infections, which has spread globally (Willems *et al*., 2001; Homan *et al.*, 2002; Leavis *et al*., 2003; Willems *et al.*, 2005). CC17 is associated with the presence of the variant *esp* gene as part of a pathogenicity island (Leavis *et al.*, 2004) and resistance to ampicillin. In 2003, another putative virulence gene, hyaluronidase (hyl_{Efm}) was described to be enriched among clinical *E. faecium* isolates (Rice *et al.*, 2003). More recently, Hendrickx and co-workers identified five surface-exposed LPXTG cell wall-

anchored proteins (CWAPs) specifically enriched in CC17 *E. faecium*, suggesting that the presence of these proteins may constitute another factor contributing to hospital adaptation (Hendrickx *et al.*, 2007).

Portuguese Hospitals report one of the major contributions to the volume of nosocomial VRE strains in Europe, with VREfm contributing to nearly 30%, according to EARSS (European Antimicrobial Resistance Surveillance System) (Werner *et al.*, 2008). However, only a few reports exist of genetic studies of VRE in Portuguese Hospitals (Melo-Cristino, 1998; Novais *et al.*, 2005; Freitas *et al.*, 2009).

The endemic nature of VRE in many healthcare institutions requires that an accurate detection of these bacteria is achieved. In Hospitals, appropriate studies must be performed in the microbiology laboratories before a decision is made on the correct antimicrobial therapy. These studies include susceptibility testing. VITEK system is used and constitutes a fast and reliable system for identification of enterococcal species and detection of VRE. This system has been reported as highly sensitive and no false positive results have been described in detection of VRE (van Den Braak *et al.*, 2001). It is common to find enterococci in polymicrobial infections with other genera. Although less frequently, it is also possible to isolate, from the same infection site, two different enterococcal strains from the same species.

In this work we had three major aims. The first one was to contribute to the general knowledge on distribution of enterococcal strains in Portuguese Hospitals. Leiria Hospital was never target of such a study and we believe it is crucial to cover as many clinical settings as possible to know better the strains circulating in Portuguese hospitals. Considering this we investigated the genetic relatedness of 22 enterococcal strains (*E. faecalis* and *E. faecium*) and the

genetic nature of the vancomycin resistance in four VREfm strains. Secondly, we report a case of misdetection of VREfm due to a mixed culture with a VSEfm and want to call attention to such cases, which can pass undetected and can contribute to the unnoticed spread of VRE. We also report the isolation of two related *E. hirae* strains that caused hospital infection in two different patients. To our knowledge this is the first time *E. hirae* species is reported as an etiologic agent in the hospital setting in Portugal.

MATERIALS AND METHODS

BACTERIAL STRAINS AND GROWTH CONDITIONS

During one year (between 2004 and 2005) 24 enterococcal isolates were collected from Leiria Public District Hospital, Portugal, and sent to our Laboratory. Identification to the species level was carried out by the hospital, using Vitek 120 (Biomerieux). Overall, we received ten *E. faecalis* strains and twelve *E. faecium* strains and two *E. hirae* strains, from different biological products (Table 1). Four of the *E. faecium* isolates were reported by the hospital (using Vitek system) as vancomycin resistant.

Strains were grown at 37ºC without shaking, in BHI medium (Oxoid). In the particular case of strain 72362, when specified, the strain was grown in parallel in BHI-drug free and in BHI supplemented with 2 µg/ml of vancomycin (Sigma).

ANTIBIOTIC SUSCEPTIBILITY DETERMINATION

After arrival at our laboratory, all isolates were screened by the disk diffusion method for resistance towards fifteen antibiotics, namely rifampicin (RD), ampicilin, (AMP), penicillin B (P), imipenem (IPN), cefoperazone (CFP), vancomycin (VA), nitrofurantoin (F), bacitracin (B), streptomycin (S), gentamicin (CN), kanamycin (K), tetracycline (TE), clindamycin (DA), erythromycin (E) and ciprofloxacin (CIP) according to the Clinical and Laboratory Standards Institute (CLSI, former NCCLS) guidelines and used the recommended breakpoints to define resistance (Clinical and Laboratory Standards Institute, 2000), as described elsewhere (Lopes *et al.*, 2005). The antibiotic susceptibility of the two *E. hirae* strains was tested for a broader group of antibiotics. Their susceptibility towards 30 antibiotics was tested as previously described by Lopes *et al.* (2005).

MIC values for both vancomycin and teicoplanin were determined for the four isolates that were considered vancomycin-resistant enterococci (VRE) by the hospital. E-test (AB Biodisk) was used according to manufacturer's instructions. Each plate was inoculated with a suspension of cells equivalent to #2 in the McFarland turbidity standard. *E. faecalis* DSMZ 2570 was used as a control strain. Strains were classified as resistant (MIC≥ 32 µg/ml), intermediate (MIC≥ 8 µg/ml and MIC≤ 16 µg/ml) and susceptible (MIC≤ 4 µg/ml) according to the CLSI document (Clinical and Laboratory Standards Institute, 2000).

PFGE TYPING

To analyze clonal relatedness among the isolates, total DNA agarose discs were prepared and DNA digestion with *Sma*I was performed as described in (Chung *et al.*, 2000), except for lysis solution, which contained only RNase A and lysozyme. Agarose discs were electrophoresed on a 1% Seakem GTG agarose gel (FMC Bioproducts) in 0.5× TBE buffer low EDTA (50 mM Tris, 50 mM boric acid, 0.2 mM EDTA, pH 8.0) in a CHEF apparatus (Pharmacia) at 12ºC for 18 hours at 220V with pulse times of 5 s rising to 35 s during the electrophoresis period. Gels were stained by immersion in ethidium bromide (1 µg/ml) for 30 minutes and photographed with a UV light source. Genetic relatedness of the strains was determined according to Tenover criteria (Tenover *et al.*, 1995). Fragment patterns were analyzed by the software package BioNumerics version 1.5 (Applied Maths). Clustering was performed with the unweighted average linkage (UPGMA) with the Dice similarity coefficient.

PCR SCREENING OF *vanA* OPERON OF VREfm

Vancomycin resistance genotype was investigated using PCR for detection of both *vanA* and *vanB* genes as described by Ribeiro *et al.* (2007). Total DNA was extracted from cells as described elsewhere (Lopes *et al.*, 2003). Since only *vanA* was detected in the four strains, *vanA* operons, using Tn*1546* transposon as the source sequence, were partially mapped. *vanR*, *vanS*, *vanH*, *vanX*, *vanY* and intergenic regions SH, XY and YZ were investigated using PCR as previously described (Ribeiro *et al.,* 2007). Sequencing was performed by Baseclear.

PCR SCREENING OF RESISTANCE AND VIRULENCE DETERMINANTS ON VREfm AND *E. hirae* STRAINS

The four *E. faecium* isolates reported by the hospital as VRE were also screened for the presence of the bifunctional gene *aac(6')-aph(2'')*, responsible for high-level gentamicin resistance, as described previously (Lopes *et al.*, 2003).

PCR for *esp* gene was carried out using specific primers for *E. faecium esp* gene, using the primers espefm_f (5'-TTGCTAATGCTAGTCCACGACC-3') and espefm_r (5'-GCGTCAACACTTGCATTGCCGA-3').

The presence of virulence traits (namely Cyl operon, Fsr and GelE-SprE operons and *ace*, *asa1*, and *efaA* genes) in the *E. hirae* strains was screened by PCR using primers described on Table 2 and Immolase DNA polymerase (Bioline), according to the manufacturer´s instructions.

MLST TYPING

VREfm were genotyped by MLST (multilocus sequence typing) as described by Homan *et al.* (2002). Internal fragments of seven housekeeping genes were amplified by PCR with the set of primers described in (Homan *et al.*, 2002). PCR

products were purified with a Qiagen kit and both strands were sequenced (Baseclear). MLST alleles and sequence types (ST) were identified using the available database (http://efaecium.mlst.net/).

SOUTHERN DOT BLOT HYBRIDIZATION

In order to investigate if the *vanA* operon was located in the chromosome or in a plasmid, *vanA* amplicon obtained by PCR, was used as a probe. Both total DNA and plasmid DNA was prepared as described previously (Lopes *et al.*, 2003), spotted onto a Hybond–N+ membrane (Amersham Biosciences) and fixed to the membrane by heating to 80ºC for 2 hours. The Southern blot hybridization was performed with the ACL™ Direct Nucleic Acid Labelling and Detection System (Amersham Biosciences), according to manufacturer's instructions. Signal was detected after 30 min of exposure of the film (Hyperfilm™ MP, Amersham Biosciences).

RESULTS AND DISCUSSION

ANTIMICROBIAL SUSCEPTIBILITY AND VIRULENCE TRAITS

Among the strains received from the hospital, 27% were isolated from blood infections, 55% from pus, 5% from catheters and the remaining 14% from other infections (Table 1). From the six strains responsible for blood infections, five were identified as *E. faecium*.

All isolates were screened for antibiotic resistance to fifteen antimicrobial agents. Results of this study are presented in Table 1. The *E. faecium* strains were resistant to a higher number of antibiotics as compared to *E. faecalis* isolates. In fact, nine out of ten *E. faecium* strains were resistant to eight to ten antibiotics; as opposed to seven, out of nine *E. faecalis*, which were resistant to six to eight antibiotics. 95% (18 out of 19) strains were bacitracin resistant, regardless of the species, and vancomycin resistance was only detected in three of the four *E. faecium* isolates reported as possible VRE by the Hospital. All isolates were resistant to kanamycin and resistance to erythromycin was detected in 79% of the assayed strains. Resistance to both ampicillin and penicillin was detected in 47% of the strains, but only in *E. faecium* isolates.

The four strains reported as VRE by the Hospital (21428, 57182, 60921 and 72362) were further analyzed and MIC values for both vancomycin and teicoplanin were determined. As shown in Table 3, three strains were confirmed to be VRE, as predicted by the disk diffusion assay, but strain 72362 behaved as susceptible to both antibiotics. Thus, VRE isolates represent 14% (3/22) of the hospital isolates, and vancomycin-resistant *E. faecium* (VREfm) sum up to 25% (3/12).

Table 1. Identification, source of isolation and antibiotic resistance of the enterococcal strains from Leiria Hospital.

Strain	Species	Date of isolation	Source	Antibiotic resistance profile[b]
113373	*E. faecalis*	26-11-04	Sputum	nd
118103	*E. faecalis*	28-11-04	Pus from wound	RD, CFP, B, S, CN, K, E
125944	*E. faecium*	20-12-04	Pus from wound	nd
128555	*E. faecium*	30-12-04	Blood	nd
2692	*E. faecium*	11-01-05	Blood	RD, AMP, P, CFP, B, S, CN, K, TE, E
5534	*E. faecalis*	18-01-05	Vaginal secretions	B, CN, K, TE
9308	*E. faecalis*	21-02-05	Pus from wound	RD, B, S, CN, K, TE
12499	*E. faecalis*	21-02-05	Pus from cicatriz scar	B, S, CN, K, TE
19015	*E. faecium*	22-02-05	Blood	RD, AMP, P, CFP, B, CN, K, E
19864	*E. faecalis*	22-02-05	Bronchoalveolar aspiration	RD, B, S, CN, K, TE, E
127417	*E. faecalis*	21-02-05	Pus from wound	CFP, B, S, CN, K, TE
44132	*E. faecalis*	28-04-05	Blood	CFP, B, S, CN, K, TE, E
44805	*E. faecalis*	29-04-05	Pus from wound	RD, B, S, CN, K, TE, E
21011	*E. faecium*	07-03-05	Pus from peritoneal absess	RD, AMP, P, CFP, B, S, CN, K, TE, E
21428[a]	*E. faecium*	07-03-05	Blood	RD, AMP, P, CFP, IPN, VA, F, B, CN, K, TE, DA, E
24285	*E. faecium*	07-03-05	Pus	RD, AMP, P, CFP, B, S, CN, K, TE, E
27461	*E. faecalis*	29-03-05	Pus from scab	CFP, B, S, CN, K, TE, E
29379	*E. faecium*	29-03-05	Catheter	RD, AMP, P, CFP, B, S, CN, K, TE, E
41058	*E. faecium*	19-04-05	Pus from wound	RD, AMP, P, CFP, B, S, CN, K, TE, E
57182[a]	*E. faecium*	03-06-05	Pus from surgical wound	RD, AMP, P, CFP, VA, B, K, E
60921[a]	*E. faecium*	16-06-05	Pus from surgical wound	RD, AMP, P, CFP, VA, B, K, E
72632[a]	*E. faecium*	24-08-05	Blood	RD, CFP, B, K, E
SAVE H1	*E. hirae*		Wound Infection	MET, K, NET, DA, LIN, COL, PB
SAVE H2	*E. hirae*		Wound Infection	MET, K, NET, DA, LIN, COL, PB

[a] reported as VRE according to the Vitek method used in the Hospital; [b] results of the disk diffusion method obtained after receiving the strains from the Hospital; , rifampicin (RD), ampicilin, (AMP), penicillin B (P), imipenem (IPN), cefoperazone (CFP), vancomycin (VA), nitrofurantoin (F), bacitracin (B), streptomycin (S), gentamicin (CN), kanamycin (K), tetracycline (TE), clindamycin (DA), erythromycin (E), ciprofloxacin (CIP), methicillin (MET), netilmycin (NET), lincomycin (LIN), colistin (COL), polymyxin B (PB); nd, not determined.

In fact, *E. faecium* strains were not only associated with vancomycin resistance, but were also multiresistant. This result is in accordance to increasing observed tendency that point to *E. faecium* as the most resistant enterococcal species, in particular to vancomycin (Wade, 1997). One of these isolates, strain 21428 from blood, was the most resistant isolate among the 22 strains, showing resistance towards fourteen of the fifteen antibiotics tested. Resistance to both penicillin and ampicillin was associated with *E. faecium*, which is in agreement with another study from Italy (Stampone *et al.*, 2005). The co-resistance of vancomycin and ampicillin/penicillin is widely described in *E. faecium* (Fontana *et al.*, 1994; Rice, 2001; Rice *et al.*, 2005). In several countries the endemicity of

ampicillin resistant *E. faecium* (Suppola *et al.*, 1999; Harthug *et al.*, 2002; Torell *et al.*, 2003) is documented and events of acquisition of vancomycin resistance have been published (Suppola *et al*., 1999; Harthug *et al.*, 2000).

In a previous study, we did not detect any VRE neither among *E. faecalis* nor *E. faecium* isolates from human infections in Portuguese Hospitals in the Lisbon and Porto areas (Lopes *et al.*, 2005). In that same study, *E. faecalis* was the prevalent species among 39 isolates. In the present study, among 22 isolates, *E. faecalis* and *E. faecium* were found to be equally responsible for nosocomial infections. Recent publications report also the presence of VREfm from Portuguese hospitals, although not from the same areas of Portugal (Novais *et al*., 2005; Mato *et al.*, 2009). Considering what was said, the present work represents the first report of VRE strains from the area of Leiria, Portugal. The number of VRE detected in this study (14% among 22 isolates and 25% among *E. faecium* only) is considerably high, but fits into the percentages reported by the EARSS for Portugal during 2007 (Werner *et al*., 2008).

Both *E. hirae* isolates were susceptible to most of the antibiotics tested. They presented resistance to 7 antibiotics (methicillin, kanamycin, netilmycin, clindamycin, lincomycin, colistin and polymyxin B) described as associated to the genus *Enterococcus* (Lopes *et al*., 2005). Although not carrying clinically relevant antibiotic resistance determinants, these strains had the genes coding for three of the most relevant virulence factors described in the genus *Enterococcus*, namely the *cyl* operon, coding for the citolysin, the *gelE-spreE*, coding for gelatinase and a serine protease, and *fsr*, reported as controlling both gelatinase and serine protease expression and, among others, biofilm development in *E. faecalis* (Bourgogne *et al*., 2006). The *cyl* operon was not complete, since *cylR1* was not detected (Table 2). However, these results demonstrate that other species then

E. faecalis, namely *E. hirae*, are acquiring the same virulence genes which are most probably responsible for the *E. hirae* success as an infectious agent. Other studies reporting *E. hirae* as the etiologic agent responsible for causing severe infection did not characterize the strains regarding their virulence traits (Gilad *et al*., 1998; Poyart *et al*., 2002). However, these studies describe *E. hirae* strains able to cause aortic-valve endocarditis (Poyart *et al*., 2002), bacteremia and septicaemia (Gilad *et al*., 1998) which require that the strains are able to adhere, invade and infect the host cells in order to cause disease. As the two *E. hirae* isolates that we report in this study, these strains described as causing severe infections, most likely carry several virulence factors.

Table 2. Virulence determinants found in *E. hirae* strains and primers (f-forward; r-reverse) used in the PCR reactions to detect the described genes (+, detected; -, not detected).

Gene	Primer (5′→3′)	Amplicon size (bp)	SAVE H1	SAVE H2
cylR1	GCTATTTTTCTTTTTCTACCTAA-f TATGAAGTCCTCTTGTTGAAA-r	128	-	-
cylR2	GCTTTATTAGAAGTTAGCAGACAA-f TGGAGTATTCAGGTAGTAAGCAA-r	105	+	+
cylLL	AAATGGAAGCGATTCAAGGT-f CAGCCACAAGCAGCACTACT-r	100	+	+
cylLS	AACTTGTTGGTCCTTCTTTTGA-f CCTATGGTAAAACATGCTGGAG-r	106	+	+
cylM	TGGTGTAAGGGAACTGTAGGTG-f ATGGCACAGACAATCTTTATTTTTA-r	117	+	+
cylB	GGAGAATTAGTGTTTAGAGCG-f TATCCCTAAAAAAAGACTATCTATTA-r	99	+	+
cylA	TGCTCGTGAAATGATGATGAC-f CGCTTACTTCTGGAGTTG-r	125	+	+
cylI	TGATGATGATGAAATAAGCCA-f AACCCATTGTTTTTCTGCTAT-r	103	+	+
fsrA	GCATCCAGATTTGATTAGAGTC-f TGGTACTTCCGTTCCGTC-r	112	+	+
fsrB	AAGTCAGTGATGGTGTGGGAA-f ATTCTTTTTAGCTTTTTCAGTTTGTC-r	98	+	+
fsrC	TAATACCGCAAAGCAAGCAA-f AGCCAACAAACGAATCACAA-r	103	+	+
gelE	TCTTCACTTTTTGTAGCCGC-f TGCCTGAGAAATTGCCTC-r	96	+	+
sprE	ACAGGCTTTGTTGTTGGAA-f ATCACGACCTGGATAAAACC-r	123	+	+
ace	AATCAAAATGTGGAAATGCC-f CACTTGTTTCTGTTGCCTGTT-r	109	+	+
asa1	AGCCAATGTGGTTCCTGTTC-f AGTTCTGTCGTATCCCCTGTC-r	126	+	+
efaA	ACCTGAACAAATGACCACGA-f TCTTTTGAGACCCGTTCCAT-r	108	+	+

MOLECULAR TYPING OF THE *E. faecium*, *E. faecalis* AND *E. hirae* ISOLATES

Dendrograms based on the PFGE *Sma*I restriction pattern of *E. faecium* and *E. faecalis* isolates are presented in Figures 1A and 1B, respectively. In general, diversity among isolates was observed, although a higher heterogeneity was

present among *E. faecalis* isolates. However, some clones were detected. Analysis of *E. faecium* isolates by PFGE showed three groups of isolates that had identical or similar profiles, differing by six or fewer bands (Figure 1A). According to the dendrogram, the coefficient of similarity (CS) can be fixed to 0.92.

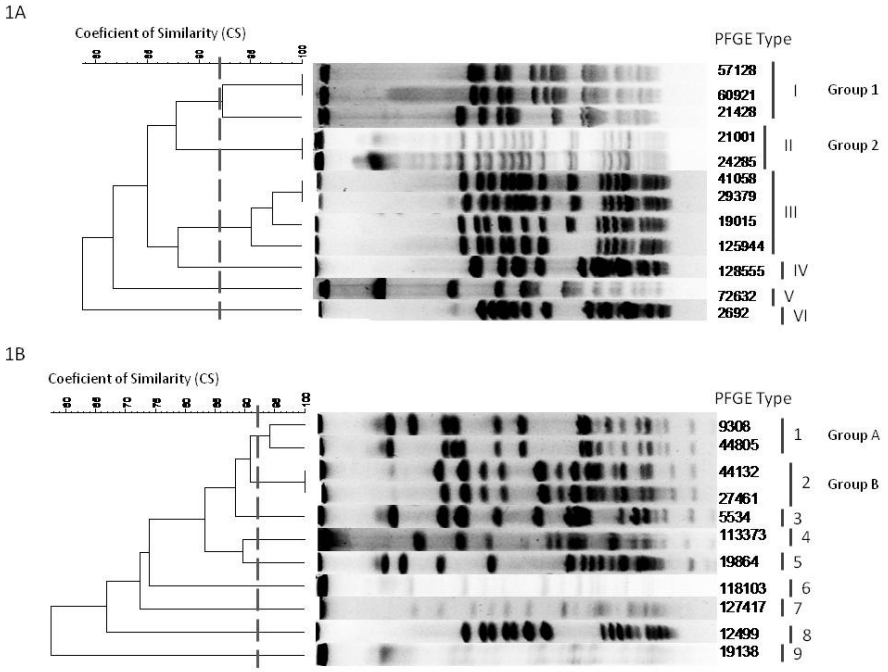

Figure 1. Dendrograms of *E. faecium* (1A) and *E. faecalis* (1B) strains, based on PFGE *Sma*I restriction pattern analysis.

Strains in Group 3 were collected in the Hospital over a span of four months from different biological sources, namely pus, blood and catheter (Table 1). Among these strains, one clone is distinguishable (Clone 3). Group 2 is itself a clone, Clone 2. Among strains in Group 1 there is also a clone, Clone 1. Within the *E. faecium* clones (Clone 1, strains 57182 and 60921; Clone 2, strains 21011 and 24285; Clone 3, strains 41058 and 29379) the same antibiotic resistance pattern was observed (Table 1).

E. faecium Clone 1 (assigned to Group 1) corresponds to two VREfm isolates. These strains were both collected from pus from surgical wounds, but in different days and from different patients. This PFGE pattern is similar to the PFGE profile of the other VREfm, strain 21428 (Figure 1A), differing by six bands. The strain 21428 was isolated from blood three months before the other two VREfm strains and is resistant to more antibiotics. Its antibiotic resistance profile (Table 1) differs from the other two VREfm strains by resistance to imipenem, tetracycline, clindamycin and nitrofurantoin.

Strain 72632, also reported by the Hospital as VRE, but confirmed as VSE by MIC determination in the lab (Table 2), had a PFGE profile differing from the Group 1 profile by more than seven bands. Strain 72632 was resistant to a lower number of antibiotics, and was susceptible to ampicillin and penicillin, differing from strains in Group 1. This strain was isolated from blood two months after Clone 1 isolates and five months after VREfm strain 21428, also isolated from blood (Table 1).

Among *E. faecalis* isolates, two groups with CS higher than 0.92 were observed by PFGE analysis (Figure 1B) and nine PFGE types could be distinguished. Strains in Group A, although identical, differed in resistance to erythromycin (Table 1).

Among *E. faecium* strains, six different PFGE types were observed and vancomycin resistance was associated with PFGE type I. Among *E. faecalis* strains, nine PFGE types were detected. These results show that *E. faecium* clinical isolates are more clonal, which is particularly true for the VREfm isolates.

The fact that the only three VREfm isolates belong to the same PFGE type and considering that they were collected over three months from different patients

and different biological samples (pus and blood) shows that the same PFGE type remains in the hospital for days and is able to cause infection in different patients.

The PFGE profiles of the two *E. hirae* strains (Figure 2) indicate that they are genetically related but not clones as they present distinct macrorestriction patterns, differing in less than 7 bands. The two strains were isolated from different patients with just one week apart and were both isolated from the same kind of biological product (pus). Therefore, the PFGE results suggest that the first isolated strain (SAVE H1) was able to persist in the hospital, suffer a few genetic events that originated the second strain (SAVE H2) which was again able to cause an infection in another patient. However, the distinct PFGE patterns clarify that they are two distinct strains. Consequently we can rule out the hypothesis that the isolates could belong to the patient's own commensal flora in the case of the second strain. It is widely known that enterococcal infections are most frequently caused by patient's endogenous flora, which might be the case of the first infection, although we are not able to prove it.

Figure 2. PFGE of *Sma*I digested total DNA of *E. hirae* strains SAVE H1 and SAVE H2.

MLST TYPING

The genetic relatedness of VREfm observed by PFGE genotyping was confirmed by MLST. All three VREfm belong to ST18 (*atp* 7, *ddl* 1, *gdh* 1, *purk* 1, *gyd* 5, *pstS* 1, *adk* 1), which is part of the Hospital-adapted clonal lineage, CC17 (Willems *et al.*, 2005). As described for strains that form CC17, strains 21428, 57182 and 60921 were all ampicillin resistant (Table 1). Ciprofloxacin resistance

was also associated with the studied VREfm, which is consistent with data that co-relates CC17 strain adaptation to the Hospital environment and ciprofloxacin resistance (Leavis *et al*., 2006).

Other studies with strains from different Portuguese Hospitals have reported the presence of *E. faecium* belonging to CC17, although not all are VRE (Freitas *et al*., 2009; Mato *et al*., 2009).

Strain 72362 had a different PFGE profile from the other VREfm strains, and the following MLST allele sequence: *atpA* 46, *ddl* 18, *gdh* 10, *purK* 23, *gyd* 1, *pstS* 46, *adk* 11, representing anew ST356. This strain was susceptible to ampicillin, penicillin and ciprofloxacin.

MOLECULAR ANALYSIS OF VANCOMYCIN RESISTANCE IN VREfm STRAINS

The genotype responsible for the vancomycin resistance in the strains reported by the hospital as VREfm was checked by PCR. Both *vanA* and *vanB* genes were searched. *vanB* gene was not detected but *vanA* was found in four strains (Table 3). A partial mapping of the VanA genetic element based on the sequence of the Tn*1546* transposon revealed the presence of all the essential genes for expressing resistance, namely *vanR*, *vanS*, *vanH*, *vanA* and *vanX* (Table 3). *vanY* was detected in the three confirmed VREfm strains, but with a smaller size then the expected 486 bp (Figure 3). In the same strains the PCR product of the PCR amplification of the *vanX-vanY* intergenic region had a higher molecular weight (2 kb) then the expected 594 bp (Figure 3) and was due to the presence of the insertion sequence IS*Ef1*. IS*Ef1* has also been found in *vanX-vanY* intergenic region in VREfm from other Portuguese Hospitals, namely from the areas of Coimbra, Viseu and Porto (Novais *et al*., 2005; Novais *et al*., 2008). This IS element is one of three predominant types found in the *E. faecalis* V583

genome (Paulsen *et al.*, 2003). It thus appears that IS*Ef1* element is common among nosocomial VREfm in Portugal.

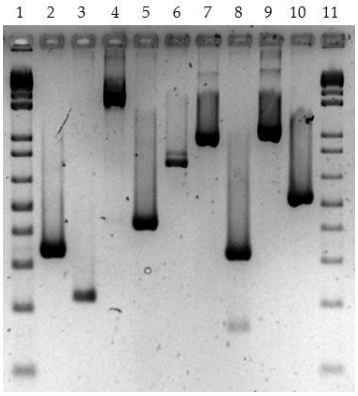

Figure 3. Electrophoresis on agarose gel of the PCR products of amplification of IR *vanY-vanZ* (2), *vanY* (3), IR *vanX-vanY* (4), *vanX* (5), *vanA* (6), *vanH* (7), IR *vanS-vanH* (8), *vanS* (9) and *vanR* (10) of strain 21428. Lanes 1 and 11, 1Kb Plus ladder purchased from GibcoBRL.

Gentamicin resistance was also evaluated in the strains reported as VRE by the hospital. The bifunctional gene *aac(6')-aph(2'')*, responsible for HLGR, was detected in all of the four strains, but only strain 21428 was HLGR (Table 3) by phenotypic analysis. The fact that only 21428 behaved accordingly, suggests that the gene may not be functional in the other two true VREfm strains. Similar results have been described in a previous work, but with another gene conferring HLGR (Lopes *et al*., 2003). Further studies are needed to clarify this behavior. The appearance of an increasing number of HLGR enterococci in the clinical environment makes it difficult to treat severe cases of endocarditis because the synergism with cell-wall active agents (like ampicillin, penicillin G or vancomycin) no longer works. HLGR is also associated with CC17 *E. faecium* Hospital-adapted lineage (Leavis *et al*., 2006).

In contrast to *E. faecalis*, little is known about the virulence of *E. faecium* (Gilmore *et al*., 2002). The enterococcal surface protein gene, *esp*, is part of the

E. faecalis PAI (Shankar *et al.*, 2002), is a major putative pathogenicity marker in clinical isolates of *E. faecium* and *E. faecalis*, and a marker for epidemic strains of *E. faecium (Willems et al., 2001)*. We detected the presence of the *esp* gene in the true VREfm strains (Table 3).

Table 3. Characterization of the four *E. faecium* isolates reported as VRE by the Hospital. Respective results of the PCR screening of genes and intergenic regions (IR) of the *vanA* operon, antibiotic resistance and *esp* gene.

Strain	Antibiotic resistance profile[a]	*esp*	MIC (μg/ml)			*vanR*	*vanS*	IR SH *vanH vanA vanX*	IR XY	*vanY*	IR YZ
			VA	TE	CN						
21428	RD, **AMP**, **P**, CFP, IPN, **VA**, F, B, CN, K, TE, DA, E, **CIP**	+	> 256	24	> 1024	+	+	+	+[b]	+[c]	+
57182	RD, **AMP**, **P**, CFP, VA, B, K, E, **CIP**	+	> 256	16	12	+	+	+	+[b]	+[c]	+
60921	RD, **AMP**, **P**, CFP, VA, B, K, E, **CIP**	+	> 256	16	12	+	+	+	+[b]	+[c]	+
72362	RD, CFP, B, K, E, F	-	2	1.5	32	+	+	+	+	+	+

[a], results of the disk diffusion method obtained right after receiving the strains from the Hospital, rifampicin (RD), ampicilin, (AMP), penicillin B (P), imipenem (IPN), cefoperazone (CFP), vancomycin (VA), nitrofurantoin (F), bacitracin (B), streptomycin (S), gentamicin (CN), kanamycin (K), tetracycline (TE), clindamycin (DA), erythromycin (E), ciprofloxacin (CIP), teicoplanin (TE), in bold are the antibiotic resistances that are strongly associated with CC17; [b], the amplicon had 2 kb instead of the expected 594 bp. Sequencing of this amplicon revealed the existence of the insertion sequence IS*Efl*; [c], the amplicon had 230 bp instead of the expected 486 bp.

Leavis *et al*. (2003) propose an evolutionary scheme for *E. faecium* genotypes and phenotypes from an ancestral *E. faecium* type. and argue that the hospital-adapted genogroup of *E. faecium* developed and spread unnoticed, thus creating a pool of strains with epidemic potential that only become recognized and relevant after becoming vancomycin resistant (Willems *et al*., 2001; Leavis *et al*., 2003). The scheme is based on the presence of ampicillin and vancomycin resistance, presence of the variant *esp* gene, and the type 1 allele for the *purK*

gene (Leavis *et al*., 2003). Considering the results obtained for the three VREfm strains (Amp^R, Van^R, esp^+, *purK* 1^+) these strains fulfill all the requirements to be part of this well adapted epidemic hospital genogroup. The stress-inducing conditions in hospitals, such as antimicrobial drug use, may favour the selection of this CC17 genogroup, with enhanced antibacterial resistance, virulence and ability to spread.

Since 2002, nine sporadic cases have been reported in the United States (Perichon & Courvalin, 2009). Spread of multi-resistant *E. faecium* strains and their resistance genes will have serious implications for health care. In Portugal the transfer of vancomycin resistance determinants to MRSA was not yet reported however, our results indicate that is possible that this transfer events occurs, since CC17 clones are becoming more frequent.

LOCALIZATION OF THE ELEMENT CARRYING *vanA* OPERON ON VREfm STRAINS

Hybridization of plasmid DNA, using *vanA* amplicon as a probe, gave positive reactions in all four strains reported by the hospital as VRE (Figure 4). Thus, our findings point to the presence of vanA genotype in a plasmid, in all of the *vanA*-carrying strains. The presence of vancomycin resistance in mobile elements makes transfer and dissemination of resistance more prone.

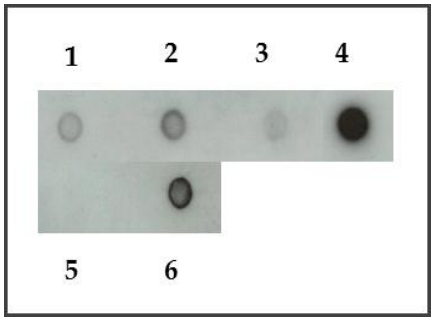

Figure 4. Southern hybridization of plasmid DNA, fixed on a Hybond–N+ membrane, of strains 57182 (1), 60721 (2), 72632 (3), V583 (5) and 21428 (6) with *vanA* PCR amplicon as probe. (4), *vanA* PCR amplicon.

INCONGRUENCE PHENOTYPE *VERSUS* GENOTYPE FOR *E. faecium* 72362 STRAIN

VITEK is a fast and reliable system used in hospitals for identification of enterococcal species and detection of VRE. This system has been reported as highly sensitive and no false positive results have been described in detection of VRE (van Den Braak *et al*., 2001). However, strain 72362 that arrived to our Laboratory described as VREfm, using VITEK system, was found to be vancomycin susceptible by MIC determination (MIC 2 μg/ml), although carrying the *vanA* gene. The MIC for this isolate was confirmed by E-test several times and never changed from susceptible. We thus decided to investigate further this isolate in order to understand the discrepancy between phenotype and genotype and the reason for the apparent misidentification with VITEK system. The entire *vanA* operon (from nucleotide 3983 to 10200) of isolate 72362 was sequenced (Baseclear) and no mutations were found in this operon when compared to the published Tn*1546* sequence from BM4147 (Genbank Accession Nº M97297). We then decided to retest 72362 in a similar VITEK 2 system, and it came identified as *E. faecium*, but susceptible to both vancomycin and teicoplanin. Assuming the reliability of the VITEK system, and the fact that VITEK protocol requires picking of a few colonies, an explanation for our findings could lay on the presence of a mixed culture, namely of two *E. faecium* strains, one VRE and another VSE (vancomycin-susceptible enterococci). The opposite results obtained in the two VITEK assays could result from the choice of a colony: in the first time, at the Hospital and before arriving at our Laboratory, a VRE colony could have been selected; and in the second assay, a VSE colony could have been selected.

We thus decided to grow the original 72362 isolate in parallel in the absence and in the presence of 2 μg/ml vancomycin and perform E-test for both cultures.

Results are shown in Figure 5. It is clear from analysis of Figure 5a and 5b in particular, because of the presence of several small colonies inside the halo (Figure 5b), that 72632 behaves as VRE if previously grown in the presence of the antibiotic in a concentration that eliminates, or prevents, the growth of the VSE strain (which has a MIC of 2 μg/ml). We then picked four colonies inside the halo (In colonies) and four outside the halo (Out colonies) and re-grew each of the eight colonies in parallel in the absence and in the presence of 2 μg/ml vancomycin. For the sake of easier understanding, we name the isolation outside the halo as Out colonies, although we are aware that we were not able to pick just one colony, because outside the halo cultures grow as a film.

As shown in Figures 5c and 5d, all In colonies were resistant to vancomycin even if not grown in the presence of the antibiotic. Moreover, it seems that these four cultures were now homogeneous. The PFGE profile of these four In colonies (Figure 6) was the same for all, in the presence and absence of vancomycin, but different from the original 72362 profile. In1 colony was selected for typing with MLST and confirmed to belong to a different ST from the original 72362 culture. In1 belongs to the ST18 and thus to CC17, as 21428, 57182 and 60921 strains. In the case of the Out colonies our results suggest that after one passage, we are still in the presence of mixed cultures. In fact, when grown in the presence of vancomycin, the cultures behaved as resistant to the antibiotic (Figure 5e) but in the absence of vancomycin a halo of growth inhibition was detected. Analysis of the PFGE profile of these cultures (Figure 6) revealed that the profile selected by prior growth in vancomycin is the same of the In colonies, and different from the original 72362 culture, which in turn is the same of the Out colonies grown without vancomycin.

After growth in the absence of the antibiotic, the Out colonies showed two different behaviours: Out 1, 2 and 4 colonies presented colonies inside the inhibition halo (Figure 5g); and Out3 colony had no colonies inside the inhibition halo, exactly as 72362 isolate (Figure g). These results suggests that Out 1, 2 and 4 isolates, were enriched in VRE when compared with original culture 72362, but Out 3 would be similar to 72362. It is likely that if these four Out colonies would have been passed again through vancomycin, VRE enrichment could be achieved and the culture would behave as the In colonies.

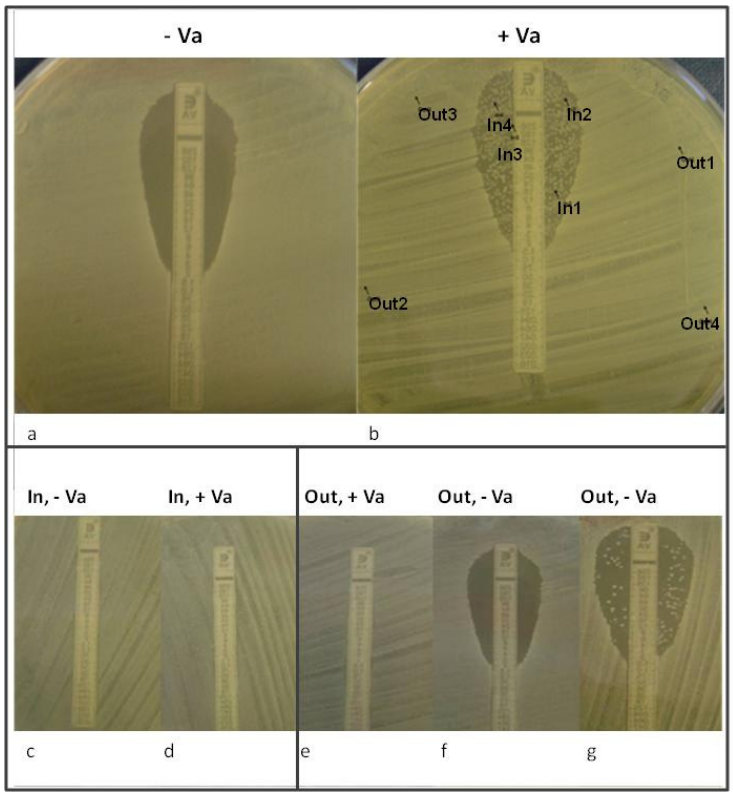

Figure 5. E-test plates containing vancomycin strips on BHI agar: 5a, 72362 original culture grown in the absence of vancomycin; 5b, 72362 original culture grown in the presence of 2 μg/ml vancomycin (small arrows indicate the position of the eight selected isolated "colonies" In1 to In4 and Out1 to Out4); 5c, colony In1 grown in the absence of vancomycin (results for In2 to In4 were the same); 5d, colony In1 grown in the presence of 2μg/ml vancomycin (results for In2 to In4 were the same); 5e, colony Out1 grown in the presence of 2 μg/ml vancomycin (results for Out2, Out3 and Out4 were the same); 5f, colony Out3 grown in the absence of vancomycin; 5g, Out1 grown in the absence of vancomycin (results for Out2 and Out 4 were the same).

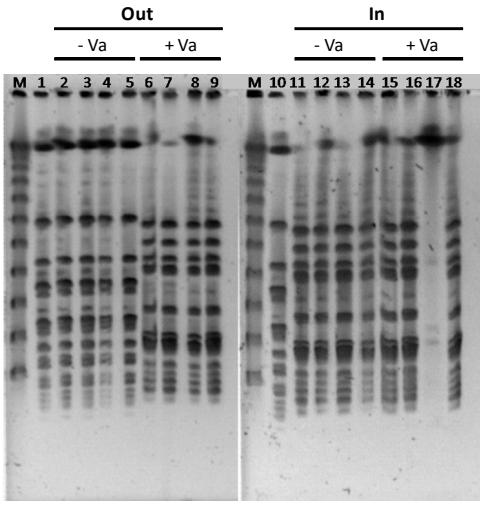

Figure 6. PFGE of *Sma*I digested total DNA from In and Out colonies: M, Lambda Ladder PFG marker (NEB); lanes 1 and 10, original strain 72362; lanes 2 to 9, Out colonies as depicted in Figure 5b; lanes 11 to 18, In colonies as depicted from Figure 5b, grown in the presence (+ VA) or absence (- VA) of 2 μg/ml of vancomycin.

Our results clearly demonstrate that 72362 is a mixed culture of two *E. faecium* strains, with different susceptibilities to vancomycin. In the absence of the antibiotic in the medium, the VRE strain grows poorly or becomes nearly absent, thus leading to a susceptible outcome in E-test. However, if DNA is extracted from this mixed culture, and because PCR needs only (in theory) one copy of the gene to allow detection, we could detect the entire *vanA* operon, belonging to the VRE strain. In the same way, when MLST was performed, and because both strains are *E. faecium*, the primers used to amplify the housekeeping genes would amplify in both strains. The fact that the VSE strain grew much better, and was thus in much higher amounts, would obviously lead to the amplification of genes from this VSE strain, and therefore the ST found was not ST18.

In Hospitals the use of the VITEK system is very useful and reliable, although, as shown in this report, indolent pathogens such as VRE can pass undetected if in mixed cultures with faster growing strains. The use of PCR, in

parallel with the use of the VITEK system, to detect similar situations as the one reported in this manuscript, is probably not viable. One option could be to screen enterococci recovered from blood cultures, or other significant biological products, with a vancomycin screening agar in parallel with the VITEK or other commercial system. Recently, in a broad study by Huang *et al.* (2007), assessment of VRE in United States Hospitals was proven to be improved by routine screening performed with media containing 6 μg/ml vancomycin (Huang *et al*., 2007). We strongly believe that this same measure could easily be implemented during infection control and allow a better knowledge about the strains causing infection, thus enabling both a correct selection of antimicrobial therapy and epidemiology. In summary, it is important that physicians are aware that similar cases can occur during treatment of patients. A better assessment of VRE can be achieved easily, as we propose, improving the success of the therapy outcome. This is particularly striking when CC17 strains can pass undetected, because of being indolent pathogens when in competition with faster growing strains.

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PART 2:

RESPONSE TO VANCOMYCIN: PLAYERS AND THEIR ROLES IN RESPONSE TO VANCOMYCIN

Chapter 4

Transcriptomic Profile of *Enterococcus faecalis* V583 in Response to Vancomycin Reveals Bacterial Support for Full Expression of High-Level Vancomycin Resistance

Tânia Ribeiro, Axel Hartke, Michael S. Gilmore, Fatima Lopes. "Transcriptomic Profile of *Enterococcus faecalis* V583 in response to vancomycin reveals bacterial support for full expression of high-level vancomycin resistance" (in preparation).

The author had a major contribution in this manuscript, namely in the planning of the experimental work and in the performance of the experiments.

CONTENTS

ABSTRACT

Enterococci are harmless in healthy individuals but constitute a serious threat to patients with compromised immune system or under prolonged antibiotic treatments. Vancomycin, a glycopeptide antibiotic, used to treat multiresistant enterococcal infections is now close to become obsolete due to increasing isolation of vancomycin-resistant enterococci. Vancomycin resistance genes have been identified in *Enterococcus faecalis*. However, mutants in regulators have been shown to be essential for high-level resistance to vancomycin, revealing that much is yet to know on *E. faecalis* response to this antibiotic. The vancomycin stress response was studied in *E. faecalis* V583, a *vanB* carrying clinical strain, using a therapeutic (10 µg/ml) and a subinhibitory vancomycin dose (0.3 µg/ml). The study was done at two different exposure times (t_{10} and t_{30}) to resolve antibiotic stress effects occurring on two biologically relevant time scales. The subinhibitory dose strictly and specifically induced the vancomycin resistance genes. In contrast, exposure to the therapeutic dose affected drastically the transcriptional profile of the cells. About 14% of the V583 genes were found to be differentially transcribed and are enrolled mainly in regulatory/signal transduction functions, transport and binding and cell envelope synthesis/modification. The differential expression of genes involved in transport and binding and in cell envelope metabolism suggests that transport and maintenance of cell integrity are key factors in stress response to vancomycin. The two-component systems (TCS) *vanRS*, *croRS* and *ehk06-err06* responded to antibiotic treatment. The upregulated TCSs may display an important role on the specific response of V583 to vancomycin as well as in the regulation of multiple genes and metabolic pathways that have been altered under vancomycin exposure. The fact that the subinhibitory dose selectively induced the *vanB* operon genes suggests a dose

threshold from which other genes and metabolic pathways start to be differentially expressed under vancomycin exposure.

In conclusion, microarray data demonstrated that *E. faecalis* responds globally to therapeutic vancomycin doses, suggesting that survival/resistance to this antibiotic may involve other cell elements than just the *van* resistance genes. It seems also clear that, in order to express vancomycin resistance the cells alter they metabolism focusing on making available the metabolites that are required for cell wall synthesis. This knowledge might help future research on alternative ways to fighting enterococcal infections.

INTRODUCTION

Antibiotics are associated with treatment of many infectious diseases and their therapeutic action relies on the use of high concentrations. During treatment, a range of concentrations below the inhibition one occurs in different tissues of the treated host. Such range of low concentrations remains also present in the host for long periods of time after treatment has finished. Therefore, microorganisms, both the targets of the antibiotic treatment and the others, are exposed to a range of subinhibitory (SI) antibiotic concentrations in the host, during and after treatment with the therapeutic dose. Work performed in recent years has shown that SI antibiotic concentrations, i.e, those which do not affect growth of the organism, influence considerably and widely, gene transcription in microorganisms. SI concentrations of antibiotics modulate the expression of a vast number of cellular functions such as SOS pathways and DNA repair systems (Mesak *et al.*, 2008; O'Sullivan *et al.*, 2008; Lopez & Blazquez, 2009), cell wall stress response (Sass *et al.*, 2008), virulence genes (Shen *et al.*, 2008; Weir *et al.*, 2008), quorum sensing mechanisms (Skindersoe *et al.*, 2008), flagella (Horii *et al.*, 2003) and biofilm formation (Hoffman *et al.*, 2005). Transcriptomic approaches using SI antibiotic concentrations have been used mainly for the purpose of understanding the mode of action of the molecule and for the discovery of new bioactive compounds. However, we can get further information with these kind of studies, namely if we apply these assays for antibiotics which are on the way of becoming obsolete due to significant resistance among the target organisms. Knowing the effects of low concentrations of the soon-to-become-obsolete antibiotics can provide relevant information for finding either new ways of dosing the chemical or new targets for the resistant microorganism.

One of the most relevant examples of soon-to-become obsolete antibiotics is vancomycin, a glycopeptide. Vancomycin inhibits cell wall synthesis by forming complexes with the peptidyl-D-alanyl–D-alanine (D-Ala–D-Ala) termini of peptidoglycan (PG) precursors at the cell surface (Reynolds *et al.*, 1994). Vancomycin was first introduced into the clinical practice in the 50s and in 1986 the first resistant enterococcal strain was isolated (Leclercq *et al.*, 1988). Since then, vancomycin resistant enterococci (VRE), responsible for several diseases such as endocarditis, bacteremia, urinary tract infections and wound infections, have emerged as one of the major nosocomial agents in Hospitals. Their prevalence as nosocomial opportunistic pathogens correlates with the increasing use of glycopeptide antibiotics (Shepard & Gilmore, 2002). The control of glycopeptide resistant enterococci spread within and between hospitals and an effective therapeutic are the major challenges concerning enterococci.

Enterococcus genus constitutes the major reservoir of vancomycin resistance genotypes, although acquired resistance to this antibiotic is mostly due to two types of gene clusters, designated *vanA* and *vanB*. Both confer resistance by the same mechanism and encode related enzymes (Reynolds *et al.*, 1994). In both cases, resistance is due to synthesis of peptidoglycan precursors ending in the depsipeptide D-alanyl–D-lactate (D-Ala–D-Lac) that binds glycopeptides with reduced affinity (Evers *et al.*, 1996). The *vanB* operon contains the $vanY_BWH_BBX_B$ resistance genes, being the last three essential for the resistance phenotype. $vanH_B$ encodes a dehydrogenase that reduces pyruvate to D-Lac, *vanB* encodes a ligase that synthetize the depsipeptide D-Ala-D-Lac and $VanX_B$ hydrolyses the D-Ala-D-Ala dipeptide synthetized by the Ddl ligase. Expression of the resistance genes is regulated by the $vanR_BS_B$ two-component regulatory system, composed by a membrane-associated sensor kinase ($VanS_B$) and a cytoplasmic response

regulator ($VanR_B$) that acts as a transcriptional activator (Arthur *et al.*, 1992; Evers & Courvalin, 1996). The regulatory and resistance genes are transcribed from distinct promoters that appear to be coordinately regulated (Arthur *et al.*, 1997).

E. faecalis has an extraordinary ability to survive to a wide range of environmental stresses. This ability makes them reliable indicators of faecal contaminations of food products and drinking water (Franz *et al.*, 1999; Giraffa, 2002). *E. faecalis* is a commensal microorganism of the intestinal tract of humans and animals, is harmless in healthy individuals but constitute a serious threat to patients with compromised immune system or under prolonged antibiotic treatments.

Recent studies on susceptible bacteria, namely *Staphylococcus aureus* (Kuroda *et al.*, 2003; McAleese *et al.*, 2006; McCallum *et al.*, 2006), *Bacillus subtilis* (Cao *et al.*, 2002), *Bacillus licheniformis* (Wecke *et al.*, 2006), *Streptococcus pneumoniae (Haas et al., 2005)* and *Mycobacterium tuberculosis* (Provvedi *et al.*, 2009), have shown that SI vancomycin concentrations elicit a cell wall stress stimulon, which seems to be predominantly regulated by TCSs and extracytoplasmatic function σ factors (Jordan *et al.*, 2008).

Based on what is described for other genus we are led to believe that vancomycin may act directly on other genes, either via a $vanR_B$-dependent pathway or indirectly and that the knowledge of the transcriptional changes induced by this antibiotic may help us to understand how vancomycin acts on *E. faecalis* cells. We thus designed a study aimed to understand the extent of the effects of SI vancomycin concentrations in *E. faecalis*. We used V583 strain, carrying the *vanB* operon, and strain $V583Ery^S\Delta vanB$, which is erythromycin susceptible. We investigated the response to the exposure to a therapeutic and a

subinhibitory dose of vancomycin using a genome-wide microarray based on the V583 genome sequence.

MATERIALS AND METHODS

BACTERIAL STRAINS AND GROWTH CONDITIONS

This study was performed with *E. faecalis* V583 (V583), V583Ery^S and V583Ery^{S}Δ*vanB*. V583 and V583 Ery^S are isogenic strains and V583Ery^S has a deletion of the gene that confers erythromycin resistance (EFA0007, *ermB*) (V583Ery^S strain was provided by A. Benachour).

The strains were grown overnight in Brain Heart Infusion (BHI) medium, at 37°C. Cultures were then diluted 20 times and grown in BHI until an optical density of 0.4-0.45 at 600 nm was reached. Cultures were split in two and vancomycin (Sigma) was added to one of the cultures to a final concentration of 10 µg/ml (therapeutic dose) in case of V583 or 0.3 µg/ml (subinhibitory dose) in the case of V583Ery^S and its isogenic deletion mutant Δ*vanB*. The two cultures (BHI and BHI plus vancomycin) were then further incubated, and 5 ml samples of each culture were collected after 10 (t_{10}) and 30 minutes (t_{30}) past vancomycin addition. Samples were immediately suspended in RNA Protect solution (QIAGEN) and centrifuged for 10 min at 4°C. Pellets were conserved at 4ºC until RNA extraction. Growth of strains with and without vancomycin was monitored by measuring the optical density of the cultures in BHI media at 600 nm.

CONSTRUCTION OF *vanB* MUTANT BY HOMOLOGOUS RECOMBINATION

A fragment containing the *vanB* gene was amplified by PCR using primers 5'-ATCAGGCATGCGATATCGGCAGACAAC-3' (forward) and 5'-GCAGGGAGCTC GGTACAGAGT AAAGTTCTG-3' (reverse), digested with *Sph*I and *Sal*I, and cloned into vector pUCB30 (Benachour *et al.*, 2007). The deletion was created by inverse PCR from the sequence cloned into pUCB30 using primers 5'-CTTGCATAGCTTCCATACGCCG-3' and 5'-CCAGCAGACATTCCGGTCGAG-3',

which hybridize inside the *vanB* gene. The PCR product obtained was ligated and reamplified with primers 5'-CGCAACCATGGCC GCTGGCAATCAATGC-3' and 5'-TTTCTGTCGACAAGAG AGTAATTGGTCTG-3', containing *Nco*I and *Sal*I restriction sites, underlined respectively, and cloned into pMAD vector. The resulting plasmid was introduced into *E. faecalis* V583. Integrations by double-crossover recombination within *vanB* gene were checked by PCR and sequencing. The v*anB* gene deletion mutant was created in the strain V583Ery^S due to clone selection convenience since pMAD carries erythromycin resistance gene.

TOTAL RNA EXTRACTION

Total RNA extraction was performed with RNeasy Mini columns (QIAGEN). DNA digestion was done on the columns by adding RNase-free DNase (QIAGEN) and incubating at room temperature for 30 min. 10 µg of each RNA sample was used for hybridization experiments.

DNA MICROARRAY CONSTRUCTION

The Affimetrix microarrays used were previously designed and described in McBride *et al*. (2007). Briefly, the microarray was designed to contain a total of 3582 probes including 3182 predicted ORFs from the chromosome of strain V583; additional pathogenicity island genes of strain MMH594, known to be absent in V583; and *E. faecalis* plasmid and antibiotic resistance genes or clusters from other *E. faecalis* strains for which nucleotide sequences had been reported, namely the *vanA* operon, *blaZ*, *bcr* operon, *vanG* operon, *vanE* operon, *tetM*, pRE25 plasmid/cat, as well as the genes present on the V583 endogenous plasmids pTEF1, pTEF2 and pTEF3. Additionally microarrays included 111

Affymetrix designed eukaryotic and prokaryotic negative control probe sets (McBride *et al.*, 2007). A full list of probe sets including genes represented and excluded from the microarray is available in the ArrayExpress public repository at http://www.ebi.ac.uk/ under accession number E-MEXP-1090. Details of the algorithm used in construction of custom Affymetrix GeneChips are available at the manufacturer's website (www.affymetrix.com/technology/index.affx).

DNA MICROARRAY REPLICATES AND DATA ANALYSIS

For each condition three batches of RNA were obtained in three separate growth experiments to provide true biological replicates. Each set of independent RNA samples was used to perform the microarray hybridizations. cDNA synthesis, fluorescent labelling, oligonucleotides array hybridization and preliminary data analysis was performed by the company Genome Explorations (Memphis, Tennesse).

Average relative fold changes were calculated from the average of the signal log ratio (SLR) from three separate experiments by using the following equations: for an SLR of ≥ 0, average relative fold change = 2^{SLR}; for an SLR of <0, average relative fold change $=-1\times 2^{(-1\times SLR)}$ (Ng *et al.*, 2003).

SEMI-QUANTITATIVE REVERSE TRANSCRIPTASE PCR (RT-PCR)

To independently confirm the differential expression observed by DNA microarray experiments some genes were selected for analysis by semi-quantitative RT-PCR. The genes selected and the primers used are listed on Table 1 and were synthesized and purchased from Eurofins (Ebersberg, Germany).

cDNA was synthesized from RNA samples isolated from cells grown in presence and absence of vancomycin (10 µg/ml) and after 10 and 30 minutes of antibiotic exposure. cDNA synthesis was performed using Transcriptor High Fidelity cDNA Synthesis Sample (Roche) according to the manufacturer's instructions and using 4 µg of RNA as template.

Table 1. List of genes and primers (5´→3´) used for semi-quantitative RT-PCR

Locus (gene)	Function	Primer ID	Sequence (5´→3´)	Reference
EF2299 (*vanR$_B$*)	*vanB* operon genes	EF2299_for EF2299_rev	AGCGATAAAATACTTATTGTGGA TGCGAGCTAACCACATTC	(Miele *et al.*, 1995)
EF2298 (*vanS$_B$*)		EF2298_for EF2298_rev	AACGACTATTCCAAACTAGAAC GCTGGAAGCTCTACCCTAAA	(Miele et al., 1995)
EF2297 (*vanY$_B$*)		EF2297_for EF2297_rev	ACTTAGGTTATGACTACGTTAAT TCGTCAAGCTTGATCCTA	(Miele et al., 1995)
EF2296 (*vanW*)		EF2296_for EF2296_rev	ATGAACAGAAAAAGATTGACACAG TCATTGGTTCGCCTCCTG	This study
EF2295 (*vanH*)		EF2295_for EF2295_rev	ATCGGCATTACTGTTTATGGAT TTACGTCATGCTCCTCTGAG	(Miele et al., 1995)
EF2294 (*vanB*)		EF2294_for EF2294_rev	ATGGGAAGCCGATAGTC GATTTCGTTCCTCGACC	(Dutka-Malen *et al.*, 1995)
EF2293 (*vanX$_B$*)		EF2293_for EF2293_rev	ACTTGGGATAATTTCACCGG TGCGATTTTGCGCTTCATTG	(Manson *et al.*, 2003)
EF0680 (*pbp2A*)	Penicillin binding proteins	EF0680_for EF0680_rev	TGAAGATCGCGGGTTTTATC CGCATCTTGATACCCCTGAT	This study
EF0746		EF0746_for EF0746_rev	GCACCAGATAGTGGCGAACT CCAAATTCATGGGCAAAACTAGC	This study
EF2429 (*guaC*)	GMP reductase	EF2429_for EF2429_rev	CATGCATCGGTTTGATGAAG TCCCTTTATGGCGTAACCAG	This study
EF1645 (*codY*)	Transcriptional regulator	EF1645_for EF1645_rev	GTCAAGTGAACGAGCTTTTGC GCATTCACAATTACGGAACGAG	This study
EF1260	DNA binding regulators	EF1260_for EF1260_rev	CTTAAATGAAGGGTGGGAAGC ACCGTTTGGATGACTTGAGG	This study
EF3289 (*croR*)		EF3289_for EF3289_rev	AAGGATACGAAGTCGTGAAAGC TCCAACTCCCCACACTGTTTG	This study
EF1646 (*hsIU*)	Heat shock protein	EF1646_for EF1646_rev	CGGGATGTCGAATCAATGGTG GACAAGTGGAAAGCACCTGAAG	This study
EF0802	Hypothetical proteins	EF0802_for EF0802_rev	GTGATAGTTCATTAAAGGAGG GTTATGACTGAAAGCAAGGCG	This study
EF1231		EF1231_for EF1231_rev	CTATGCGTGGAAGATTGAGC GTACCCGTACCAACAAATGC	This study
EF1813		EF1813_for EF1813_rev	TTACATGCCAGGCTATACGC TGGACTGTCGTTTGATTGCC	This study
EF2292		EF2292_for EF2292_rev	TCTGCTTTATTATGCGGGCG GAATCAGCAGAGCAAGAACG	This study
EF2896		EF2896_for EF2896_rev	ATGGCCTTGGCTTTGTTTGC ACTCTGACGAACCAGATACC	This work

RESULTS

Vancomycin therapeutic dose choice (10 µg/ml) was based on what is described in the literature. Therapeutic peak levels of 20-40 µg/ml and trough serum levels of 5-10 µg/ml have been identified for vancomycin and are accepted worldwide (Rybak *et al.*, 2009). The vancomycin subinhibitory concentration (0.3 µg/ml) choice was based on growth curves performed in BHI media with a V583Ery^S isogenic mutant, V583 Ery^{S}Δ*vanB*, which is intermediate to vancomycin with a MIC of >12 µg/ml determined by microdilution. A vancomycin dose of 0.3 µg/ml corresponds to the highest dose that does not affect the growth of the intermediary strain.

GROWTH OF *E. faecalis* STRAINS UNDER EXPOSURE TO VANCOMYCIN

Figure 1 shows the growth curves of the strains used in this study, in the absence and presence of vancomycin stress. It is clear that, at both times of collection of RNA (t_{10} and t_{30} post vancomycin addition), growth was not yet affected in none of the strains. In fact, exposure to 10 µg/ml of vancomycin reduced slightly V583 growth when compared with the untreated control. However, this reduction in growth was only perceptible after 30 minutes of antibiotic exposure. The same behaviour was seen for the Δ*vanB* mutant. Therefore, any observations drawn from the results presented are mainly consequences of the action of the antibiotic and less a result of the growth inhibition. A SI concentration corresponds, by definition, to 1-10% of the MIC (Yim *et al.*, 2006). The therapeutic dose used in this work is thus SI for V583. 0.3 µg/ml is a true SI concentration for both V583Ery^S and Δ*vanB*. For the sake of easier writing, 10 µg/ml will be referred to as therapeutic dose (and used for V583) and

0.3 µg/ml will be called SI concentration (used for both V583Ery^S and its Δ*vanB* isogenic deletion mutant).

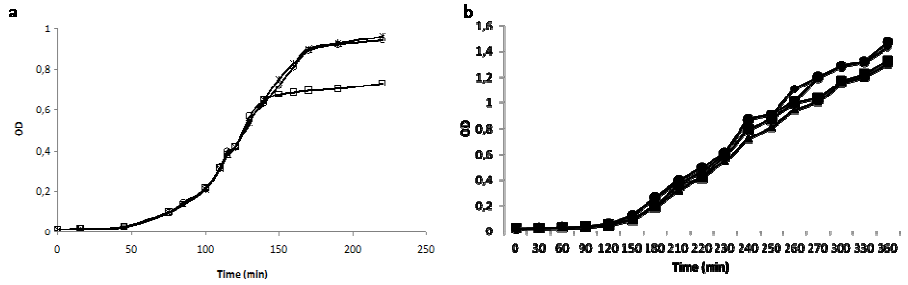


Figure 1. a) growth curve of *E. faecalis* V583 in BHI without vancomycin (∗), with a therapeutic vancomycin dose (10 µg/ml) (□) and a subinhibitory vancomycin dose (0.3 µg/ml) (○); b) growth curve of *E. faecalis* V583Ery^S (♦), V583Ery^S with 0.3 µg/ml of vancomycin (●), V583Ery^{S}Δ*vanB* (■) and V583Ery^{S}Δ*vanB* with 0.3 µg/ml of vancomycin (▲).

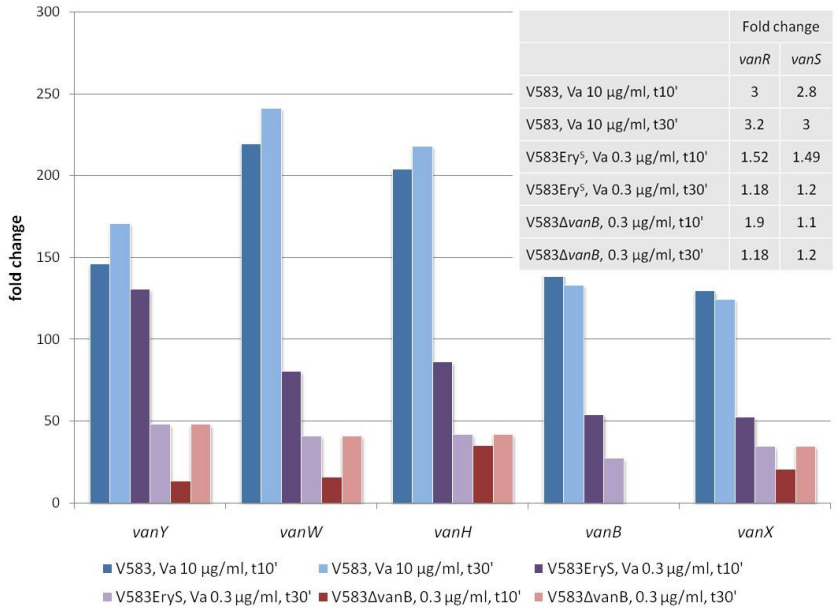
CRITERIA FOR ANALYSIS OF MICROARRAY RESULTS

We only present and discuss here genes that were differentially transcribed in all three independent replicate experiments, with fold change higher than 2.0 in, at least, one of the sampling times. For change p-values higher than 0.9975, probes were considered downregulated and for p-values lower than 0.0025, probes were considered upregulated. Since the typical cell division time of *E. faecalis* in BHI medium is 30 minutes, cells were collected at two time points after the addition of vancomycin, 10 and 30 minutes, to resolve antibiotic stress effects occurring on two biologically relevant time scales.

EXPRESSION OF SPECIFIC VANCOMYCIN RESISTANCE GENES

All genes from vancomycin resistance operon *vanR$_B$S$_B$Y$_B$WH$_B$BX$_B$* (EF2299 to EF2293) were, as expected, upregulated in the presence of vancomycin, both at t_{10} and t_{30}, with both vancomycin concentrations (Figure 2). *vanY$_B$*, *vanW*,

vanH$_B$, *vanB* and *vanX*$_B$ were the most strongly induced genes by 10 µg/ml of vancomycin. *vanR*$_B$*vanS*$_B$ had a discrete increment on expression, of about 3-fold.



	Fold change	
	vanR	*vanS*
V583, Va 10 µg/ml, t10'	3	2.8
V583, Va 10 µg/ml, t30'	3.2	3
V583Ery^S, Va 0.3 µg/ml, t10'	1.52	1.49
V583Ery^S, Va 0.3 µg/ml, t30'	1.18	1.2
V583Δ*vanB*, 0.3 µg/ml, t10'	1.9	1.1
V583Δ*vanB*, 0.3 µg/ml, t30'	1.18	1.2

Figure 2. *vanB* operon genes fold changes under exposure to therapeutic vancomycin dose (10 µg/ml) and subinhibitory vancomycin dose (0.3 µg/ml). *vanR*$_B$ and *vanS*$_B$ were not included on the graphic due to the significant difference between fold change values for these genes and the other genes from the operon.

Although the fold change values observed with the SI concentration of vancomycin are lower for *vanY*$_B$, *vanW*, *vanH*$_B$, *vanB* and *vanX*$_B$ genes, compared to the ones observed with the therapeutic dose, they are still high in strains V583Ery^S and Δ*vanB*. For the same two strains *vanR*$_B$*vanS*$_B$ genes presented fold changes lower than the ones observed for the therapeutic dose.

EXPOSURE TO A VANCOMYCIN SUBINHIBITORY DOSE

With 0.3 µg/ml of vancomycin the only genes differentially expressed were the genes from the *vanB* operon, together with a single hypothetical protein

(EF2292), in both V583Ery^S and Δ*vanB* strains. For these strains, the fold change values for $vanR_B$ and $vanS_B$ are below the cut-off value of 2. We only consider them here because they are directly involved in vancomycin response. However, despite the low level of induction, it is enough to induce the rest of the *van* operon to similar levels in both strains.

EXPOSURE TO A VANCOMCYIN THERAPEUTIC DOSE

As opposed to the SI concentration of vancomycin, the therapeutic dose was able to produce an overall differential expression in genes assigned to various roles in the cell (Figure 3).

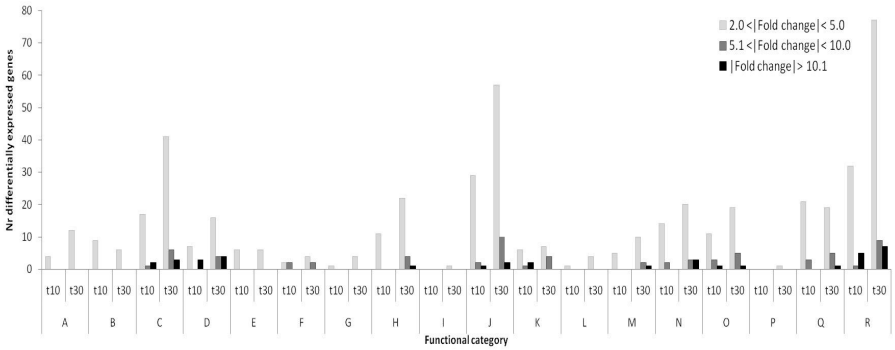


Figure 3. Functional classification of *E. faecalis* V583 genes showing differentially expressed levels, represented according to their absolute fold change value (light grey 2.0<|fold change|<5.0, medium grey 5.1<|fold change|<10.0, dark grey |fold change|>10.1), in response to exposure to 10 µg/ml vancomycin. Functional classification refers to TIGR annotation. Differentially expressed plasmid-encoded genes are also included; genes that have been assigned with more than one cellular role were counted twice. A) Amino acid biosynthesis, B) Biosynthesis of cofactors/prosthetic groups/carriers, C) Cell envelope, D) Cellular processes, E) Central intermediary metabolism, F) DNA metabolism, G) Mobile and extrachromosomal element functions, H) Energy metabolism, I) Fatty acid and phospholip metabolism, J) Hypothetical proteins, K) Protein fate, L) Protein synthesis, M) Purine/pyrimidine/nucleoside and nucleotides, N) Regulatory functions, O) Signal transduction, P) Transcription, Q) Transport and binding proteins, R) Unknown function proteins.

In total, 441 different genes, corresponding to 14% of the V583 chromosomal genes, were differentially transcribed. At t_{10}, 205 genes (6%) were differentially transcribed, of which 127 genes were significantly upregulated and 78 genes

were downregulated. At t_{30}, 445 genes were differentially transcribed, corresponding to about 14% of V583 genes, which indicates a general increase in transcriptional activity by exposure to the therapeutic dose of vancomycin. Although the transcriptional profile is different at both time points there is a considerable overlap since 129 genes are common. Only one plasmid encoded gene was upregulated, EFA0067, encoding a PTS system protein. Figure 3 represents all the functional categories of genes that were differentially expressed under the conditions tested. It is worth mentioning that 5% of the differentially expressed genes present fold change values higher than |3| and that 2% have fold change values higher than |5|. These results show that transcriptional events in *E. faecalis* V583 are significantly altered by vancomycin exposure, concerning the number of genes involved and the fold change values that these genes present.

Some functional classes of genes show no relevant changes at the transcription level under vancomycin exposure, namely the categories of genes involved in fatty acid and phospholipid metabolism, mobile and extrachromosomal element functions, protein synthesis and transcription. Cell envelope, energy metabolism, cellular processes, transport and binding and regulatory and signal transduction are the most affected functional categories under vancomycin therapeutic dose. Among the most upregulated categories are genes encoding for hypothetical proteins and proteins with unknown function followed by genes involved in synthesis of cell envelope components and genes with regulatory functions (Figure 3). At t_{30} a considerable number of genes encoding for transport and binding proteins were also upregulated. Considering the downregulated genes, the categories with more genes involved are again those encoding for

hypothetical and unknown function proteins, transport and binding proteins, followed by genes involved in signal transduction and cell envelope-related proteins.

A list of all genes found to be differentially expressed under exposure to 10 µg/ml of vancomycin, sorted by locus order, is presented in supplementary table S1. In this table we distinguish, by different grey tones, genes differentially expressed at t_{10} and t_{30} from genes only expressed at one of the assayed time points. It is reasonable to assume that the first category of genes is responding either to vancomycin or to the immediate changes introduced by the high upregulation of the *vanB* operon genes and are directly responding to the stress. Since it is maintained through the time of the experiment, these genes also maintain their differential expression. Genes only up- or downregulated at 10 minutes post-stress induction are likely involved in the immediate response to the presence of vancomycin. As soon as the cell changes, other responses are turned on, namely those elicited by the expression of genes which are turned on or off only 30 minutes post-stress induction.

Among the genes which expression is regulated at both time points we find some which encode for hypothetical proteins and unknown function (50 genes), the *vanB* operon genes and genes with assigned roles in cell envelope (17 genes, of which 4 code for PBPs), energy or central intermediary metabolism (13 genes), regulatory functions (14 genes, among which we found three two-component systems, namely TCS05, TCS06 and TCS15), transport and binding (10 genes) and also some involved in DNA metabolism (3 genes), amino-acid, nucleotide, cofactor, prosthetic groups and carrier biosynthesis (9 genes) and protein fate (6

genes). Eight genes were found to be upregulated at one time point and downregulated at the other and encode proteins putatively enrolled in cell envelope (2 genes), nucleotide metabolism (1 gene), hypothetical proteins (1 gene) and signal transduction/transport and binding (4 genes).

INDEPENDENT ANALYSIS OF GENE EXPRESSION BY RT-PCR

Semi-quantitative RT-PCR was performed for 19 genes (Table 1) to confirm results obtained with the microarray experiments. The choice of the genes was made to include vancomycin resistance genes, genes from different functional categories and different levels of expression. The transcriptional profiles from semi-quantitative RT-PCR and microarray analysis correlate well.

DISCUSSION

E. faecalis V583 was the first vancomycin-resistant clinical isolate reported in the United States (Sahm *et al.*, 1989) and until recently it was the only *Enterococcus* isolate which genome sequence has been completed. Recently the sequence of 28 more enterococcal genomes, 16 of which are *E. faecalis*, became available (Palmer *et al.*, 2010).

Scarce information is available concerning gene expression in enterococci. The few transcriptomic studies have dealt with responses of *E. faecalis* to erythromycin (Aakra *et al.*, 2005), chloramphenicol (Aakra *et al.*, 2010), SDS and bovine bile salts (Solheim *et al.*, 2007), growth in blood (Vebo *et al.*, 2009) and in urine (Vebo *et al.*, 2010) and exposure to copper (Reyes-Jara *et al.*, 2010). The harsh nature of this bacterium and its relevance as a nosocomial agent clearly evidences that *E. faecalis* possesses important mechanisms for dealing with both biotic and abiotic stresses. The establishment of transcriptional profiles of *Enterococcus* under stress conditions is fundamental to understand and define the complex pathways triggered under stress. This may be a useful tool to identify, for instance, new targets for the development of alternative strategies to prevent or irradicate enterococcal severe infections.

As mentioned above, SI antibiotic concentrations are in fact the real concentrations that most bacteria face after and even during antibiotic treatment. Therefore it is extremely relevant to understand the extent of changes induced by SI antibiotic concentrations, especially in *E. faecalis*, a bacterium intrinsically resistant to most antibiotics used in therapy and tolerant to vancomycin. Vancomycin is a last resort antibiotic on the edge of becoming clinically obsolete for treatment of enterococcal and other clinically relevant Gram-positive bacteria.

Vancomycin induced stress is relevant also because it is a cell-wall active antibiotic and the cell-wall is in fact the great sensory organ of the bacterial cell. Somehow, understanding the cell envelope stress response (CESR) imposed by vancomycin may shed light into the process of how *E. faecalis* senses the outside environment. Some work as been done on CESR using transcriptomics and SI or sublethal antibiotic concentrations in other Gram-positive bacteria (Jordan *et al*., 2008). However, comparisons are hard to draw from the published results. Therefore, we designed this study in order to allow three different comparisons: we compared the same strain but with different vancomycin susceptibilities (namely the Δ*vanB* mutant, which is Van^S, and the Van^R strain V583) and using different concentrations adjusted to the same concentration relative to the MIC (we used approximately 2.5% MIC, which corresponds to 0.3 µg/ml and 10 µg/ml, respectively). Additionally, we compared the same strain when challenged with different vancomycin concentrations (for the Van^R strains, we used two sublethal concentrations, namely 0.3 µg/ml and 10 µg/ml), and we looked for both the initial and transient stress responses (after 10 minutes) and the long term stress response (after 30 minutes) using a therapeutic dose.

GLOBAL ADAPTATION OF THE V583 TRANSCRIPTOME REVEALS CHANGES COMPRISING MOST FUNCTIONAL GENE CATEGORIES

Vancomycin was able to induce the *van* genes at both concentrations and in both Van^S and Van^R strains. However the level of induction was related with the absolute vancomycin concentration, and not to the concentration relative to MIC, since both Van^R and Van^S strains responded similarly to 0.3 µg/ml of the antibiotic. Moreover, at this low vancomycin concentration, only the *van* genes were differentially transcribed, thus implying that this low dose does not impose

any stress response on the cell, nor shows any of the overall transcriptomic changes precluded from the hormesis hypothesis (Yim *et al.*, 2006). If one assumes that the hormesis hypothesis is true, then we can say that what induced, in fact, the overall cell response seen in V583 with 10 µg/ml was not just vancomycin per se, but also the extensive changes it induces in the cell wall of a resistant strain, i. e. the large substitution of the D-Ala-D-Ala precursors by D-Ala-D-Lac. This substitution is likely small when the Van^R cells are challenged with 0.3 µg/ml, thus not inducing any other response in the cell. Those changes may alter in turn the substrates of many enzymes and thus induce a series of chain reactions leading to a stress response by the cell. This stress response may be responsible for the growth arrest seen in V583 after the 30 min challenge with the vancomycin therapeutic dose.

STRESS RESPONSE OF V583 CAUSED BY VANCOMYCIN EXPOSURE

Recent efforts to unravel the mechanisms involved in antibiotic mediated cell killing or growth arrest have revealed two common mechanisms: oxidative stress response and SOS DNA stress response (Dwyer *et al.*, 2009). When we challenged V583 with the vancomycin therapeutic dose, genes belonging to both stress mechanisms were found to be differentially regulated. We found *katA* (EF1597) gene to be upregulated under vancomycin exposure. This gene encodes a heme-containing catalase. The physiological role of catalase is to remove the hydrogen peroxide formed under oxic conditions, which is toxic to cells. The *E. faecalis katA* gene is expressed when the cells are grown under aerobic conditions (Shepard & Gilmore, 1999). Although the gut is mainly an anoxic environment, enterococci are frequently in oxic environments, such as live tissues during colonization. Under these conditions, enterococci may produce

large amounts of superoxide and hydrogen peroxide. It is also possible that *E. faecalis* respiratory chain, which is active in the presence of oxygen and heme, produces hydrogen peroxide. Production of hydrogen peroxide has been suggested to be an important factor in virulence and bacterial competition (Frankenberg *et al.*, 2002). *E. faecalis* cells can also encounter oxidative stress mediated by exogenous superoxide and hydrogen peroxide produced by host defense cells, like neutrophils. Conceivably, both bacterial hydrogen peroxide production and the expression of catalase are virulence factors. Catalase production, as well as the ability to use externally supplied heme, has been implicated in virulence in several pathogenic microorganisms, e.g., *Staphylococcus aureus* (Clements & Foster, 1999), *Helicobacter pylori* (McGee & Mobley, 1999), and *Candida albicans* (Wysong *et al.*, 1998). Possibly, heme acquisition and catalase production by *E. faecalis* can be similarly incriminated as virulence determinants (Frankenberg *et al.*, 2002). Of the 17 genes predicted in V583 to play a role in oxidative stress response (Riboulet *et al.*, 2007) only *katA* was found, in our study, to be upregulated in presence of vancomycin. In a recent study by Bizzini *et al*. (2009) concerning vancomycin tolerance, superoxide dismutase was found to be essential for *E. faecalis* tolerance to vancomycin (Bizzini *et al.*, 2009). In this study JH2-2, which is a vancomycin susceptible strain, was challenged with a vancomycin concentration 20 times its MIC value. We thus cannot compare the two studies, since we used a resistant strain and a SI vancomycin concentration. However, our results suggest, at least, the induction of catalase, which means that peroxide mediated stress was induced by vancomycin under the tested conditions.

Concerning the SOS DNA stress response, genes involved in these pathways were also upregulated in the presence of a vancomycin therapeutic

dose. All together, the induction of these genes suggests a strong mutagenic pressure induced by vancomycin. One of these genes, EF1587, codes for MutT, a Nudix family protein. MutT hydrolyses 8-oxo-dGTP to 8-oxo-dGMP, an event that can prevent the misincorporation of 8-oxoguanine opposite adenine in DNA. 8-oxoguanine (8-oxoG), a damaged form of guanine (G) generated by reactive oxygen species, is known to have highly mutagenic potency because of its mispairing with adenine. Somehow, the induction of EF1587 suggests the involvement of oxygen reactive species in the intracellular cascade of events induced by vancomycin challenge.

When cells are exposed to some environmental stress, there is a rapid increase in synthesis of a group of evolutionary conserved proteins known as heat-shock proteins (HSPs), or stress proteins, consisting mainly of chaperones and proteases that are involved in protein (re)folding and protein degradation (Laport *et al.*, 2004). We observed two HSPs upregulated under vancomycin exposure, EF1646 (*hslU*) and EF1647 (*hslV*), which are related to adaptation to atypical conditions and to protein folding and stabilization, respectively. Clp-HSP100 ATPases are a widespread family of ubiquitous proteins that occur in both prokaryotes and eukaryotes and play important roles in the folding of newly synthesized proteins and refolding of aggregated proteins. We found it upregulated under vancomycin exposure (EF2355). This family is considered a class of stress proteins and it has been shown to participate in the virulence of several pathogens (Chastanet *et al.*, 2004).

Several authors hypothesize that *gidA* (EF3311) could be an important regulatory molecule, controlling virulence-associated factors of enteric pathogens

(Kinscherf & Willis, 2002; Sha *et al.*, 2004) and there is some evidence that g*idA* mutants are avirulent in mice (Sha *et al.*, 2004). We found this gene to be downregulated by vancomycin at t_{30}, with an expression 2.1-fold decreased.

EXPOSURE TO VANCOMYCIN AFFECTS THE EXPRESSION OF REGULATORY GENES AND TCSs

Regulatory genes constitute one of the most important groups of induced genes under vancomycin treatment, which indicates that the response to vancomycin is a highly regulated process and is triggered early in the stress response.

Two-component systems are employed by bacteria to sense and respond to environmental stimuli and typically consist of a membrane-bound histidine protein kinase and a cytoplasmic effector termed response regulator (Stock *et al.*, 2000). The available genome sequence for *E. faecalis* V583 allowed the identification of 17 TCSs Ehk-Err (enterococcal histidine kinase – enterococcal response regulator) and an orphan RR (Hancock & Perego, 2002). Studies on enterococcal TCSs have been performed with *E. faecalis* JH2-2, OG1RF and V583 (Teng *et al.*, 2002; Le Breton *et al.*, 2003; Hancock & Perego, 2004). These studies have implicated some of the enterococcal TCSs in virulence, biofilm formation, antibiotic resistance and stress response (Hancock & Perego, 2002; Le Breton *et al.*, 2003).

The best characterized TCSs in *Enterococcus* are the VanRS and $VanR_BS_B$ systems Err11-Ehk11), which regulate VanA and VanB glycopeptide resistance, respectively (Arthur *et al.*, 1992). Until very recently this TCS have not been associated to response to other antibiotics or different stresses, suggesting that

they are highly specific to vancomycin response. However, the recent work of Aakra *et al*. (2010) states that $vanR_BS_B$ is induced under exposure to chloramphenicol (Aakra *et al.*, 2010). Also, the fact that a *vanRS* homolog was found in a vancomycin susceptible strain (OG1RF) (Bourgogne *et al.*, 2006), suggests that VanRS systems might have a broader role than exclusively vancomycin resistance.

The histidine kinase EF3290 (*croS*) and its cognate response regulator EF3289 (*croR*) were both upregulated at t_{10} and t_{30}. This TCS designated as *croRS* (ceftriaxone resistance), is known as Err05-Ehk05 by Hancock and Perego (2004). A mutant in *rr05* in V583 background showed increased susceptibility to vancomycin (Hancock & Perego, 2002) and we determined that the vancomycin MIC for the same mutant is 32 µg/ml, suggesting a role in vancomycin resistance and/or in response to vancomycin stress. A *croR* mutant also shows increased susceptibility to bacitracin and to second- and third-generation cephalosporins (Hancock & Perego, 2004). Considering the present data together with Hancock and Perego observations, it seems that *croR* is involved in resistance to a wide range of cell wall-active agents, indicating a role in regulating cell-wall synthesis or stress. Le Breton *et al*. reported that *croR* mutation in *E. faecalis* JH2-2 led to significant growth defects and cell morphology alterations (Le Breton *et al.*, 2003). The observed defects were ascribed to a deregulation of *sagA* (renamed *salB*), as a similar phenotype was observed for a *sagA* mutant (Breton *et al.*, 2002). However, other authors did not observed any growth defect when analysing a *croR* mutant, but in *E. faecalis* V583 strain (Comenge *et al.*, 2003; Hancock & Perego, 2004). The role of croRS remains to be clarified and might be strain specific. We found *salB* (EF0394) downregulated at t_{30} under vancomycin

exposure. *SalB* expression has been described dependent on CroR, supporting the thesis of a direct involvement of the CroRS two-component system in the activation of *salB* under environmental stresses such as high osmolarity and temperature (Muller *et al.*, 2006). The fact that we found *salB* with reduced expression (-2.5-fold change at t_{30}) might indicate that either *salB* responds differentially under a different type of stress (e. g. antibiotic exposure) or that depending on the stress type *salB* might be under regulation of a negative regulator, instead of *croR*. *SalB* was also found to be downregulated during growth in blood (Vebo *et al.*, 2009). We also found differentially expressed other genes previously described as being under croR regulation, such as EF0443 (coding for a surface protein of unknown function), EF3245 (coding for a cell envelope associated acid phosphatase, unknown specificity enzyme), EF0746 (coding for a putative penicillin binding protein) and EF2222 (coding for an ABC transporter) (Muller *et al.*, 2006). Interestingly, these genes were described as negatively regulated by CroR, however under vancomycin exposure they are all induced, once again indicating that stress response is strongly dependent on the stress-type imposed to the cells or is strain-dependent.

We detected the upregulation of other response regulator (*rr06*) at both t_{10} and t_{30} but its cognate histidine kinase was only detected at t_{30}. The TCS Err06-Ehk06 is homologous to the *B. subtilis* YclJ-YclK TCS, which is activated under oxygen limitation (Kobayashi *et al.*, 2001; Hartig *et al.*, 2004). In *E. faecalis* JH2-2 this TCS has also been implied in the response to oxidative stress and in the expression of the catalase gene (*kat*) (Muller *et al.*, 2008), which was found to be induced as previously mentioned on this discussion but only at t_{30}, as observed for *ehk06*, confirming that *katA* expression is dependent on TCS06 in V583 and stress. Mutants in *E. faecalis err06* were shown to be more susceptible than the

wild-type to heat and SDS (Hancock and Perego, 2004) and more sensitive in macrophages (Muller *et al.*, 2008). The vancomycin induction of TCS06 may be related with the oxidative stress by H_2O_2 production in prolonged stress.

The EF1820 (*fsrC*) which is the histidine kinase from TCS Err15-Ehk15 was downregulated in response to vancomycin treatment. The operon fsr/gelE/sprE regulated by this TCS is described as a virulence operon. *fsr* and *gelE-sprE* are more than virulence factors in *E. faecalis*. Recently, they have been assigned a role in autolysis activation and regulation by modulating the activity of cell wall murein hydrolases (Thomas *et al.*, 2008). These enzymes are crucial for the correct growth of bacteria by allowing the cell-wall turnover. It is thus not surprising that a cell-wall active antibiotic, such as vancomycin, can substantiate changes in the expression of *fsr* and *gelE-sprE* operons.

There are some data indicating that this operon is under the regulation of *codY*, which interacts with *gelE/sprE* promoter (Pascal Serror, personal communication). In this study *codY* was found to be upregulated, and as it is a negative regulator, genes under its regulation are repressed. Assuming that *codY* negatively regulated the *fsr* system, it does make sense that *fsrC* (and all the remaining detected genes of the operon) are downregulated.

codY (EF1645) is described as a pleiotrophic regulator at it was first identified in *B. subtilis* (Slack *et al.*, 1995; Serror & Sonenshein, 1996) as a repressor of several gene categories namely extracellular degradative enzymes, transport systems, catabolic pathways, genetic competence, antibiotic synthesis, flagellin, early esporulation functions and virulence. We found that *codY* is upregulated under vancomycin exposure (2.2-fold at t_{10}/2.6-fold at t_{30}). In our study, we actually detected downregulation of some genes from these categories, namely genes involved in virulence (*fsr* and *cps* operons, *gelE*, *salB*), degradative

enzymes (*sprE*), many genes involved in transport (ABC transporters and PTS systems) (Figure 2). This study associates *codY* induction to vancomycin stress for the first time, suggesting that this regulator can also play roles in CESR.

In *B. subtilis* codY is part of a 4-cistronic operon (*codVWXY*) (Slack *et al.,* 1995*)* as well as in *S. aureus* (*xerC-clpQ-clpY-codY*) (Frees *et al.*, 2005). We believe that in *E. faecalis* V583 *codY* is also transcribed as part of an operon – EF1648-EF1647 (*hslV*)-EF1646 (*hslU*)-*codY*. All the putative *codY* operon genes are induced under vancomycin exposure at similar fold change rates. EF1647 (*hslV*) is homologous to *B. subtilis codW* and *S. aureus clpQ* and EF1646 (*hslU*) is homologous to *B. subtilis codX* and *S. aureus clpY.*

TCS03 and TCS08, homologous to vraRS of *S. aureus* and *vncRS* of *S. pneumoniae*, respectively, were not differentially regulated by vancomycin in *E. faecalis* V583. It is thus highly possible that those TCSs do not play the same roles in the three species.

We have observed between 16 regulator genes (t_{10}) to 26 regulator genes (t_{30}) being differentially expressed under vancomycin exposure, which indicates a complex network of mechanisms involved in glycopeptide resistance/response.

psr regulator (EF1569) is the PBP5 synthesis repressor of *E. faecium, E. faecalis* and *E. hirae* and was found upregulated in response to vancomycin exposure. It shares high sequence identity and similarity to *S. aureus msrR* and *B. subtilis lytR* and they all belong to the LytR-CpsA-Psr family of cell envelope-related transcriptional regulators. *msrR* is strongly expressed in glycopeptide resistant strains of *S. aureus* and its expression is induced by cell wall related antibiotics including vancomycin and oxacillin (Rossi *et al.*, 2003; Utaida *et al.*, 2003; Cui *et al.*, 2005). We detected the upregulation of *pbp4* (EF2476,

equivalent to *pbp4* of *E. faecium* and *E. hirae*), *pbp2a* (EF0680) and EF0746, a putative penicillin-binding protein. Opposed to what is described for *E. faecium* and *E. hirae* (Ligozzi *et al.*, 1993; Massidda *et al.*, 1998), *E. faecalis* JH2-2 *pbp4* regulation seems to be *psr*-independent (Duez *et al.*, 2001). There are evidences that *E. hirae psr* might be involved in the regulation of autolysis and of other cell surface-related properties or functions and not directly on the overproduction of PBP5 or on the levels of resistance to penicillin G (Massidda *et al.*, 1996).

Four out of the seven transcriptional regulators with LysR domains annotated on V583 genome were highly induced by vancomycin, namely EF1302 (6.4-fold at t_{10}/10.1-fold at t_{30}) and EF1303 (7.2-fold at t_{10}/13.4-fold at t_{30}) which are co-transcribed, according to Biocyc database prediction, EF1815 (3.3-fold at t_{10}/9.2-fold at t_{30}) and EF2703 (11.6-fold at t_{30}), indicating a relevant role of LysR-type regulators on vancomycin stress response.

EXPOSURE TO VANCOMYCIN ALTERS THE EXPRESSION LEVELS OF GENES INVOLVED IN CELL-WALL ASSEMBLY

As far as the genes involved in cell envelope are concerned, we observed that *dlt* operon (*dltABCDX*, genes EF2746-2750) is upregulated under vancomycin exposure. This operon is responsible for D-alanylation of the teichoic acids (Peschel *et al.*, 1999; Lai *et al.*, 2007; Li *et al.*, 2007a). The gene EF2749 is not annotated in the V583 genome, although it shares 40% identity with the homologous gene *dltA* of *Staphylococcus epidermidis* RP62A. In this species the *dlt* operon together with *mprF* gene (also known as *fmtC*), which is responsible for the incorporation of lysyl-phosphatidylglycerol in the cytoplasmic membrane, and a putative transporter system of unknown function that has homology to the

Staphylococcus aureus vraF and *vraG* genes, have been proven to control resistance to cationic antimicrobial peptides and were found to be under aps (antimicrobial peptide sensor) control in *S. epidermidis* (Li *et al.*, 2007b).

Li *et al.* (2007) also identified a similar peptide-sensing system in *S. aureus* though this system seems to have significant interspecies differences (Li *et al.*, 2007a). *mprF* has no similarity with genes of known function, but related genes were identified in the genomes of several pathogens including *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, and *E. faecalis* (Peschel *et al.*, 2001).

In the genome of V583 there is a homologue to *S. aureus vraF* sharing 49% of identity (EF2752, not detected in this study), but there is no homologue to *vraG*. The aps system is homologous to the two component system *vraRS* previously described (Kuroda *et al.*, 2000) and apparently important for vancomycin resistance.

Peschel *et al.* (2000) showed that structural changes of teichoic acids in the cell envelope have a considerable influence on the susceptibility to vancomycin and other glycopeptides. *S. aureus* cells lacking D-alanine esters in teichoic acids exhibited at least three fold-increased sensitivity to glycopeptide antibiotics (Peschel *et al.*, 2000).

Since glycopeptide antibiotics bind to D-alanyl-D-alanine dipeptides, it is tempting to speculate that in order to resist to vancomycin exposure *E. faecalis* cells upregulate *dlt* operon likely as parallel resistance mechanism, probably common amongst enterococci. The teichoic acid D-alanine esters incorporated by *dlt* operon contain positively charged amino groups while the terminal D-alanine residues of murein peptides contain negatively charged carboxyl groups. This may constitute an alternative pathway for the cell wall biosynthesis to avoid the direct binding of vancomycin, a cationic antibiotic, to the D-alanine esters of

teichoic acids. *dlt* operon together with *oatA* gene (EF0783) contributes partially to lysozyme resistance in *E. faecalis* JH2-2, by altering the cell envelope net charge and in the case of *oatA* through the modification of different sites of the PG structure (Le Jeune *et al.*, 2010). *oatA* gene is upregulated under vancomycin exposure in *E. faecalis* V583.

Interestingly, also the capsule operon (*cps* operon, EF2485-EF2492) is downregulated both under vancomycin exposure and when cells are grown in blood (Vebo *et al.*, 2009). We could assume that the capsule operon genes are downregulated in order to let available the substrates necessary for cell wall synthesis (Figure 4).

Several other genes involved in cell wall synthesis and modification respond to vancomycin. GlmS activity has a critical role in the initiation of PG synthesis. In the Embden-Meyerhof pathway, this enzyme converts fructose 6-phosphate into glucosamine 6-phosphate, from which murein monomer precursor is synthesized. We observed that the *glmS* gene (EF2151) was downregulated under vancomycin exposure, suggesting that cell wall precursor synthesis is shifted towards the glycolysis pathway (Figure 4).

It has also been found that just a few specific cell wall synthesis enzymes are upregulated, namely *nagB* (EF0466), *nagA* (EF3044), *pbp2B* (EF2857), *pbp2a* (EF0680), *pbp4* (EF2476), EF0746 (penicillin binding protein, putative), *murB* (EF2733), *murM* (EF2658) and EF2585, a Mur ligase family protein. The MurB enzyme catalyzes the synthesis of UDP-N-acetylmuramic acid (UDP-MurNAc) from EP-UNAG (enolpyruvyl-UDP-*N*-acetylglucosamine). The absence of a homologue in eukaryotic cells makes *murB* an attractive target for small molecule inhibitors with the potential to have broad antibacterial activity (Yang *et al.*, 2006). However, from the metabolic scheme presented on Figure 4, it seems that the cell

alters its metabolism in a way that favours the cell wall synthesis through the induction or repression of different metabolic pathways.

Three proteins involved in cell division, belonging to the FtsW/RodA/SpoVE family (EF1300, EF1301, EF2502), were differentially induced under vancomycin exposure, as EF2502 is induced at both time points and the other two are only activated after 30 min of exposure to vancomycin. The enterococcal divisome is not well established but from what is known from *Bacillus* sp. and E. *coli*, FtsW family proteins are likely involved in the translocation of the lipid-linked precursor for PG synthesis from the inside to the outside of the membrane and delivery to its cognate PG-synthesizing complex. Also, these proteins are frequently close to the gene for a class B PBP (Errington *et al.*, 2003) but its activity has to be concerted with at least one class A PBP. FtsK is a large protein with several distinct roles in the late stages of cell division since is required to complete final closure of the septum (Errington *et al*., 2003). EF2533 belongs to the FtsK/SpoIIIE family and is downregulated only after 30 min of exposure to vancomycin. As V583 growth slows down and enters stationary phase with vancomycin exposure, it is correct to speculate that this gene is repressed while cell division process is ongoing and it might be induced later when the division process gets on the later stages. Despite the lack of knowledge on the activity of enterococcal Fts proteins our results evidence that cell metabolism shifts towards the synthesis of peptidoglycan and thus to the cell wall synthesis and assembly.

The cell shape determining complex MreBCD also belongs to this morphogenic network and is ubiquitous in rod-shaped bacteria. MreB is a bacterial actin that forms dynamic filaments located beneath the cell membrane. MreC and MreD are both membrane-associated proteins. MreC interacts with MreD as well as with several of the high-molecular-weight murein synthases

(PBPs). *mreC* is an essential gene in *B. subtilis* (Lee & Stewart, 2003). Cells lacking sufficient quantities of MreC undergo morphological changes, namely, swelling and twisting of the cells, which is followed by cell lysis. *mreC* and *mreD* may be involved in the regulation of cell-shape-related protease expression. In *E. faecalis* we found that genes *mreC* (EF3062) and *mreD* (EF3061) were induced by vancomycin, and *mreB* is absent in *E. faecalis*. As described in the literature, *mreB* orthologs are present, almost exclusively, in species with cylindrical or helical shaped cells, and are usually absent in species with round-shaped cells (Margolin, 2003).

ADAPTATIVE METABOLIC SHIFT DURING VANCOMYCIN EXPOSURE

Some operons involved in specific metabolic pathways were affected by vancomycin exposure, which is the case of pyrimidine metabolism, mevalonate and citrate pathways. Figure 4 resumes some of the more relevant transcriptional changes observed at the metabolic level.

An overall downregulation of enzymes involved in pyrimidine metabolism was observed after 30 minutes. The pyrimidine biosynthetic genes (*pyr* genes) have been found to constitute a single operon in a number of different gram-positive organisms including *Bacillus subtilis* (Quinn *et al.*, 1991), *Bacillus caldolyticus* (Ghim & Neuhard, 1994), *Enterococcus faecalis* (Li *et al.*, 1995), and *Lactobacillus plantarum* (Elagoz *et al.*, 1996). The operon *pyrRPBCaAaB* and the genes *pyrDII*, *pyrD-2* were downregulated at t_{30}. *pyrD-2*, *pyrE* and *pyrF* genes were upregulated at t_{10}. We found other genes related to pyrimidine pathways scattered on the chromosome of *E. faecalis*, namely *pyrG* coding for a CTP synthase (EF1147) and *pyn* gene that codes for a pyrimidine-nucleoside phosphorylase (EF0173), both of them downregulated (Figure 4). The *de novo*

pyrimidine biosynthetic pathway includes five enzymes that results in the production of UMP. The five enzymes are aspartate transcarbamoylase (EF1719), dihydroorotase (EF1718), dihydroorotate dehydrogenase (EF1714), orotate phosphoribosyltransferase (EF1712) and orotidine 5′-monophosphate decarboxylase (EF1713). This pathway is tightly regulated as it directly influences DNA metabolism, and thus replication. It is possible that aspartate is being directed to L-alanine synthesis for cell-wall through upregulation of L-aspartate beta-decarboxylase (EF1037) and thus does not enter the pyrimidine de novo synthesis pathway. The increased flux to L-alanine can thus provide enough substrate for the upregulated enzymes encoded by EF2658 and EF2150, responsible for L-alanine addition to UDP-MurNAc-pentapeptide, thus allowing an increase of substrates for the upregulated transglycosylase and transpeptidase activities (EF0746, EF0680, EF2476 and EF2857), and thus increase peptidoglycan synthesis.

Isopentenyl diphosphate (IPP) is the building block of all isoprenoids in all forms of life (Doun *et al.*, 2005). Isoprenoids participate in processes as diverse as cell-wall biosynthesis (Reusch, 1984), photosynthetic light harvesting (Johnson & Schroeder, 1996), lipid membrane structure, intracellular signalling and electron transport (Hedl & Rodwell, 2004). Synthesis of IPP can occur either by the mevalonate pathway or by the glyceraldehyde-3-phosphate-pyruvate, or non-mevalonate, pathway (Doun *et al.*, 2005). However, mevalonate pathway is the exclusive route to IPP synthesis in Gram-positive cocci (Wilding *et al.*, 2000). Inhibitors directed against the enzymes of this pathway might serve as antibiotics against these pathogens (Doun *et al.*, 2005), and there are evidences that mevalonate pathway knockout mutants have an attenuated virulence (Wilding *et*

al., 2000). Some of the genes coding for enzymes of the mevalonate pathway were induced in this work, namely EF0902 encoding for a phosphomevalonate kinase, EF0903 (*mvaD*) that codes for a mevalonate diphosphate decarboxylase and EF0904 (*mvk*) coding for a mevalonate kinase (Figure 4). They are only induced at t_{10}, suggesting that they might be involved in an immediate, but transient, response to vancomycin exposure. In a transcriptomic study of the response of *S. aureus* to cell-wall active antibiotics (oxacillin, bacitracin and D-cycloserine), the mevalonate pathway genes were also found to be induced. EF0902 is induced by oxacillin exposure and EF0904 is induced by bacitracin (Utaida *et al.*, 2003).

We have detected the downregulation of several genes coding for proteins involved in the citrate pathway - *citCDEFX* genes (EF3319-EF3322). We also found some genes that code for enzymes involved in glucose metabolism, namely EF1503 (codes for a fructose-1,6-biphosphate, putative), EF2646 (codes for a glycerate kinase, putative) to be downregulated.

As mentioned in this discussion, many genes involved in metabolism have altered expression by the presence of the therapeutic dose of vancomycin. Figure 4 resumes some of those genes and intends to support, together with this discussion, the hypothesis that in order for V583 to express full high-level vancomycin resistance (HLVR), many metabolic genes are activated/deactivated in order to help the *vanB* operon activated genes. We also suggest that there are some key metabolites which concentrations must be tightly regulated in order to achieve HLVR. HLVR appears to rely on increased cell-wall synthesis and modification and we believe that the metabolic fluxes in the cells are reorganized in order to help the cell to perform this. We suggest that acetyl-CoA, a key

metabolite, does not enter the citrate pathway and is thus fluxed into UPP synthesis, used for cell-wall synthesis. Two other key metabolites are D-frutose-6P and GlcN-6P. Frutose-1,6-bophosphatase (EF1503) is highly downregulated at both 10 and 30 minutes after vancomycin addition. Its downregulation will enable D-frutose-6P to enter the glycolysis pathway to provide the cell with enough pyruvate for D-lactate production, by VanH (EF2295), which will be used for cell-wall synthesis by VanB (EF2294). Another enzyme downregulated at both 10 and 30 minutes is GlmS (EF2151). Decreased activity of this enzyme, together with upregulation of *nagA* (EF3044) and *nagB* (EF0466), will direct the flux of GlcN-6P into D-frutose-6P and this in turn will feed glycolysis for pyruvate production and cell-wall synthesis by the *vanB* operon enzymes. *nagA*, *nagB* and *glmS* genes have also been identified as key genes controlling cell-wall synthesis in *S. aureus* and D-frutose-6P and GlcN-6P as key metabolites (Komatsuzawa *et al.*, 2004). Our results also show that, together with the suggested tight regulation of carbon flux for cell-wall synthesis, amino acids also appear relevant in this reorganization of the cell metabolism and some nitrogen containing metabolites seem to have key roles in this process, namely aspartate, alanine, glutamine and glutamate. L-aspartate is fluxed into L-Ala synthesis, by both upregulation of EF1037 and downregulation of EF1719 (*pyrB*). This will likely provide cell-wall synthesis with L-Ala for production of UDP-MurNAc-pentapeptide. Both glutamate and glutamine play key roles in cell-wall synthesis in *S. aureus* (Cui *et al.*, 2000). Glutamate is used for cell-wall pentapeptide synthesis and glutamine serves as the NH_4^+ donor in the amidation reaction of the iso-D-glutamate in the stem pentapetide of the murein monomer precursor and for the reaction catalyzed by GlmS, which converts D-frutose-6P to GlcN-6P. This reaction is likely downregulated in the presence of vancomycin and glutamine synthase (EF2159),

which converts glutamate into glutamine, is downregulated. All together, this suggests that glutamine pool is either reduced under vancomycin exposure or is being directed to glutamate synthesis by EF2560, which in turn is used for cell-wall synthesis. It is also possible that amidation of muropeptides is changed by vancomycin.

VANCOMYCIN EXPOSURE AFFECTS TRANSPORTER GENES

Transport and binding proteins is another category of genes with major differences in expression. Several genes encoding transport and binding proteins are highly induced, namely EF1053 (10.2-fold), EF1054 (16.8 fold) both encoding for an ABC transporter, EF1304 encoding for a magnesium-translocating P-type ATPase (15.7-fold) and EF1814 encoding a drug resistance transporter (15.3 fold). Some of the transport genes were downregulated such as EF0082 (a major facilitator ABC transporter), EF0760 and EF2649 (amino acid transporters). Interestingly, with exception for EF2649, all these transporter genes are only induced or repressed at t_{30} revealing that their induction is not a first line response to vancomycin exposure. Many of the transporters differentially expressed in this study are ABC transporters, which belong to MDR (multidrug resistance) superfamily of transporters (Lubelski *et al.*, 2007). ABC transporters are membrane proteins responsible for the uptake and secretion of a wide range of substrates, from ions and small molecules such as amino acids, sugars, xenobiotics and vitamins up to polymers such as peptides, proteins, and polysaccharides. Due to their wide substrate range, ABC transporters have been implicated in a range of cellular processes such as nutrition uptake, xenobiotic protection, extrusion of cellular waste products, bacterial immunity and virulence, osmotic stress, lipid transport, and export of macromolecules during biogenesis,

differentiation and pathogenesis (Lubelski *et al.*, 2007). Bioinformatic analysis of *E. faecalis* genome revealed the presence of 34 putative MDR transporters, 23 of which belong to the ABC transporters family. Systematic gene inactivation studies in *E. faecalis* confirmed the involvement of ABC-type MDR transporters in drug resistance (Lubelski *et al.*, 2007). We found 17 ABC-type transporters differentially transcribed under vancomycin exposure, 7 of which were induced. The role of ABC transporters in vancomycin resistance is however poorly understood.

Genes encoding hypothetical and unknown functions proteins are the most representative groups of differentially expressed genes. In total 180 of these genes were differentially expressed under vancomycin exposure, corresponding to about 41% of the total number of genes found in this study. This finding reveals the limitations of the current understanding of genes involved in major bacterial processes. Many of these genes are located next to genes with homology to proteins with known functions and that were also differentially expressed in this study.

There is no significative overlap between *E. faecalis* transcriptional profile under vancomycin exposure and the other transcriptomic studies involving exposure to stress conditions (Aakra *et al.*, 2005; Solheim *et al.*, 2007; Vebo *et al.*, 2009; Aakra *et al.*, 2010; Vebo *et al.*, 2010). However, *gelE* is the only gene that presents the same behaviour under vancomycin and erythromycin exposure and growth in blood, being downregulated in the three conditions. The only other genes that are common to these three stress conditions, although with different behaviour, are *dltA*, *dltB*, *croR*, *croS*, and EF3245 encoding for a cell envelope

associated acid phosphatase with unknown substrate. It is likely that these genes might have a role in general stress response. The only genes common to vancomycin, erythromycin, bile salts and SDS exposure are EF2049 and EF2050 encoding for an ABC transporter. There are two gene clusters that are differentially expressed under vancomycin and chloramphenicol treatment and exposure to bile salts and SDS, EF1491-EF1500 (V-type ATPase) and EF1712-EF1721 (Pyr). The V-type ATPase gene cluster is induced with vancomycin, chloramphenicol, SDS and bile salts pointing the importance of optimal redox conditions in the treated/stressed cell cultures, as discussed by Solheim *et al*. (2007). The *pyr* operon is downregulated under vancomycin exposure but induced under exposure to chloramphenicol and bovine bile.

Recently a study using a proteomic approach to evaluate the effects of vancomycin exposure was published (Wang *et al.*, 2010). Only six genes were common to both studies which is reasonable since the design of the two experiments differs greatly, including the growth phase at which the cultures are sampled, the vancomycin concentration and the exposure period of time to vancomycin.

We have described here a profile of transcriptional events that occur in V583 cells treated with a therapeutic and with a subinhibitory dose of vancomycin. The response to the subinhibitory dose was restricted to *vanB* operon genes both in V583 and on its isogenic mutant V583Δ*vanB*. However, under exposure to a therapeutic dose the transcriptional profile of V583 presents major changes. In this latter case, the genes that responded to vancomycin presence are not, for sure, all directly related to the resistance mechanism, but should correspond to adaptation to environmental changes. Some of the genes may be in fact relevant

for vancomycin susceptible enterococci tolerance to low vancomycin concentrations.

It is logical to assume that *E. faecalis* vancomycin resistance and/or stress tolerance is a multi-factorial phenomenon, particularly because V583 differentially expressed genes found to have several distinct biological functions. Some of the processes which are induced or repressed are responding to vancomycin and may be general amongst enterococcal isolates, others are induced in order to support the cell in its full expression of resistance by providing the cell with what it needs to keep the *vanB* operon with substrate, other may even be consequences of all the two other categories. This suggests that multiple genes and pathway adjustment may be needed to give rise to the diverse changes, which would then together give rise to vancomycin resistance and/or tolerance (Figure 4).

Vancomycin-induced changes occurred mainly at the level of transcription of genes encoding cell envelope proteins, transporters and regulatory proteins, suggesting that *E. faecalis* respond to vancomycin regulating a panoply of mechanisms by increasing the transcription of several regulators. It became clear with this work that expression of vancomycin resistance expression is the final product of an orchestrated expression and fine-tuning regulation of many genes and major metabolic pathways. The establishment of a vancomycin stimulon would constitute a valuable tool for antibacterial drug development with the recognition of new cellular targets.

In V583, a vancomycin resistant strain, vancomycin stress response has little in common with other stress responses, both in the same and other *E. faecalis* strains, as obtained either by transcriptomic or proteomic approaches. It has also little in common with *S. aureus* vancomycin resistance mechanisms, although

some features of enterococcal vancomycin resistance overlap with previous studies on CESR.

We believe that the fact that we are challenging a vancomycin resistant strain with the inducer of the resistance mechanism can account for the differences mentioned. Moreover, we believe that some of the observed changes in expression are required for the full activity, and thus full expression of resistance phenotype (high-level vancomycin resistance), of the *vanB* operon. In Figure 4 we show some of the key genes involved in metabolic pathways which we propose are crucial for sustaining the expression of high vancomycin resistance in V583. Further work is required to test and establish the role of these metabolic steps which we propose are key in vancomycin resistance expression, but not directly regulated by $vanR_BS_B$ system. Thus it is likely that many regulators and other genes shown to be induced or repressed by vancomycin are in fact helping the *vanB* operon to fully fulfil its role.

This work constitutes a valuable contribution to the knowledge of the transcription events that *E. faecalis* undergoes under stress, and hopefully could be used in future to elucidate the biology of this bacterium.

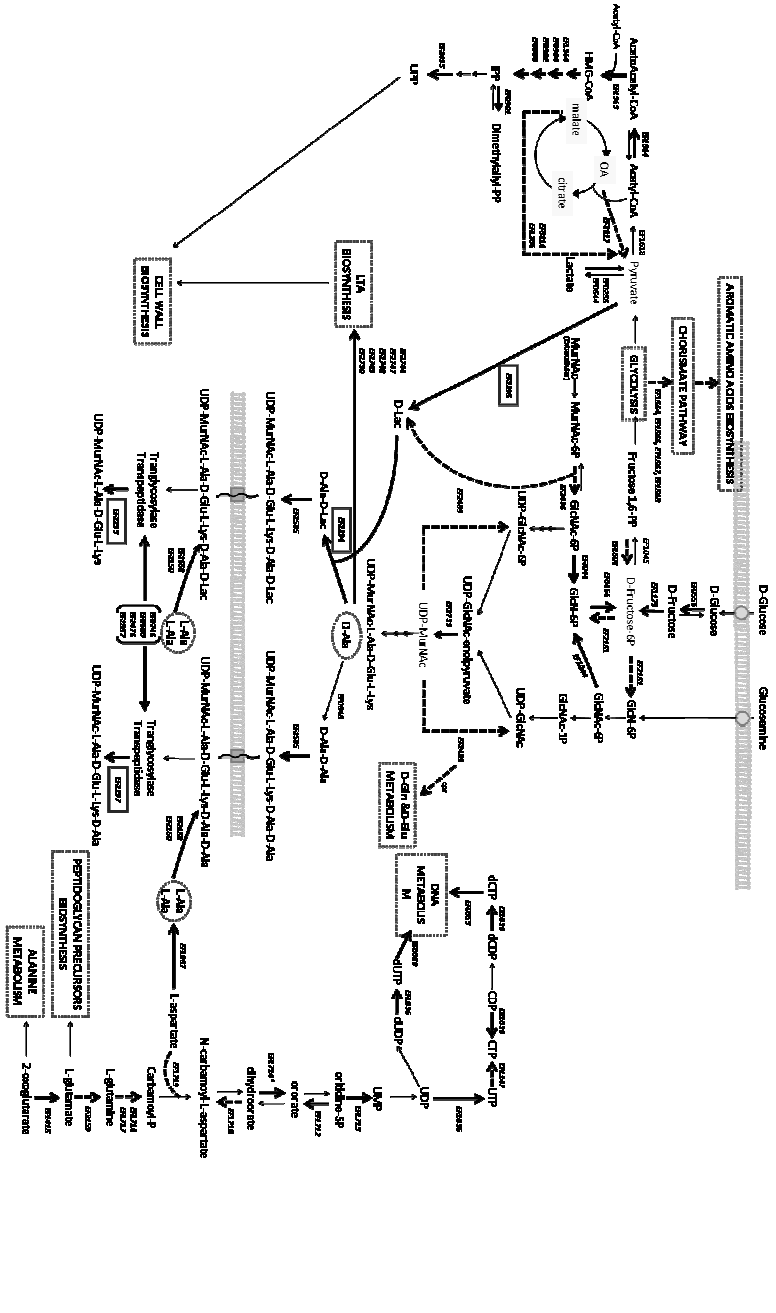


Figure 4. Proposed metabolic pathways with altered transcription for V583 under exposure to 10 µg/ml of vancomycin, as determined by microarray analysis. Induced genes are indicated in solid bold arrows. Repressed genes are indicated by bold dashed arrows. HMG-CoA, 3-hydroxy-3-methyl-glutaryl-CoA; OA, oxaloacetate; GlcN, glucosamine; GlcNAc, N-acetylglucosamine; MurNAc, N-acetylmuramic acid; UPP, undecaprenyl pyrophosphate; IPP, isopentenyl pyrophosphate; LTA, lipotheicoic acid; UTP, uridine triphosphate; UDP, uridine diphosphate; UMP, uridine monophosphate; CTP, cytidine triphosphate; CDP, cytidine diphosphate.

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Supplementary Table S1. Average relative fold change of genes with differential expression profiles under 10 µg/ml vancomycin exposure, sorted by functional category

Locus (a)	Gene	Descriptions	Role (a)	Fold change	
				t_{10}	t_{30}
EF0019	-	PTS system, IIB component	Signal transduction/Transport and binding proteins	-2.5	NDE
EF0020	-	PTS system, mannose-specific IIAB components	Signal transduction/Transport and binding proteins	-2.7	NDE
EF0021	-	PTS system, mannose-specific IIC component	Signal transduction/Transport and binding proteins	-3.0	NDE
EF0022	-	PTS system, mannose-specific IID component	Signal transduction/Transport and binding proteins	-2.7	NDE
EF0025	-	membrane protein, putative	Cell envelope	-2.4	NDE
EF0026	-	conserved hypothetical protein	Hypothetical proteins	3.3	5.5
EF0028	-	PTS system, IIBC components	Signal transduction/Transport and binding proteins	NDE	-2.7
EF0029	-	aminotransferase, class II	Unknown function	NDE	-2.5
EF0032	-	membrane protein, putative	Cell envelope	NDE	2.6
EF0038	*proB*	glutamate 5-kinase	Amino acid biosynthesis	NDE	3.1
EF0043	*gltX*	cytolysin regulator R1	Protein synthesis	NDE	2.7
EF0053	*dnaQ*	DNA polymerase III, epsilon subunit	DNA metabolism	NDE	2.4
EF0054	-	hypothetical protein	Unknown function	-2.3	3.5
EF0062	-	5-nucleotidase family protein	Purines, pyrimidines, nucleosides, and nucleotides	-3.1	-2.8
EF0071	-	lipoprotein, putative	Cell envelope	NDE	3.7
EF0082	-	major facilitator family transporter	Transport and binding proteins	NDE	-6.7
EF0083	-	hypothetical protein	Unknown function	NDE	-4.8
EF0095	-	lipoprotein, putative	Cell envelope	-2.7	4.2
EF0108	-	C4-dicarboxylate transporter, putative	Transport and binding proteins	-2.2	NDE
EF0124	-	hypothetical protein	Unknown function	NDE	-2.2
EF0156	-	conserved hypothetical protein	Hypothetical proteins	NDE	-2.1
EF0157	-	conserved domain protein	Hypothetical proteins	NDE	-2.1
EF0171	*adD*	adenosine deaminase	Purines, pyrimidines, nucleosides, and nucleotides	NDE	2.2
EF0173	*pyn*	pyrimidine-nucleoside phosphorylase	Purines, pyrimidines, nucleosides, and nucleotides	NDE	-2.5
EF0176	-	basic membrane protein family	Cell envelope	NDE	-2.5
EF0177	-	basic membrane protein family	Cell envelope	NDE	-2.1
EF0178	-	ABC transporter, ATP-binding protein	Transport and binding proteins	NDE	-2.5
EF0179	-	ABC transporter, permease	Transport and binding proteins	NDE	-2.8

		protein			
EF0180	-	ABC transporter, permease protein	Transport and binding proteins	NDE	-2.8
EF0248	-	hypothetical protein	Unknown function	-2.0	NDE
EF0256	*pth*	peptidyl-tRNA hydrolase	Protein synthesis	NDE	2.3
EF0257	*mfd*	transcription-repair coupling factor	DNA metabolism	2.0	NDE
EF0266	-	chaperonin, 33 kDa	Protein fate	2.2	NDE
EF0267	-	zinc-binding TIM-barrel protein, nifR3 family, putative	Unknown function	2.5	NDE
EF0292	-	PTS system, IIC component	Signal transduction/Transport and binding proteins	NDE	-3.3
EF0305	-	conserved domain protein	Hypothetical proteins	NDE	-2.1
EF0314	-	hypothetical protein	Unknown function	NDE	-2.3
EF0317	-	transcriptional regulator, Cro/CI family	Regulatory functions/Mobile and extrachromosomal elemen functions	NDE	-2.2
EF0318	-	conserved hypothetical protein	Hypothetical proteins	NDE	-2.1
EF0326	-	conserved hypothetical protein	Hypothetical proteins	NDE	-2.1
EF0327	-	hypothetical protein	Unknown function	NDE	-2.4
EF0329	-	conserved hypothetical protein	Hypothetical proteins	NDE	-2.5
EF0361	-	chitinase, family 2	Cell envelope	NDE	-5.6
EF0365	-	conserved hypothetical protein	Hypothetical proteins	NDE	2.1
EF0366	-	conserved hypothetical protein	Hypothetical proteins	NDE	2.1
EF0367	-	conserved hypothetical protein	Cell envelope	NDE	2.0
EF0369	-	hydrolase, haloacid dehalogenase-like family	Unknown function	NDE	-2.5
EF0376	-	hypothetical protein	Unknown function	NDE	2.2
EF0394	*salB*	secreted antigen, putative	Protein fate	NDE	-2.5
EF0397	-	conserved hypothetical protein	Hypothetical proteins	-2.0	NDE
EF0402	*nhaC-1*	Na+H+ antiporter	Transport and binding proteins	NDE	-2.6
EF0411	-	PTS system, mannitol-specfic IIBC components	Signal transduction/Transport and binding proteins	NDE	-2.4
EF0423	*eda-1*	2-dehydro-3-deoxyphosphogluconate aldolase4-hydroxy-2-oxoglutarate aldolase	Amino acid biosynthesis/Energ metabolism	NDE	-2.7
EF0428	-	conserved hypothetical protein	Hypothetical proteins	NDE	-2.3
EF0429	-	TRAP dicarboxylate transporter, DctP subunit	Transport and binding proteins	NDE	-2.8
EF0432	-	transcriptional regulator, AraC family	Regulatory functions	-2.7	NDE
EF0439	-	immunity protein PlnM, putative	Cellular processes	NDE	2.6
EF0443	-	LysM domain protein	Unknown function	3.5	4.2

EF0455	-	PTS system, IIC component	Signal transduction/Transport and binding proteins	NDE	-6.6
EF0466	*nagB*	glucosamine-6-phosphate isomerase	Central intermediary metabolism	NDE	2.6
EF0468	-	LemA family protein	Unknown function	NDE	5.7
EF0469	-	conserved domain protein	Hypothetical proteins	NDE	6.0
EF0516	-	membrane protein, putative	Cell envelope	-2.4	NDE
EF0539	-	phosphosugar-binding transcriptional regulator, RpiR family	Regulatory functions	3.1	4.0
EF0542	-	conserved hypothetical protein	Hypothetical proteins	NDE	2.2
EF0543	-	membrane protein, putative	Cell envelope	NDE	2.4
EF0554	-	PTS system, IIB component	Signal transduction/Transport and binding proteins	NDE	2.2
EF0556	-	xylose isomerise	Energy metabolism	NDE	2.1
EF0559	-	polysaccharide biosynthesis family protein	Cell envelope	4.6	9.5
EF0562	-	hypothetical protein	Unknown function	-2.6	NDE
EF0574	-	hypothetical protein	Unknown function	NDE	2.0
EF0662	-	conserved hypothetical protein	Hypothetical proteins	NDE	2.9
EF0664	-	hypothetical protein	Unknown function	NDE	-7.1
EF0680	-	penicillin-binding protein 2A	Cell envelope	4.0	5.9
EF0692	-	phosphosugar-binding transcriptional regulator, RpiR family, putative	Regulatory functions	2.4	4.5
EF0708	-	conserved hypothetical protein	Hypothetical proteins	3.6	4.0
EF0711	-	conserved hypothetical protein	Hypothetical proteins	NDE	-2.2
EF0713	-	conserved hypothetical protein	Hypothetical proteins	NDE	-2.9
EF0714	-	hypothetical protein	Unknown function	NDE	-3.1
EF0720	-	voltage-gated chloride channel family protein	Transport and binding proteins	NDE	-2.4
EF0723	-	hypothetical protein	Unknown function	NDE	-2.2
EF0737	-	amidase, putative	Unknown function	NDE	2.3
EF0746	-	penicillin-binding protein, putative	Cell envelope	8.5	13.2
EF0747	-	conserved hypothetical protein	Hypothetical proteins	3.2	4.6
EF0760	-	amino acid ABC transporter, ATP-binding protein	Transport and binding proteins	NDE	-2.3
EF0783	*oatA*	acyltransferase, putative	Unknown function	2.9	3.2
EF0789	-	ABC transporter, ATP-binding permease protein	Transport and binding proteins	NDE	-2.7
EF0790	-	ABC transporter, ATP-binding permease protein	Transport and binding proteins	NDE	-2.3
EF0802	-	hypothetical protein	Unknown function	19.3	26.3

EF0803	-	conserved hypothetical protein	Hypothetical proteins	NDE	-2.1
EF0815	-	PTS system, IIAB components	Signal transduction/Transport and binding proteins	NDE	-2.7
EF0816	-	PTS system, IIC component	Signal transduction/Transport and binding proteins	NDE	-4.4
EF0817	-	PTS system, IID component	Signal transduction/Transport and binding proteins	NDE	-3.0
EF0819	-	conserved hypothetical protein	Hypothetical proteins	4.0	6.6
EF0833	-	conserved hypothetical protein	Hypothetical proteins	NDE	-2.7
EF0844	-	hypothetical protein	Unknown function	-2.5	NDE
EF0860	-	membrane protein, putative	Cell envelope	NDE	-2.4
EF0871	-	cation-transporting ATPase, E1-E2 family	Transport and binding proteins	NDE	-2.8
EF0901	-	isopentenyl diphosphate delta isomerase, putative	Biosynthesis of cofactors, prosthetic groups, and carriers	2.4	NDE
EF0902	-	phosphomevalonate kinase	Biosynthesis of cofactors, prosthetic groups, and carriers/Central intermediary metabolism	2.4	NDE
EF0903	*mvaD*	mevalonate diphosphate decarboxylase	Biosynthesis of cofactors, prosthetic groups, and carriers/Central intermediary metabolism	2.5	NDE
EF0904	*mvk*	mevalonate kinase	Biosynthesis of cofactors, prosthetic groups, and carriers/Central intermediary metabolism	2.5	NDE
EF0932	-	hypothetical protein	Unknown function	3.4	7.6
EF0953	-	hypothetical protein	Unknown function	NDE	-2.1
EF0972	-	DNA repair exonuclease family protein	DNA metabolism	7.6	11.7
EF1006	-	conserved hypothetical protein	Hypothetical proteins	2.9	3.7
EF1012	-	PTS system, IIB component	Signal transduction/Transport and binding proteins	-5.9	5.2
EF1013	-	PTS system, IIC component	Signal transduction/Transport and binding proteins	NDE	-5.6
EF1017	-	PTS system, IIB component	Signal transduction/Transport and binding proteins	-6.3	7.0
EF1018	-	PTS system, IIA component	Signal transduction/Transport and binding proteins	-6.4	5.9
EF1019	-	PTS system, IIC component	Signal transduction/Transport and binding proteins	-3.7	4.5
EF1035	-	lipoprotein, putative	Cell envelope	NDE	1.8
EF1036	-	nucleoside diphosphate kinase	Purines, pyrimidines, nucleosides, and nucleotides	NDE	11.4
EF1037	-	L-aspartate beta-decarboxylase, putative	Amino acid biosynthesis	2.8	3.8

EF1038	-	lipoprotein, putative	Cell envelope	NDE	2.5
EF1039	-	hydrolase, haloacid dehalogenase-like family	Unknown function	2.4	3.2
EF1041	-	xanthineuracil permease family protein	Transport and binding proteins	NDE	-2.1
EF1053	-	ABC transporter, ATP-binding protein	Transport and binding proteins	NDE	10.2
EF1054	-	ABC transporter, permease protein	Transport and binding proteins	NDE	16.8
EF1067	-	hypothetical protein	Unknown function	NDE	3.6
EF1097	-	hypothetical protein	Unknown function	-7.1	-12.5
EF1098	-	conserved hypothetical protein	Hypothetical proteins	-2.0	NDE
EF1102	-	conserved hypothetical protein TIGR01655	Hypothetical proteins	NDE	3.2
EF1103	-	amino acid permease family protein	Transport and binding proteins	NDE	-2.6
EF1128	*sgaB*	phosphotransferase enzyme II, B compnent SgaB	Transport and binding proteins	NDE	4.0
EF1129	-	hexulose-6-phosphate synthase, putative	Energy metabolism	NDE	3.0
EF1130	-	hexulose-6-phosphate isomerase SgbU, putative	Energy metabolism	NDE	5.4
EF1131	*araD*	L-ribulose-5-phosphate 4-epimerase	Energy metabolism	NDE	2.7
EF1132	-	CBS domain protein	Unknown function	NDE	4.7
EF1133	*dapD*	2,3,4,5-tetrahydropyridine-2-carboxylate N-succinyltransferase	Amino acid biosynthesis	2.1	4.3
EF1134	-	peptidase, M20/M25/M40 family	Protein fate	NDE	6.6
EF1139	-	glutamine amidotransferase, class I	Unknown function	NDE	-2.1
EF1147	*pyrG*	CTP synthase	Purines, pyrimidines, nucleosides, and nucleotides	NDE	-2.6
EF1149	*recU*	recombination protein U	DNA metabolism	NDE	2.1
EF1156	-	transcriptional regulator, GntR family	Regulatory functions	NDE	2.1
EF1158	-	N4-(beta-N-acetylglucosaminyl)-L-asparaginase, putative	Energy metabolism	NDE	-2.8
EF1173	-	glycosyl transferase, WecB/TagA/CpsF family	Cell envelope	NDE	-2.3
EF1174	-	hypothetical protein	Unknown function	NDE	-2.8
EF1179	*cscK*	Fructokinase	Energy metabolism	NDE	2.5
EF1192	-	aquaporin Z	Transport and binding proteins	-2.6	NDE
EF1198	-	conserved hypothetical protein	Hypothetical proteins	4.2	6.7
EF1199	-	conserved hypothetical protein	Hypothetical proteins	3.9	5.9

EF1206	-	malate dehydrogenase, decarboxylating	Energy metabolism	NDE	-3.1
EF1212	-	transcriptional regulator	Regulatory functions	2.2	5.1
EF1217	-	lipoprotein, putative	Cell envelope	NDE	-2.5
EF1224	-	transcriptional regulator, Cro/CI family	Regulatory functions	NDE	4.6
EF1228	-	hypothetical protein	Unknown function	NDE	2.1
EF1229	-	conserved hypothetical protein	Hypothetical proteins	NDE	2.1
EF1231	-	conserved hypothetical protein	Hypothetical proteins	29.8	49.3
EF1241	-	hypothetical protein	Unknown function	NDE	-2.0
EF1247	-	conserved hypothetical protein	Hypothetical proteins	NDE	-2.1
EF1248	-	hypothetical protein	Unknown function	NDE	-2.5
EF1258	-	hypothetical protein	Unknown function	2.5	3.1
EF1260	-	DNA-binding response regulator	Regulatory functions/Signal transduction	2.0	2.7
EF1261	-	sensor histidine kinase	Signal transduction	NDE	2.2
EF1262	-	hypothetical protein	Unknown function	3.0	3.2
EF1263	-	hypothetical protein	Unknown function	3.0	3.4
EF1264	-	sulfatase domain protein	Unknown function	NDE	2.2
EF1268	-	cation-transporting ATPase, E1-E2 family	Transport and binding proteins	4.5	6.4
EF1300	-	cell division protein, FtsW/RodA/SpovE family	Cellular processes	NDE	7.6
EF1301	-	cell division protein, FtsW/RodA/SpovE family	Cellular processes	NDE	8.5
EF1302	-	transcriptional regulator, putative	Regulatory functions	6.4	10.1
EF1303	-	transcriptional regulator, LysR family	Regulatory functions	7.2	13.4
EF1304	-	magnesium-translocating P-type ATPase	Transport and binding proteins	3.5	15.7
EF1314	-	aspartate aminotransferase, putative	Amino acid biosynthesis	2.6	3.8
EF1322	-	conserved hypothetical protein	Hypothetical proteins	-2.2	NDE
EF1339	-	conserved hypothetical protein	Hypothetical proteins	2.3	2.4
EF1340	-	pheromone cAM373 precursor lipoprotein	Cell envelope/Cellular processes	2.7	2.5
EF1344	-	sugar ABC transporter, permease protein	Transport and binding proteins	NDE	-3.5
EF1347	-	glycosyl hydrolase, family 13	Energy metabolism	NDE	-2.9
EF1351	-	hypothetical protein	Unknown function	NDE	2.8
EF1352	-	magnesium-translocating P-type ATPase	Transport and binding proteins	NDE	2.6
EF1357	-	transcriptional regulator, AraC family	Regulatory functions	NDE	2.4

EF1358	-	glycerol dehydrogenase, putative	Central intermediary metabolism	NDE	3.4
EF1361	-	dihydroxyacetone kinase family protein	Unknown function	NDE	2.6
EF1363	-	hydroxymethylglutaryl-CoA synthase	Biosynthesis of cofactors, prosthetic groups, and carriers/Central intermediary metabolism	3.0	3.3
EF1364	-	acetyl-CoA acetyltransferase hydroxymethylglutaryl-CoA reductase, degradative	Biosynthesis of cofactors, prosthetic groups, and carriers/Central intermediary metabolism	2.5	3.1
EF1396	-	molybdenum cofactor biosynthesis family protein, putative	Biosynthesis of cofactors, prosthetic groups, and carriers	NDE	-2.6
EF1403	-	conserved hypothetical protein	Hypothetical proteins	NDE	2.1
EF1412	-	conserved domain protein	Hypothetical proteins	NDE	3.7
EF1415	*gdhA*	glutamate dehydrogenase	Amino acid biosynthesis	NDE	2.6
EF1449	-	conserved hypothetical protein	Hypothetical proteins	NDE	2.9
EF1486	*ply-2*	Endolysin	Cell envelope/Mobile and extrachromosomal element functions	NDE	2.1
EF1492	-	V-type ATPase, subunit F	Energy metabolism/Transport and binding proteins	NDE	3.4
EF1493	-	V-type ATPase, subunit I	Energy metabolism/Transport and binding proteins	NDE	3.0
EF1494	-	V-type ATPase, subunit K	Energy metabolism/Transport and binding proteins	NDE	2.9
EF1495	-	V-type ATPase, subunit E	Energy metabolism/Transport and binding proteins	NDE	2.5
EF1496	-	V-type ATPase, subunit C	Energy metabolism/Transport and binding proteins	NDE	2.3
EF1501	-	hypothetical protein	Unknown function	NDE	2.9
EF1502	-	beta-lactamase, putative	Cellular processes	NDE	-4.8
EF1503	-	fructose-1,6-bisphosphatase, putative	Energy metabolism	-4.8	-9.3
EF1518	-	conserved hypothetical protein	Hypothetical proteins	5.1	8.3
EF1533	-	conserved hypothetical protein	Hypothetical proteins	NDE	2.6
EF1534	-	peptidyl-prolyl cis-trans isomerase, cyclophilin-type	Protein fate	2.4	3.0
EF1564	*aroC*	chorismate synthase	Amino acid biosynthesis	NDE	-2.1
EF1566	*aroA*	3-phosphoshikimate 1-carboxyvinyltransferase	Amino acid biosynthesis	NDE	-2.5
EF1567	*aroK*	shikimate kinase	Amino acid biosynthesis	NDE	-2.9
EF1568	-	prephenate dehydratase	Amino acid biosynthesis	NDE	-2.4
EF1569	*psr*	transcriptional regulator, PSR protein	Regulatory functions	2.3	2.4

EF1571	-	hypothetical protein	Unknown function	2.4	4.1
EF1572	-	hypothetical protein	Unknown function	2.6	3.6
EF1582	-	major facilitator family transporter	Transport and binding proteins	NDE	-2.9
EF1587	-	MutT/nudix family protein	DNA metabolism	5.4	8.1
EF1589	-	acetyltransferase, GNAT family	Unknown function	NDE	3.2
EF1591	-	transcriptional regulator, AraC family	Regulatory functions	NDE	3.6
EF1597	*katA*	Catalaseperoxidase	Cellular processes	NDE	2.1
EF1599	-	TPR domain transcriptional regulator, Cro/CI family	Regulatory functions	NDE	2.2
EF1622	-	conserved domain protein	Hypothetical proteins	-3.0	NDE
EF1627	*eutC*	ethanolamine ammonia-lyase small subunit	Energy metabolism	NDE	2.4
EF1644	-	lacX protein, putative	Unknown function	2.5	2.8
EF1645	*codY*	transcriptional regulator CodY	Regulatory functions	2.2	2.6
EF1646	*hslU*	heat shock protein HslVU, ATPase subunit HslU	Cellular processes	2.4	3.1
EF1647	*hslV*	heat shock protein HslV	Protein fate	2.5	3.2
EF1648	*xerC*	site-specific recombinase, phage integrase family	DNA metabolism	2.4	3.2
EF1665	-	conserved hypothetical protein	Hypothetical proteins	NDE	2.9
EF1668	-	transcriptional regulator, MarR family	Regulatory functions/Cellular processes	NDE	-2.2
EF1669	-	glyoxylase family protein	Unknown function	NDE	-2.5
EF1672	-	permease protein, putative	Transport and binding proteins	NDE	2.1
EF1673	-	ABC transporter, ATP-binding protein	Transport and binding proteins	NDE	2.6
EF1685	*hlyIII*	hemolysin III	Cellular processes	NDE	4.6
EF1708	-	conserved hypothetical protein	Hypothetical proteins	NDE	2.4
EF1712	*pyre*	orotate phosphoribosyltransferase	Purines, pyrimidines, nucleosides, and nucleotides	2.5	NDE
EF1713	*pyrF*	orotidine 5`-phosphate decarboxylase	Purines, pyrimidines, nucleosides, and nucleotides	2.1	NDE
EF1714	*pyrD-2*	dihydroorotate dehydrogenase	Purines, pyrimidines, nucleosides, and nucleotides	2.1	-3.6
EF1715	*pyrDII*	dihydroorotate dehydrogenase electron transfer subunit	Purines, pyrimidines, nucleosides, and nucleotides	NDE	-5.2
EF1716	*pyraB*	carbamoyl-phosphate synthase, large subunit	Purines, pyrimidines, nucleosides, and nucleotides	NDE	-3.9
EF1717	*pyraA*	carbamoyl-phosphate synthase, small subunit	Purines, pyrimidines, nucleosides, and nucleotides	NDE	-4.3
EF1718	*pyrC*	Dihydroorotase	Purines, pyrimidines, nucleosides, and nucleotides	NDE	-4.8
EF1719	*pyrB*	aspartate carbamoyltransferase	Purines, pyrimidines, nucleosides, and nucleotides	NDE	-4.1

EF1720	-	uracil permease	Transport and binding proteins	NDE	-4.1
EF1721	*pyrR*	pyrimidine operon regulatory protein PyrR	Purines, pyrimidines, nucleosides, and nucleotides/Transcription	NDE	-4.6
EF1727	*ebsa*	ebsA protein	Cellular processes	NDE	3.6
EF1751	-	membrane protein, putative	Cell envelope	NDE	2.7
EF1752	-	conserved hypothetical protein	Hypothetical proteins	NDE	2.6
EF1753	-	conserved hypothetical protein	Hypothetical proteins	2.8	4.7
EF1793	*ilvE*	branched-chain amino acid aminotransferase	Amino acid biosynthesis	NDE	2.2
EF1812	-	hypothetical protein	Unknown function	3.1	8.2
EF1813	-	sulfatase domain protein	Unknown function	4.1	14.1
EF1814	-	drug resistance transporter, EmrB/QacA family protein	Transport and binding proteins/Cellular processes	3.2	15.3
EF1815	-	transcriptional regulator, LysR family, putative	Regulatory functions	3.3	9.2
EF1817	*sprE*	serine proteinase, V8 family	Protein fate	-5.0	-9.6
EF1818	*gelE*	Coccolysin	Cell envelope/Protein fate/Cellular processes	-3.6	-8.0
EF1820	*fsrC*	histidine kinase, putative	Signal transduction	-2.7	-2.9
EF1821	*fsrB*	agrBfs protein	Unknown function	-2.3	-2.9
EF1825	-	conserved domain protein	Hypothetical proteins	NDE	3.4
EF1826	-	alcohol dehydrogenase, zinc-containing	Energy metabolism	NDE	2.2
EF1833	-	hypothetical protein	Unknown function	NDE	4.3
EF1841	-	HD domain protein	Unknown function	2.9	5.4
EF1906	-	conserved hypothetical protein	Hypothetical proteins	NDE	2.8
EF1912	-	ROK family protein	Unknown function	NDE	2.5
EF1947	-	hypothetical protein	Unknown function	NDE	2.4
EF1988	-	hypothetical protein	Unknown function	NDE	4.7
EF2018	-	conserved hypothetical protein	Hypothetical proteins	NDE	2.5
EF2047	-	amino acid permease family protein	Transport and binding proteins	NDE	-2.1
EF2049	-	ABC transporter, permease protein, putative	Transport and binding proteins	NDE	3.2
EF2050	-	ABC transporter, ATP-binding protein	Transport and binding proteins	NDE	4.0
EF2067	-	conserved hypothetical protein TIGR00481	Hypothetical proteins	2.9	3.3
EF2068	-	multidrug resistance protein, putative	Cellular processes/Transport and binding proteins	2.6	NDE
EF2125	-	hypothetical protein	Unknown function	NDE	-2.1
EF2126	-	hypothetical protein	Unknown function	NDE	-2.2
EF2127	-	hypothetical protein	Unknown function	NDE	-2.1

EF2128	-	conserved hypothetical protein	Hypothetical proteins	NDE	-2.0
EF2132	-	recT protein, putative	DNA metabolism/Mobile and extrachromosomal element functions	NDE	-2.1
EF2133	-	hypothetical protein	Unknown function	NDE	-2.4
EF2135	-	hypothetical protein	Unknown function	NDE	-2.9
EF2148	-	hypothetical protein	Unknown function	NDE	2.3
EF2150	-	FemAB family protein	Cell envelope/Cellular processes	NDE	2.2
EF2151	*glmS*	glucosamine-fructose-6-phosphate aminotransferase, isomerizing	Central intermediary metabolism	-3.1	-4.8
EF2152	-	cobalt transport family protein	Transport and binding proteins	NDE	-2.7
EF2153	-	ABC transporter, ATP-binding protein	Transport and binding proteins	-2.0	-2.8
EF2154	-	conserved hypothetical protein	Hypothetical proteins	-2.1	NDE
EF2159	*glnA*	glutamine synthetase, type I	Amino acid biosynthesis	-3.6	NDE
EF2160	*glnR*	regulatory protein GlnR	Regulatory functions	-2.8	NDE
EF2196	-	glycosyl transferase, group 2 family protein	Cell envelope	NDE	2.1
EF2197	*epaB*	glycosyl transferase, group 2 family protein	Cell envelope	NDE	2.3
EF2205	-	conserved hypothetical protein	Hypothetical proteins	NDE	3.3
EF2213	-	PTS system, IIBC components	Signal transduction/Transport and binding proteins	-3.3	3.3
EF2214	-	glyoxylase family protein	Unknown function	3.5	6.9
EF2215	-	conserved hypothetical protein	Hypothetical proteins	3.6	6.3
EF2217	-	alpha-1,2-mannosidase, putative	Cell envelope	NDE	5.3
EF2221	-	ABC transporter, substrate-binding protein	Transport and binding proteins	NDE	2.9
EF2222	-	ABC transporter, permease protein	Transport and binding proteins	NDE	4.9
EF2223	-	ABC transporter, permease protein	Transport and binding proteins	NDE	6.7
EF2236	-	conserved hypothetical protein	Hypothetical proteins	NDE	-2.9
EF2237	-	lipoprotein, putative	Cell envelope	NDE	-5.8
EF2247	-	transcriptional regulator	Regulatory functions	NDE	-2.1
EF2292	-	hypothetical protein	Unknown function	131.5	176.9
EF2293	*vanX*	D-alanyl-D-alanine dipeptidase	Protein fate	129.5	123.9
EF2294	*vanB*	D-alanine-D-lactate ligase	Protein fate	188.0	132.9
EF2295	*vanH*	D-specific alpha-keto acid dehydrogenase	Cell envelope/Cellular processes	203.5	217.8
EF2296	*vanW*	vancomycin B-type resistance protein VanW	Cellular processes/Signal transduction	219.1	241.1

EF2297	*vanY$_B$*	D-alanyl-D-alanine carboxypeptidase	Cell envelope/Cellular processes	145.6	170.5
EF2298	*vanS$_B$*	sensor histidine kinase VanS$_B$	Signal transduction	2.8	3.0
EF2299	*vanR$_B$*	DNA-binding response regulator VanR$_B$	Regulatory functions/Signal transduction	3.0	3.2
EF2303	-	conserved hypothetical protein	Hypothetical proteins	NDE	-2.5
EF2307	-	conserved hypothetical protein	Hypothetical proteins	NDE	-2.3
EF2317	-	hypothetical protein	Unknown function	NDE	-3.0
EF2342	-	hypothetical protein	Unknown function	NDE	2.1
EF2355	*clpB*	ATP-dependent Clp protease, ATP-binding subunit ClpB	Protein fate	NDE	2.1
EF2377	-	amino acid permease family protein	Transport and binding proteins	-2.3	NDE
EF2424	-	pyrroline-5-carboxylate reductase, putative	Amino acid biosynthesis	NDE	2.6
EF2427	-	hypothetical protein	Unknown function	3.0	6.5
EF2428	-	transcriptional regulator, PadR family	Regulatory functions	2.7	6.3
EF2429	*guaC*	GMP reductase	Purines, pyrimidines, nucleosides, and nucleotides	2.7	6.4
EF2431	-	chlorohydrolase family protein	Unknown function	NDE	2.3
EF2432	-	metallo-beta-lactamase superfamily protein	Unknown function	NDE	2.1
EF2433	-	phosphoglycerate mutase family protein	Energy metabolism	NDE	2.4
EF2436	-	glucokinase regulator-related protein	Unknown function	NDE	-2.2
EF2440	-	celC-related protein	Unknown function	-3.3	NDE
EF2441	-	conserved hypothetical protein	Hypothetical proteins	-3.9	NDE
EF2442	-	phosphate transporter family protein	Transport and binding proteins	-2.5	NDE
EF2445	-	2-dehydropantoate 2-reductase, putative	Biosynthesis of cofactors, prosthetic groups, and carriers	2.1	2.1
EF2447	-	DNA internalization-related competence protein ComEC/Rec2	Cellular processes	NDE	5.1
EF2470	-	HD domain protein	Unknown function	3.6	4.0
EF2476	*pbp4*	penicillin-binding protein 4	Cell envelope	2.4	3.2
EF2481	-	hydrolase, haloacid dehalogenase-like family	Unknown function	NDE	2.1
EF2484	-	conserved hypothetical protein	Hypothetical proteins	NDE	-2.5
EF2485	*cpsK*	ABC transporter, permease protein	Transport and binding proteins	NDE	-2.6
EF2486	*cpsJ*	ABC transporter, ATP-binding protein	Transport and binding proteins	NDE	-2.4
EF2487	*cpsI*	UDP-galactopyranose mutase	Cell envelope	NDE	-2.2

EF2488	*cpsH*	lipoprotein, putative	Cell envelope	NDE	-2.5
EF2489	*cpsG*	MurB family protein	Cell envelope	NDE	-2.3
EF2490	*cpsF*	conserved hypothetical protein	Hypothetical proteins	NDE	-2.5
EF2491	*cpsE*	glycosyl transferase, group 2 family protein	Cell envelope	NDE	-2.4
EF2492	*cpsD*	glycosyl transferase, group 2 family protein	Cell envelope	NDE	-2.3
EF2494	*cdsA*	phosphatidate cytidylyltransferase	Fatty acid and phospholipid metabolism	NDE	3.0
EF2495	*uppS*	undecaprenyl diphosphate synthase	Biosynthesis of cofactors, prosthetic groups, and carriers	2.2	3.1
EF2500	-	GcvH family protein	Unknown function	2.1	2.5
EF2502	-	cell division protein, FtsW/RodA/SpoVE family	Cellular processes	2.5	2.2
EF2514	-	hypothetical protein	Unknown function	NDE	-2.3
EF2519	-	conserved hypothetical protein	Hypothetical proteins	-2.4	-2.2
EF2520	-	conserved hypothetical protein	Hypothetical proteins	NDE	-2.6
EF2521	-	conserved hypothetical protein	Hypothetical proteins	NDE	-2.5
EF2524	-	sortase family protein	Cell envelope/Protein fate	NDE	-2.5
EF2532	-	hypothetical protein	Unknown function	NDE	-2.7
EF2533	-	FtsK/SpoIIIE family protein	Cellular processes	NDE	-3.1
EF2539	-	hypothetical protein	Unknown function	-2.2	-3.9
EF2567	*selD*	selenide, water dikinase	Protein synthesis	NDE	-2.6
EF2582	-	chlorohydrolase family protein	Unknown function	NDE	-4.2
EF2585	-	mur ligase family protein	Cell envelope	2.6	2.9
EF2586	-	cobyric acid synthase, putative	Biosynthesis of cofactors, prosthetic groups, and carriers	2.2	2.6
EF2623	*cadA*	cadmium-translocating P-type ATPase	Transport and binding proteins	-3.2	NDE
EF2627	-	teichoic acid glycosylation protein, putative	Cell envelope	2.2	3.1
EF2643	-	conserved hypothetical protein	Hypothetical proteins	2.1	3.4
EF2646	-	glycerate kinase, putative	Energy metabolism	-2.4	NDE
EF2647	-	permease, GntP family	Transport and binding proteins	-3.1	NDE
EF2649	-	spermidineputrescine ABC transporter, spermidineputrescine-binding protein	Transport and binding proteins	-2.3	NDE
EF2650	-	spermidineputrescine ABC transporter, permease protein	Transport and binding proteins	-2.2	NDE
EF2658	-	FemAB family protein	Cell envelope/Cellular processes	2.1	3.1
EF2681	-	hydrolase, haloacid dehalogenase-like family	Unknown function	2.6	NDE
EF2692	-	conserved hypothetical protein	Hypothetical proteins	NDE	2.2

EF2697	-	conserved domain protein	Hypothetical proteins	NDE	3.0
EF2698	-	tellurite resistance protein, putative	Cellular processes	NDE	2.8
EF2702	-	hypothetical protein	Unknown function	NDE	4.0
EF2703	-	transcriptional regulator	Regulatory functions	NDE	11.6
EF2711	-	transcriptional regulator, AraC family	Regulatory functions	NDE	4.3
EF2713	-	cell wall surface anchor family protein	Cell envelope	-2.2	2.0
EF2721	*sdhB-2*	L-serine dehydratase, iron-sulfur-dependent, beta subunit	Energy metabolism	2.4	4.0
EF2722	*sdhA-2*	L-serine dehydratase, iron-sulfur-dependent, alpha subunit	Energy metabolism	2.2	3.6
EF2733	*murB*	UDP-N-acetylenolpyruvoylglucosamine reductase	Cell envelope	2.3	2.9
EF2734	-	oxidoreductase, Gfo/Idh/MocA family	Unknown function	3.2	4.6
EF2746	*dltD*	dltD protein	Cell envelope	NDE	2.5
EF2747	*dltC*	D-alanyl carrier protein	Cell envelope	NDE	2.7
EF2748	*dltB*	basic membrane protein DtlB	Cell envelope	NDE	2.8
EF2749	*dltA*	D-alanine-activating enzyme, putative	Cell envelope	NDE	3.4
EF2750	*dltX*	conserved hypothetical protein	Hypothetical proteins	2.7	6.0
EF2771	-	conserved hypothetical protein	Hypothetical proteins	NDE	2.9
EF2784	-	conserved hypothetical protein	Hypothetical proteins	NDE	2.1
EF2796	-	hypothetical protein	Unknown function	NDE	3.6
EF2828	-	transcriptional regulator, ArpU family	Regulatory functions/Mobile and extrachromosomal elemen functions	NDE	2.6
EF2829	-	hypothetical protein	Unknown function	NDE	2.7
EF2830	-	hypothetical protein	Unknown function	NDE	2.7
EF2831	-	hypothetical protein	Unknown function	NDE	2.9
EF2832	-	conserved domain protein	Hypothetical proteins	NDE	2.7
EF2851	-	conserved hypothetical protein	Hypothetical proteins	-2.8	NDE
EF2857	-	penicillin-binding protein 2B	Cell envelope	2.3	2.8
EF2860	-	ErfK/YbiS/YcfS/YnhG family protein, putative	Unknown function	NDE	2.9
EF2862	-	conserved hypothetical protein	Hypothetical proteins	5.0	7.1
EF2891	-	glycosyl transferase, group 1 family protein	Cell envelope	NDE	2.2
EF2896	-	hypothetical protein	Unknown function	14.2	28.7
EF2899	-	oxidoreductase, pyridine nucleotide-disulfide family	Unknown function	NDE	2.0

EF2922	-	conserved hypothetical protein	Hypothetical proteins	NDE	2.7
EF2929	-	membrane protein, putative	Cell envelope	2.4	2.6
EF2930	-	conserved hypothetical protein	Hypothetical proteins	2.4	NDE
EF2965	-	conserved hypothetical protein	Hypothetical proteins	-2.3	NDE
EF2985	-	permease, putative	Transport and binding proteins	NDE	2.7
EF2986	-	ABC transporter, ATP-binding protein	Transport and binding proteins	NDE	2.9
EF2987	-	conserved hypothetical protein	Hypothetical proteins	NDE	3.3
EF3004	-	sulfate transporter family STAS domain protein	Transport and binding proteins	NDE	-3.9
EF3008	-	conserved hypothetical protein	Hypothetical proteins	-2.2	NDE
EF3023	-	polysaccharide lyase, family 8	Cellular processes	NDE	-4.0
EF3029	-	PTS system, IID component	Signal transduction/Transport and binding proteins	NDE	-3.4
EF3030	-	PTS system, IIC component	Signal transduction/Transport and binding proteins	NDE	-3.6
EF3044	*nagA-2*	N-acetylglucosamine-6-phosphate deacetylase	Central intermediary metabolism	NDE	2.0
EF3052	-	hypothetical protein	Unknown function	NDE	2.4
EF3057	-	hypothetical protein	Unknown function	2.9	5.1
EF3058	-	phosphotyrosine protein phosphatase	Regulatory functions	2.8	4.1
EF3059	-	transcriptional regulator, TetR family	Regulatory functions	2.7	3.8
EF3061	*mreD*	rod shape-determining protein MreD	Cell envelope	2.4	3.1
EF3062	*mreC*	rod shape-determining protein MreC	Cell envelope	2.3	2.5
EF3084	-	iron compound ABC transporter, permease protein	Transport and binding proteins	NDE	-2.1
EF3093	-	conserved hypothetical protein	Hypothetical proteins	NDE	2.0
EF3141	-	D-isomer specific 2-hydroxyacid dehydrogenase family protein	Unknown function	NDE	-8.7
EF3149	-	conserved domain protein	Hypothetical proteins	NDE	2.1
EF3150	-	peptidase, M16 family	Protein fate	NDE	2.4
EF3151	-	conserved hypothetical protein	Hypothetical proteins	2.4	2.5
EF3173	-	hypothetical protein	Unknown function	-2.4	NDE
EF3175	-	rrf2 family protein	Unknown function	NDE	2.3
EF3176	-	membrane protein, putative	Cell envelope	NDE	2.9
EF3189	-	hypothetical protein	Unknown function	NDE	2.5
EF3201	-	OsmC/Ohr family protein	Unknown function	NDE	-2.6
EF3202	*rpsN-3*	ribosomal protein S14	Protein synthesis	2.8	NDE
EF3222	-	hypothetical protein	Unknown function	NDE	2.1

EF3232	-	hypothetical protein	Unknown function	-2.7	NDE
EF3234	-	lipoprotein, putative	Cell envelope	NDE	2.6
EF3235	-	gluconate kinase, putative	Energy metabolism	NDE	3.4
EF3239	-	conserved hypothetical protein	Hypothetical proteins	2.3	3.3
EF3244	-	hypothetical protein	Unknown function	14.0	13.2
EF3245	-	cell-envelope associated acid phosphatase	Unknown function	10.9	10.1
EF3250	-	hypothetical protein	Unknown function	NDE	-2.5
EF3283	*ctsR*	transcriptional regulator CtsR	Regulatory functions/Cellular processes	NDE	2.2
EF3289	-	DNA-binding response regulator	Regulatory functions/Signal transduction	3.4	3.8
EF3290	-	sensor histidine kinase	Signal transduction	3.6	3.8
EF3302	-	hypothetical protein	Unknown function	NDE	3.4
EF3303	-	conserved hypothetical protein	Hypothetical proteins	3.0	3.7
EF3311	*gidA*	glucose-inhibited division protein A	Unknown function	NDE	-2.1
EF3312	*trmE*	tRNA modification GTPase TrmE	Cellular processes/Protein synthesis	NDE	-2.0
EF3313	-	hypothetical protein	Unknown function	NDE	-2.0
EF3316	-	malic enzyme family protein	Unknown function	-2.7	-3.2
EF3317	-	carboxylase, putative	Unknown function	-2.3	-3.6
EF3318	*citX*	apo-citrate lyase pyrophosphoribosyl-dephosph-CoA transferase	Energy metabolism/Protein fate	-2.8	-4.3
EF3319	*citF*	citrate lyase, alpha subunit	Energy metabolism	-2.7	-4.1
EF3320	*citE*	citrate lyase, beta subunit	Energy metabolism	-3.1	-4.9
EF3321	*citD*	citrate lyase, gamma subunit	Energy metabolism	-3.3	-5.5
EF3322	*citC*	citrate lyase ligase	Energy metabolism/Protein fate	-3.0	-5.7
EF3323	-	conserved hypothetical protein	Hypothetical proteins	-3.2	-6.3
EF3324	-	sodium ion-translocating decarboxylase, beta subunit	Transport and binding proteins/Energy metabolism	-3.5	-6.0
EF3325	-	sodium ion-translocating decarboxylase, biotin carboxyl carrier protein	Transport and binding proteins/Energy metabolism	-3.9	-11.8
EF3326	-	conserved hypothetical protein	Hypothetical proteins	-5.1	-12.7
EF3327	-	citrate transporter	Transport and binding proteins	NDE	-15.1
EFA0067	-	PTS system, IIABC components	Signal transduction/Transport and binding proteins	NDE	2.3

[(a)]gene numbers refer to TIGR locus tags and role refers to TIGR annotation; NDE, not significantly differentially expressed. In different grey tones are highlighted the genes differentially expressed at t_{10} and t_{30} from genes only expressed at one of the assayed time points.

Chapter 5

IDENTIFICATION OF A NEW GENE IN *vanB* OPERON OF *Enterococcus faecalis*

Ribeiro, T. C., Santos, S., Lopes, F. "Identification of a new gene in *vanB* operons of *Enterococcus faecalis*", International Journal of Antimicrobial Agents (Submitted for publication).

All the experimental work was carried out by T. Ribeiro with exception of *vanV* mutant construction and its MIC determination that was performed by S. Santos.

CONTENTS

ABSTRACT

We have previously found that EF2292, annotated as a hypothetical protein in the *E. faecalis* V583 genome, was highly induced under vancomycin exposure. In the present work, we provide evidence of its co-transcription with $vanY_BWH_BBX_B$ genes. We also demonstrate that its expression is under the control of $vanR_BS_B$ and propose to name it as *vanV*. This gene was found not to be required for vancomycin resistance and can code for another accessory protein in the *vanB* operon. *vanV* was detected only in some *E. faecalis* carrying the *vanB* operon, suggesting that this operon can have different composition among *E. faecalis* isolates.

INTRODUCTION

Vancomycin was clinically used for the treatment of severe infections caused by gram-positive bacteria for over 30 years before resistance was described (Courvalin, 1990). The first vancomycin resistant strain was isolated in 1986 in France (Leclercq *et al.*, 1988) and since then vancomycin resistance has emerged among enterococcal strains. Resistance emergence together with the previous knowledge that this resistance was, in some cases, transferable (Leclercq *et al.*, 1989; Nicas *et al.*, 1997), led researchers to define the resistance mechanism. In 1990 the *vanA* gene was identified and found to encode for an inducible resistance protein designated by *vanA*, with D-Ala-D-Lac activity (Dutka-Malen *et al.*, 1990). A D,D-carboxypeptidase (later designated as *vanY*) (al-Obeid *et al.*, 1990), that was not required for resistance (Arthur *et al.*, 1992a; Wright *et al.*, 1992), was found to be also induced by vancomycin and was detectable on the enterococcal membrane fractions. Later on, sequencing of another open reading frame adjacent to *vanA* gene on plasmid pIP816 has identified another protein induced by vancomycin, VanH, encoding a dehydrogenase and found to be required for resistance (Arthur *et al.*, 1991).

In 1992, *vanR* was described (Handwerger *et al.*, 1992) and it was proved that the two-component regulatory system *vanR-vanS* activates a promoter and induces co-transcription of *vanH*, *vanA*, and *vanX* (encoding a D,D-dipeptidase) resistance genes (Arthur *et al.*, 1992b; Reynolds *et al.*, 1994). An additional gene was found to be present in *vanA* operon, *vanZ*, confers low-level resistance to teicoplanin by a yet unknown mechanism (Arthur *et al.*, 1995).

The *vanB* phenotype was described shortly after (Arthur & Courvalin, 1993; Evers *et al.*, 1993). *vanB* cluster organization and functionality is similar to *vanA*, differing only in the fact that vancomycin, but not teicoplanin, is an inducer of this

cluster. The *vanB* operon proteins essential for expression of resistance, $VanH_B$, VanB and $VanX_B$, have a high level of sequence identity (67-76% identity) with the corresponding proteins of the *vanA* operon. The $VanR_BS_B$ proteins that encode the two-component system are only distantly related to VanRS (34% and 24% identity) (Evers & Courvalin, 1996).

In both VanA- and VanB-type, resistance is due to synthesis of peptidoglycan precursors ending in the depsipeptide D-Ala-D-Lac instead of the dipeptide D-Ala-D-Ala (Arthur *et al.*, 1996). Both clusters possess the so-called accessory proteins, since they do not contribute to vancomycin resistance, as is the case of VanY and VanZ on the *vanA* cluster and $VanY_B$ and VanW on the *vanB* cluster.

Only in enterococci there are, so far, eight resistance genotypes that involve related enzymatic functions. However, the resistance to glycopeptides is phenotypically and genotypically heterogeneous and can be distinguished based on the range of glycopeptides to which the strains are resistant, the resistance level, transferability, the organization of the corresponding genes and the mode of regulation of gene expression. Major advances in the understanding of the biochemical mechanisms and genetics of vancomycin resistance in enterococci have been achieved. However, the origin of the resistance genes, how they assembled together and the role of accessory proteins remains unclear and is a controversial scientific matter.

In a previous transcriptomic study *E. faecalis* V583, a *vanB*-carrying strain, was exposed to vancomycin and EF2292, located immediately upstream of the $vanX_B$ gene from the *vanB* operon and presented the same levels of induction as the vancomycin resistance genes (Chapter 4). This finding lead us to ask if EF2292 is part of the *vanB* operon and if it has any role in vancomycin resistance.

In the present study the co-transcription of EF2292 with the *vanB* operon genes in *E. faecalis* V583 was evaluated by RT-PCR and Northern blot hybridization. In order to examine the potential role of *ef2292* gene in vancomycin resistance, the gene was deleted and the resultant *ef2292* mutant strain was compared with the wild type by determining MIC to vancomycin.

MATERIALS AND METHODS

BACTERIAL STRAINS AND GROWTH CONDITIONS

This study was performed with *E. faecalis* V583. The strain was grown overnight in Brain Heart Infusion (BHI) medium, at 37°C. Culture was then diluted 20 times and grown in BHI until an optical density of 0.4-0.45 at 600 nm was reached. Culture was split in two and vancomycin (Sigma) was added to one of the cultures to a final concentration of 10 µg/ml. The two cultures (BHI and BHI with vancomycin) were then further incubated, and 5 ml samples of each culture were collected 30 minutes after vancomycin addition. Samples were immediately suspended in RNA Protect solution (Qiagen) and centrifuged for 10 min at 4°C.

RNA EXTRACTION

Total RNA extraction was performed with RNeasy Mini columns (Qiagen). DNA digestion was done using 1 U of RNase-free DNase I (Roche) and incubating reactions at 37ºC for 1 hour. When necessary the DNA digestion was done twice. The RNA was cleaned up from DNase I using the RNA clean-up kit (Qiagen). Prior to PCR reactions DNA contamination of the RNA samples was assessed by a standard (30 cycle) PCR reaction with primers targeting a chromosomal *E. faecalis* gene.

REVERSE TRANSCRIPTASE PCR (RT-PCR)

In order to perform reverse transcriptase PCR, cDNA synthesis was prepared using Transcriptor High Fidelity cDNA Synthesis Sample (Roche) according to the manufacturer's instructions and using 1 µg of RNA as template. PCR reactions were performed using the reverse primer for *ef2292* and the forward primer for

each one of the other *vanB* operon genes, namely, *vanY*$_B$, *vanW*, *vanH*$_B$, *vanB* and *vanX*$_B$. Primers are listed on Table 1.

ANALYSIS OF mRNA BY NORTHERN BLOT

For the Northern blot experiments 20 µg of each RNA sample was used to analyze *ef2292* mRNA on a 1.2% agarose MOPS/formaldehyde gel. RNA was transferred to Hybond-N$^+$ membrane (Amersham Biosciences) by capillarity using 20× SSC as a transfer buffer. RNA was UV cross-linked to the membrane. Different oligoprobes complementary to *ef2292* mRNA, *ef2292-vanX* intergenic region, *vanX* and *vanB* mRNAs were designed. Single-strand DNA probes were labeled in the presence of [γ-^{32}P]ATP (Perkin Elmer). All probes were purified on G50 Microspin columns (GE Healthcare). Hybridizations were carried out at 42°C with the PerfectHyb Plus Hybridization Buffer (Sigma). When appropriate, stripping of the membranes was done following standard protocols and membranes were re-hybridized with a different radiolabeled probe. Following a washing step, signals were visualized on a Phosphorimager STORM 860 (GE Healthcare).

Table 1 – Primers designed for RT-PCR and mutant construction, and oligoprobes designed for Northern-blot hybridization.

Primer or probe*	Sequence (5'→3')
Primers	
RT-PCR	
Forward *vanY*$_B$	TGAATCATCACAAACGGCATA
Forward *vanW*	ATGAACAGAAAAAGATTGACACAGC
Forward *vanH*$_B$	ACTGGGGGATATGACTGTGG
Forward *vanB*	GCA CAT GGA GCG TAA CAA TAT CG
Forward *vanX*$_B$	GGGATGCCAAGTACGCTACA
Mutant construction	
ef2292 internal fragment	An internal fragment was amplified using a forward primer TCTGCTTTATTATGCGGGCG and a reverse primer GAATCAGCAGAGCAAGAACG (expected size of 282 bp)
ef2292 deletion	Upstream fragment was amplified using TTCGGGCTGTGAGGTCGG and CCTTCAACGAAAACCCGAATCAAAGCAGATTCCATCAGCGC (expected size 1131 bp), and the downstream fragment was amplified using GATTCGGGTTTTCGTTGAAGG and TTTGAAGCAGGCGAGTTACC (expected size 1163 bp)
ef2292 deletion confirmation	The primes ACGGAAGAACTAAACGCTGC and CCGAATGTCCTGTTTCAAGC were used to screen the mutant clones and confirm the deleted clones (1651†/1363‡)
Probes	
EF2292	CGCCCGCATAATAAAGCAGATTCCATCAGCGC
IR_EF2292/vanX	CTGATGAAATACTAAGTAATTTGTAGCCAACTTATAC
vanX	GGTTTTCCCGTGAAGTTATCCCATGTAGCGTAC
vanB	CATGTTCCTCCGAGCAACCGCCGAAGATAATTGCGAC

†expected size for the *vanV* wild type sequence; ‡ expected size for the deleted *vanV*; IR, intergenic region; * all the primers and probes used were designed for this study, except the forward primer for *vanB* gene (Dutka-Malen *et al.*, 1995).

MUTANT CONSTRUCTION

The markerless deletion mutant was constructed by double cross-over essentially as described previously (Brinster *et al.*, 2007). Briefly, 5' and 3' flanking regions of the target gene were amplified from chromosomal DNA of V583 by PCR. The two PCR fragments were fused by PCR using the external primers and the resulting PCR fragment was cloned into the T/A cloning vector pGEM-T (Promega). The inserted PCR fragment was excised was subsequently cloned in pG+host9 plasmid (Maguin *et al.*, 1996), which was then electroporated *E. faecalis* V583. The *ef2292* double-crossover mutant was selected as described by Brinster *et al*. (2007). Successful target mutations were identified by PCR screening and confirmed by Southern blot analysis. All primers used are listed on Table 1.

MUTANT PHENOTYPE CHARACTERIZATION

To characterize the mutant phenotype the MIC value for vancomycin was determined by E-test (AB Biodisk) according to manufacturer´s instructions. *E. faecalis* DSMZ 2570 was used as a control strain.

RESULTS AND DISCUSSION

In the transcriptomic study from chapter 4, where *E. faecalis* V583 was exposed to a therapeutic vancomycin dose (10 µg/ml), EF2292, annotated as a hypothetical protein, was highly induced, with induction levels similar to those for *vanB* operon genes (Chapter 4). The EF2292 ORF is located immediately upstream of $vanX_B$ gene. Moreover, data from chapter 4 show that, under exposure to a subinhibitory vancomycin dose (0.3 µg/ml) on a V583 derivative strain and on a V583Δ*vanB* deletion mutant, the only induced genes are the *vanB* operon genes (with exception of *vanB* itself) and *ef2292* (Chapter 4). Taking together, the consistency of the induction results under vancomycin exposure and the chromosomal location of *ef2292*, underline our suspicion that EF2292 could be part of the *vanB* operon structure.

CO-TRANSCRIPTION ANALYSIS

It is widely described in the literature that $VanR_BS_B$ regulatory system controls transcription of the $vanR_BS_B$ and $vanY_BWH_BBX_B$ operons from the P_{RB} and P_{YB} promoters, respectively (Evers & Courvalin, 1996; Silva *et al.*, 1998). To analyze the co-transcription of *ef2292* with $vanY_BWH_BBX_B$, transcripts were extracted after exposure to vancomycin and analyzed by RT-PCR. PCR reactions were performed using the reverse primer for *ef2292* and the forward primer for each one of the other *vanB* operon genes, namely $vanY_B$, *vanW*, $vanH_B$ and $vanX_B$. All the searched transcripts were detected as shown in Figure 1, evidencing that EF2292 is co-transcribed together with the other *vanB* operon genes.

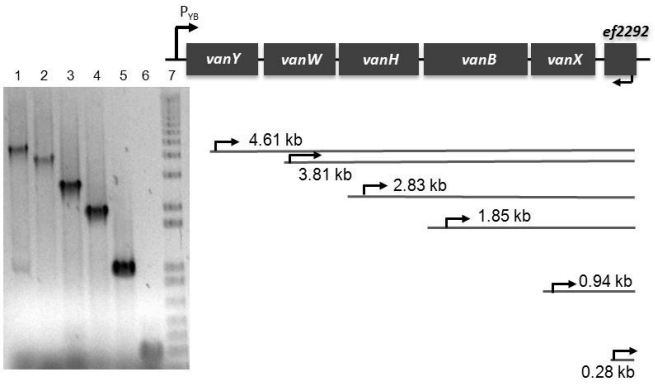

Figure 1. Agarose gel of the RT-PCR products corresponding to *vanB* operon transcripts extracted after 30 minutes of vancomycin induction. The transcripts were obtained using the reverse primer for *ef2292* and the forward primer of each one of the other *vanB* operon genes (lanes 1-6), lane 7 corresponds to 1 kb ladder (Invitrogene).

Therefore, it is likely that *ef2292* transcription occurs from the same promoter P_{YB}. The co-transcription was observed also in vancomycin uninduced cell cultures (data not shown) indicating that, although described as inducible, the *vanB* operon genes also have, in vancomycin absence, a residual constitutive transcription level.

Northern blot hybridization was performed to confirm the co-transcription of *ef2292* with the remaining *vanB* operon genes. Oligoprobes complementary to different possible mRNA transcripts were designed, namely for *ef2292*, for the intergenic region between $vanX_B$ and *ef2292*, for *vanB* and $vanX_B$ genes (Table 1). All the probes used hybridized on the same regions of the gel, corresponding to three different bands/transcripts. The transcript sizes were the expected, substantiating the existence of a single $vanY_BWH_BBX_Bef2292$ transcript as can been seen in Figure 2. The transcript with an approximate size of 4.6 kb most likely corresponds to a mRNA comprising $vanY_BWH_BBX_Bef2292$ genes. Another band with 3.0 kb, matches the $vanH_BBX_Bef2292$ transcript size. P_{YB} is the only

promoter described upstream of the *vanB* genes, it is reasonable to assume that the transcript is processed between *vanW* and $vanH_B$ transcripts. Finally, the third transcript, with approximately 2.0 kb, matches the size of the $vanBX_Bef2292$ transcript. For all the oligoprobes used, this last mentioned transcript was the most abundant, suggesting that processing occurs between $vanH_B$ and *vanB*. Smaller transcripts were not detected under the experiment conditions. Either smaller transcripts are not present or were not detected. For detection of smaller transcripts a polyacrylamide gel Northern blot would be more appropriate.

Regardless of the RNA processing events suggested by these results, Northern blot hybridization confirms *ef2292* transcription together with the other *vanB* operon genes, all transcribed from the same promoter P_{YB}.

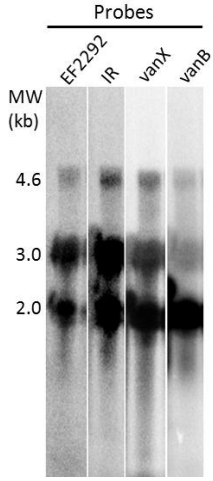

Figure 2. Northern blot analysis. mRNA from *vanB* operon genes was detected using probes for EF2292, intergenic region between EF2292 and *vanX*, *vanB* and *vanX*. The sizes of the transcripts were estimated based on the HypperLadderI (Bioline).

We know, from microarray experiments (data not shown) that in a *vanR* mutant none of the known *vanB* operon genes is induced by vancomycin, including *ef2292*. Taking all results together and considering the accepted definition of operon as a series of genes transcribed in a single mRNA, often identified by the presence of promoters and terminators, we propose a new *vanB*

operon constitution for *E. faecalis* V583 as represented on Figure 3 and to name *ef2292* as *vanV*.

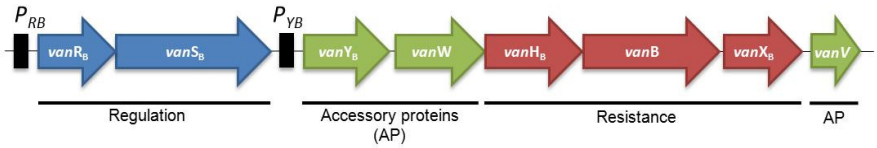

Figure 3 – Proposal for the composition of the *vanB* operon of *E. faecalis* V853 including the *vanV* gene. Using online available software no promoter regions were predicted upstream of *vanV* (http://molbiol-tools.ca/Promoters). Using the predicted terminators tool (www.jcvi.org), there is a terminator downstream of EF2298 ($vanS_B$), consistent with what is described in literature, but no terminators are predicted downstream of EF2293 ($vanX_B$) nor EF2292 (*vanV*).

vanV DISSEMINATION

In order to determine whether *vanV* gene is present in other enterococcal strains we did a blast search against the 28 recently sequenced enterococcal genomes (Palmer *et al.*, 2010). Among the newly sequenced genomes there are 9 vancomycin resistant enterococcal (VRE) strains: 4 $vanA^+$, 1 $vanB^+$ and 4 $vanC^+$, including 2 *E. faecalis*, 3 *E. faecium*, 3 *E. casseliflavus* and 1 *E. gallinarum*. Only one positive match, with 100% of sequence homology, in the *E. faecalis* Merz96 genome was detected. This is a blood isolate containing the *vanB* operon (McBride *et al.*, 2007). In Merz96, *vanV* is also located immediately upstream of the $vanX_B$ gene, revealing thus the same *vanB* operon structure as *E. faecalis* V583.

We also searched for *vanV*, by PCR, in *E. faecalis* and *E. faecium* isolates containing *vanA* or *vanB* operons. Only two strains amplified fragments with the expected size: *E. faecalis* V582 (isolated from animal calf faeces) and *E. faecalis* DSMZ 12956 (isolated from peritoneal fluid) both carrying *vanB* operon.

Despite the limited number of strains screened by PCR and available genomes, we can venture to say that *vanV* is exclusively present in *E. faecalis* strains containing the *vanB* operon, but is not ubiquous among these strains.

vanV ORIGIN

In an attempt to understand the origin of *vanV* we did a blast search against vancomycin intrinsically resistant species (namely *Lactobacillus*, *Pediococcus* and *Leuconostoc*), the producer strain *Amycolatopsis orientalis* and the vancomycin resistance biopesticide *Paenobacillus spp*.. All these species produce peptidoglycan precursors that terminate in lactate and possess ligase genes with homologies with either VanA (Marshall & Wright, 1998) or VanB ligases (Handwerger *et al.*, 1994; Patel *et al.*, 2000; Guardabassi *et al.*, 2005). No sequences similar to *vanV* were found, thus *vanV* does not seem to have origin in other genera.

V583 was isolated in 1988 and Merz96 in 2002, which rules out a linkage with time of isolation. It is true that we have not tested strains isolated prior to 1988 but we searched strains isolated in between this temporal space and the frequency is not high. Therefore, the origin of *vanV* remains to be clarified.

vanV ROLE ON VANCOMYCIN RESISTANCE

To know if *vanV* has a role on vancomycin resistance a deletion mutant V583Δ*vanV* was constructed and the MIC of the mutant and the isogenic wild type strain was determined. V583Δ*vanV* showed the same MIC as the wt V583, indicating that a deletion of *vanV* confers no phenotype related to vancomycin resistance. *vanV* is thus not required for vancomycin resistance as is the case of $vanY_B$ and *vanW* from the same operon. *vanV* thus adds to the gaps in the

current understanding of structure, function and role of some of the described proteins in *van* operons. It is described that operons can comprise functionally unrelated genes and that even conserved operons often undergo rearrangements and acquire new genes, although the mechanisms remain unclear (Price *et al.*, 2006). Price *et al.* (2006) defend that there is more likely the appending of a new gene to the end of a pre-existing operon instead of prepending a gene to the beginning, so the majority of genes retain the origin promoter instead of acquiring a new one (Price *et al*., 2006), which might be the case for *vanV*.

vanV PUTATIVE CELLULAR FUNCTION

According to KEGG database, *vanV* (EF2292) is a hypothetical protein with no paralogs on V583 genome. It has orthologs that correspond to other hypothetical proteins or to AraC family regulator proteins, mostly from *Clostridium* species. Members of AraC transcriptional regulator family have been characterized from a wide variety of prokaryotes including both Gram-negative and Gram-positive bacteria. The characteristic feature of this family is a conserved sequence of 99 amino acid, usually on the protein C-terminal end, that serves as the DNA-binding domain. Within this domain two helix-turn-helix (HTH) motifs are responsible for binding to adjacent major grooves that constitute the DNA-binding site (Gallegos *et al.*, 1997). In addition, most members contain a domain responsible for dimerization and interaction with a ligand effector molecule (Gallegos *et al.*, 1997). These proteins, primarily transcriptional activators, have three main regulatory functions, which are carbon metabolism, stress response and virulence and/or pathogenesis (Martin *et al.*, 1999; Wood *et al.*, 1999). Some regulators are involved in the response to stress conditions, such as oxidative stress, tolerance to antibiotics, organic solvents, heavy metals

and even in the transition from exponential growth to the stationary phase [(for review see (Gallegos *et al*., 1997)]. Moreover, it has recently been demonstrated that two AraC-type ORFs encoded on enterococcal pathogenicity islands regulate Esp and are involved in biofilm formation (Coburn *et al*., 2008).

Considering that *vanV* gene belongs to the *vanB* operon, as well as the fact that AraC family members are known to regulate virulence factors, antibiotic tolerance and stress response in other pathogens, we cannot rule out the possibility of a role of *vanV* in the regulation of genetic determinants related to these functions. However, *vanV* has no common motifs with AraC family of bacterial regulators, namely no HTH motifs, which weaknesses this hypothesis.

This work intends also to call attention to how much remains to be learned about these hypothetical and unknown function proteins. They definitely constitute a challenge and the characterization of such proteins will allow a better understanding of biological systems.

Further work on *vanV* gene and protein is under way and will yield information regarding the role of this hypothetical protein.

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Chapter 6

CroRS Integrity is Essential for VanRS Induction and High-Level Vancomycin Resistance Phenotype

Tânia Ribeiro, Maria de Fátima Silva Lopes. "CroRS integrity is essential for VanRS induction and high-level vancomycin resistance phenotype" (in preparation).

The author performed all the experiments presented in this chapter.

CONTENTS

ABSTRACT

VanRS is the sole two-component system (TCS) involved in resistance to vancomycin. However, we tested several mutants in *E. faecalis* response regulators of TCSs and found that both *croR* and *err06* V583 mutants were susceptible to vancomycin, in spite of the presence in V583 of an intact *vanB* operon. In order to understand the role of those TCSs in vancomycin response/resistance, we performed RT-PCR experiments, using *croR* and *err06* mutants in V583 and JH2-2, and measured the induction of those genes and *vanB* genes in response to vancomycin. Relevance of CroRS in VanA type of resistance was evaluated using JH2-2 and its *croR* deletion mutant complemented with pAT80. CroRS and TCS06 both responded to vancomycin, regardless of the presence of vancomycin resistance gene cluster. In the absence of *croR* gene, vanA^{+} JH2-2 was susceptible to vancomycin. CroRS was found to be needed for VanRS induction and for TCS06 induction by vancomycin. CroRS is likely sensing the vancomycin cell-wall perturbation and its induction probably triggers or provides the signals for vancomycin induction of both VanRS and TCS06. As TCS06 has been reported to respond to oxidative stress response and CroRS to cell-wall perturbations, the known link between cell-wall stress and oxidative stress may well be mediated by CroRS and TCS06 in *E. faecalis*.

INTRODUCTION

Two-component systems utilize the phosphotransfer cascade to alter specific gene expression profiles (West & Stock, 2001) and have been linked to tolerance and resistance to cell-wall active agents, namely resistance to β-lactams in *Listeria monocytogenes* (CesRK) (Kallipolitis *et al.*, 2003), tolerance to vancomycin (VncSR) (Novak *et al.*, 1999) and resistance to β-lactams (CiaHR) in *Streptococcus pneumoniae* (Giammarinaro *et al.*, 1999), and resistance to bacitracin in *Bacillus subtilis* (LiaSR, formerly YvqEC) (Mascher *et al.*, 2003). Many aspects of bacterial physiology are under the control of TCSs (for review see Stock *et al.*, 2000; Stortz & Hengge-Aronis, 2000; Hoch & Varughese, 2001). In *Enterococcus faecalis*, the primary form of vancomycin resistance is provided by expression of vancomycin-inducible gene cluster regulated by a TCS.

The mechanism of high-level vancomycin resistance in enterococci is well understood and the more widely spread form of resistance is mediated by *van* genes which results in the generation of D-alanyl-D-lactate muropeptide stem termini in peptidoglycan precursor molecules, for which vancomycin has a binding affinity that is several orders of magnitude lower than that for the normal D-alanyl-D-alanine termini. In *vanB* operons, expression of the $vanY_BWH_BBX_B$ resistance genes is controlled by $vanR_BS_B$ genes, which encode a two-component regulatory system (Arthur *et al.*, 1992). The regulator and resistance genes are transcribed from promoters P_{RB} and P_{YB}, respectively, which are coordinately regulated (Depardieu *et al.*, 2003; Depardieu *et al.*, 2005). In the presence of vancomycin, the membrane-bound $VanS_B$ sensor phosphorylates the cytoplasmic $VanR_B$ response regulator which then enhances the transcriptional activation of both the $vanR_BS_B$ regulatory and $vanY_BWH_BBX_B$ resistance genes (Arthur *et al.*, 1992).

Some *E. faecalis* TCSs have been shown to be involved in virulence (Teng *et al.*, 2002), biofilm formation (Hancock & Perego, 2004a), intrinsic antibiotic resistance (Comenge *et al.*, 2003; Hancock & Perego, 2004a), and/or stress responses (Teng *et al.*, 2002; Comenge *et al.*, 2003; Le Breton *et al.*, 2003; Hancock & Perego, 2004b). *E. faecalis* V583, carrying the *vanB* operon, if impaired in the enterococcal response regulator *err05*, becomes susceptible to vancomycin (Hancock & Perego, 2004b). This finding suggests that *E. faecalis* has other sensing and responding pathways to vancomycin which, if knocked-down, do not allow *vanB* genotype to fully express the respective high-level resistance phenotype, and that TCS05 is somehow involved in vancomycin resistance. The *E. faecalis* TCS05, also referred as CroRS ("ceftriaxone resistance") or Err05-Ehk05 (Hancock & Perego, 2004b), was proposed to be part of the *E. faecalis* core genome (McBride *et al.*, 2007). CroRS system is composed of the transmembranar CroS sensor histidine-kinase and the OmpR-family CroR response regulator. Its physiological role remains unclear although last decade studies have shown that CroRS is required for intrinsic β-lactam resistance (Comenge *et al.*, 2003). *CroR* deletion resulted in a 4000-fold increase in susceptibility to ceftriaxone, a third-generation cephalosporin (Comenge *et al.*, 2003). A *croR* mutant was also significantly more sensitive to bacitracin, other second- and third-generation cephalosporins, such as cefuroxime and cefotaxime, vancomycin (Hancock & Perego, 2004b), fosfomycin, D-cycloserine, moenomycin and ramoplanin (Comenge *et al.*, 2003), indicating a more general role of this TCS in enterococcal cell envelope stress response. Two target loci, encoding a secreted protein, SalB (previously referred to as SagA) (Le Breton *et al.*, 2003), and a putative glutamine/glutamate ABC transporter GlnQHMP (Muller *et al.*, 2006; Le Breton *et al.*, 2007) have been recently identified as regulated by

CroRS. Recently, in two transcriptomic studies, CroRS was also shown to be induced by bovine bile and sodium dodecyl sulfate (Solheim *et al.*, 2007) and by a therapeutic dose of vancomycin (Chapter 4). A *croR* mutation led to significant growth defects and alterations in cell morphology in *E. faecalis* JH2-2 strains (Le Breton *et al*., 2003) although no growth defect were observed in *E. faecalis* V583 *croR* mutant (Hancock & Perego, 2004b). The signal recognized by CroS remains unknown, but evidence suggests that it could be related to cell wall perturbations (Comenge *et al*., 2003).

We have recently used a microarray transcriptomic approach, based on *E. faecalis* V583 whole-genome, and exposed the vancomycin resistance *E. faecalis* V583 strain to a therapeutic dose of vancomycin (10 µg/ml) (Chapter 4). We found three TCSs induced under the conditions tested, namely VanRS (Err11-Ehk11), which regulates the expression of the *vanB* operon, CroRS (Err05-Ehk05), and Err06-Ehk06 (Chapter 4). Little is known about TCS06 (Err06-Ehk06). Err06-Ehk06 is present in different *E. faecalis* strains (McBride *et al.*, 2007) and Err06 belongs to the OmpR-family. Ehk-Err06 has homology to the *B. subtilis* YclKJ system, which is known to be activated under oxygen limitation (Kobayashi *et al.*, 2001; Hartig *et al.*, 2004). In *E. faecalis* JH2-2 strain, TCS06 has been implicated in response to oxidative stress and in expression of the catalase gene (*kat*) (Muller *et al.*, 2008). Mutants in *E. faecalis err06* were shown to be more susceptible than the wild-type to heat and SDS (Hancock & Perego, 2004b) and more sensitive in macrophages (Muller *et al.*, 2008).

In summary, recent findings show that in the absence of an active CroR, high-level vancomycin resistance phenotype is not expressed in a *vanB* resistant

strain, such as V583, and that there are two TCS is V583 which respond to vancomycin, namely CroRS and TCS06.

We thus decided to further analyze the role of CroR in vancomycin resistance by answering the question: why does the inactivation of *croR* turns a vancomycin resistant strain into a vancomycin susceptible strain? Could it be because the vancomycin resistance genes are less induced by vancomycin in the *croR* mutant? To address this question, we used a *croR* insertional mutant in V583 (Hancock & Perego, 2004b) and compared the expression of vancomycin resistance genes in the wt V583 and in the *croR* mutant. In order to see if CroR has also a role in VanA carrying strains, we introduced pAT80 plasmid (Arthur *et al.*, 1992) into wild-type *E. faecalis* JH2-2 and in its isogenic *croR* deletion mutant and looked for differences in MIC for vancomycin. We also investigated the ability of vancomycin to induce CroRS and TCS06 in a vancomycin susceptible strain, JH2-2. By doing this, we wanted to know if induction of CroRS and TCS06 was independent of the presence and expression/induction of VanRS and its regulated genes. TCS06 has not been associated to cell-wall stress. We questioned if TCS06 induction could be dependent on a cell-wall responsive two-component system, namely on CroRS. We measured induction of *err06* and *ehk06* in JH2-2 and JH2-2Δ*croR*.

MATERIALS AND METHODS

BACTERIAL STRAINS

Strains used in this study are listed in Table 1. Plasmid pAT80 (Arthur *et al*., 1992) (Table 1) was introduced into *E. faecalis* JH2-2 and JH2-2Δ*croR* cells by electrotransformation. Transformants were selected in media containing 50 µg/ml vancomycin (Sigma).

Table 1 – Strains and plasmids used on this study

Strains and Plasmids	Relevant characteristics	Reference
Strain		
V583::tet	Insertion mutant of V583 (clinical isolate, Va^R) with p3TET inserted between e*hk*02 and the adjacent open reading frame encoding a putative lipoprotein	(Hancock & Perego, 2004b)
V583*croR::tet*	*err*05 (*croR*) insertion mutant of V583, Tet^R	(Hancock & Perego, 2004b)
V583erro6*::tet*	*err*06 insertion mutant of V583, Tet^R	(Hancock & Perego, 2004b)
JH2-2	Fus^R Rif^R, clinical isolate derived strain, plasmid free	(Jacob & Hobbs, 1974)
JH2-2Δ*croR*	*croR* deletion mutant of JH2-2	(Le Breton *et al*., 2003)
JH2-2Δ*err06*	*err06* deletion mutant of JH2-2	(Le Breton *et al*., 2003)
SAVE 9	JH2-2/pAT80	This study
SAVE 11	JH2-2Δ*croR*/pAT80	This study
Plasmid		
pAT80	P_R*vanRS*P_H*HAXcat*	(Arthur *et al*., 1992)

RNA EXTRACTION

Growth was done overnight in Brain Heart Infusion (BHI) medium (Oxoid), at 37°C. Cultures were then diluted 20 times and grown in BHI until an optical density of 0.4-0.45, at 600 nm, was reached. Culture was split in two, and vancomycin (Sigma) was added to one of the cultures to a final concentration of 0.1 µg/ml, 3 µg/ml and 10 µg/ml. The split cultures (BHI and BHI plus vancomycin) were then further incubated, and 5 ml samples of each culture were collected 30 minutes past vancomycin addition. Samples were immediately

suspended in RNA Protect solution (Qiagen) and centrifuged for 10 min at 4°C. Total RNA extraction was performed with RNeasy Mini columns (Qiagen). DNA digestion was done using 1 U of RNase-free DNase I (Roche) at 37ºC for 1 hour. RNA was cleaned up from DNase I using the RNA clean-up kit (Qiagen). Prior to PCR reactions DNA contamination of the RNA samples was assessed by a standard (30 cycle) PCR reaction with primers targeting a chromosomal *E. faecalis* gene.

REVERSE TRANSCRIPTASE PCR (RT-PCR)

In order to perform reverse transcriptase PCR, cDNA synthesis was performed using Transcriptor High Fidelity cDNA Synthesis Sample (Roche) according to the manufacturer's instructions and using 1 µg of RNA as template. Semi-quantitative RT-PCR was done from serial cDNA dilutions, assuming that intensity of PCR product bands correlates to the amount of cDNA, and thus to the amount of template mRNA. 16S rRNA was used as a control.

MIC DETERMINATION

MIC values for vancomycin were determined by E-test (AB Biodisk) according to manufacturer´s instructions. *E. faecalis* DSMZ 2570 was used as a control strain.

RESULTS AND DISCUSSION

The current knowledge points to *vanRS* as the only two-component system known to confer resistance to vancomycin, although *croRS* has been previously shown to be induced by vancomycin (Comenge *et al*., 2003). Moreover, previous microarray experiments (Chapter 4) revealed that, besides VanRS and CroRS, TCS06 was also induced when *E. faecalis* V583 was exposed to 10 µg/ml of vancomycin. We determined the vancomycin MIC values for V583*croR::tet* and V583*err06::tet* insertional mutants in *E. faecalis* V583 genetic background and we found that both mutants presented a significant decrease in MIC value for vancomycin compared to the resistant wild-type strain (Table 2). JH2-2 also presented a lower MIC for vancomycin if either *croR* or *err06* were deleted. These findings suggest that both CroRS and TCS06 also play a role in vancomycin resistance and raise two questions: are *croRS* and *err06-ehk06* responding directly to vancomycin, or indirectly by sensing changes induced by the *vanB* operon induction? Why are V583*croR::tet* and V583*err06::tet* mutants susceptible to vancomycin, even in the presence of a fully functional *vanB* operon?

Table 2. MIC values for vancomycin of *E. faecalis* V583 and JH2-2 and their derivative mutants in *croR* and *err06* genes.

Strain	Vancomycin MIC (µg/ml)
V583::*tet*	>256
V583*croR::tet*	>3
V583err06::tet	>1.5
JH2-2	>3
JH2-2Δ*croR*	>1
JH2-2Δ*err06*	>1.5

BOTH CroRS AND TCS06 RESPOND TO VANCOMYCIN IN A CONCENTRATION DEPENDENT WAY

CroRS and Err06-Ehk06 systems may be induced by vancomycin either directly or indirectly by other gene(s) which expression is directly affected by vancomycin. In order to test if *croRS* and *err06* respond directly to vancomycin and are not responding to any changes induced by the presence of *vanB* genes, we evaluated the expression of these two TCSs in a susceptible strain, JH2-2. This strain has intact CroRS and TCS06 systems and carries no vancomycin resistance genes. Vancomycin doses used were 0.1 µg/ml, corresponding to nearly 3% of the MIC, 3 µg/ml which is the MIC value of JH2-2, and 10 µg/ml which is the therapeutic dose for which *croRS* and *err06-ehk06* induction was previously observed (Chapter 4). In *E. faecalis* JH2-2 strain both *croR* and *croS* were induced when exposed to vancomycin and induction levels correlate with vancomycin concentration, increasing with the increase of the antibiotic concentration (Figure 1A). This observation demonstrates that *croRS* is induced by vancomycin directly and not by any cascade events following *vanRS* induction, as JH2-2 does not have *vanRS*. Similar behavior was observed for *TCS06*, which also sensed vancomycin directly (Figure 1B). 0.1 µg/ml of vancomycin was enough to induce both TCS systems in JH2-2, MIC value - 3 µg/ml. Comenge *et al.* (2003) tested vancomycin concentration ranging from 0.01 to 5 µg/ml and have shown that the maximum induction was achieved with 2 µg/ml (Comenge *et al*. 2003). Here we show that at least until the MIC value for JH2-2 (3 µg/ml) induction is constant and shows a significant increase if a therapeutic dose (10 µg/ml) is used, in both TCSs tested.

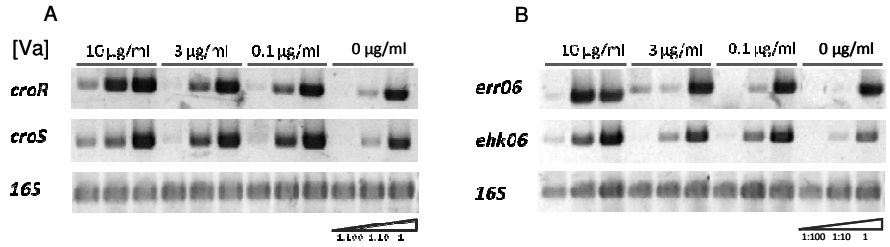

Figure 1. Semi-quantitative RT-PCR of JH2-2, A) *croR* and *croS* and B) *err06* and *ehk06* transcripts, after 30 min exposure to different vancomycin concentrations.

CroRS IS NEEDED FOR INDUCTION OF *vanB* AND *vanA* CLUSTERS

Expression levels of some *vanB* operon genes, namely $vanR_B$, $vanS_B$, *vanB* and $vanX_B$, in V583::*tet* and V583*croR::tet* strains were compared in cells grown in BHI and in BHI supplemented with 10 µg/ml of vancomycin. The aim was to know if vancomycin susceptibility in V583*croR::tet* strain was due to a lower induction of *vanB* genes by vancomycin. In the absence of vancomycin, *van*B operon genes showed similar levels of expression in both V583::*tet* and V583*croR::tet* strains (Figure 2). In the presence of 10 µg/ml of vancomycin, *vanRS* genes showed the same induction levels in V583*croR::tet* strain as found for the same strain in the absence of vancomycin. This finding suggests that CroRS is needed for induction of *vanRS* genes by vancomycin. *VanB* and *vanX* genes were induced by vancomycin in both strains, although reaching higher levels of expression in V583::*tet* strain. This fact is likely contributing to the low MIC value of V583*croR::tet* strain and supports the role and involvement of CroRS system in induction of *vanB* genes by vancomycin.

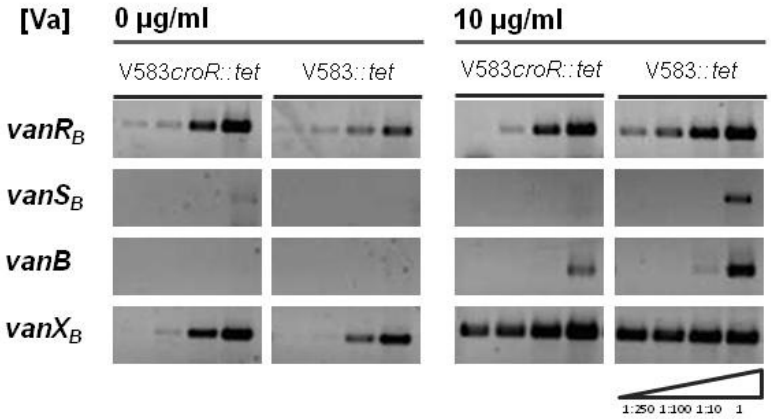

Figure 2. Semi-quantitative RT-PCR of *vanR*$_B$, *vanS*$_B$, *vanB* and *vanX*$_B$ genes in V583::*tet* and V583*croR::tet* strains after 30 minutes exposure to 10 µg/ml vancomycin.

Although the semi-quantitative RT-PCR experiments were repeated twice and gave similar results, this technique does not allow a precise quantitative analysis of induction levels of *vanB* genes. Therefore, in order to confirm the role of CroRS in *van*RS induction by vancomycin, we used another approach. JH2-2, which carries CroRS but not VanRS, and its *croR* mutant, were transformed with pAT80 plasmid (Arthur *et al*., 1992). pAT80 carries the *vanR*$_A$*S*$_A$*H*$_A$*AX*$_A$ genes and is able to confer vancomycin resistance in JH2-2 host (Arthur *et al*., 1992).

SAVE 9 strain was resistant to vancomycin (MIC >256 µg/ml), as expected. However, SAVE 11, although carrying the same pAT80 of SAVE 9 strain, was susceptible to vancomycin (MIC was 4 µg/ml). This result obtained for *vanA* type of resistance confirms our findings with *vanB* type of resistant and reveals that CroRS is essential also for expression of *vanA* genes.

CroRS MEDIATES TCS06 RESPONSE TO VANCOMYCIN

TCS06 has been implied in response to oxidative stress and in the expression of the catalase gene (*kat*) (Muller *et al.*, 2008) in *E. faecalis* JH2-2. Mutants in *err06* gene were shown to be more susceptible than the wild-type to heat and SDS (Hancock & Perego, 2004b) and more sensitive in macrophages (Muller *et al.*, 2008). Our findings show that TCS06 responds to vancomycin and, although the mechanism remains unknown, it is evident that it plays a role in resistance to vancomycin. We have shown that CroRS is also involved in vancomycin resistance and that its presence is essential for high induction of vancomycin resistance genes in *E. faecalis*. Previous works have already attributed a role for CroRS in response to cell-wall perturbations. We thus asked if CroRS could be the linker between cell-wall perturbations and TCS06 induction, as this system had never been correlated with cell-wall stress.

We decided to test the expression levels of *err06* and *ehk06* genes in JH2-2Δ*croR* strain, under exposure to vancomycin. JH2-2Δ*croR* strain has no *vanRS* and no *croR*. Results are shown in Figure 3. Both *err06* and *ehk06* genes responded to vancomycin lowest concentration, 0.1 µg/ml. However, further induction was not observed for higher vancomycin concentrations, and was absent when therapeutic vancomycin dose was used. Comparing these results with the ones shown in Figure 1B, obtained with JH2-2, which harbors an active CroRS system, we are lead to conclude that the presence of an intact CroRS system is essential for vancomycin induction of the TCS06 system. It is likely that CroRS senses the cell-wall perturbations induced by vancomycin and in turn induces, directly or indirectly, TCS06 expression. The link between these two TCSs suggests a link between cell-wall and oxidative stresses, which has been

already established, and provides clues for further studies involving the cross-talk between the two players in the linkage of cell-wall perturbation and induction of oxidative stress.

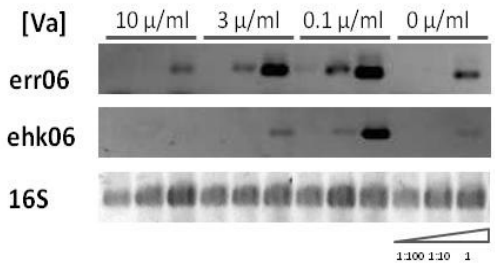

Figure 3. Semi-quantitative RT-PCR of *err06* and *ehk06* genes in JH2-2Δ*croR* strain after 30 minutes of exposure to vancomycin.

There is a restrict understanding of the signals/ligands sensed by well-characterized histidine kinases, reflecting a drawback in the understanding of this systems. For example, VanRS is the best studied enterococcal TCS however, the signal to which *vanS* responds and initiates the phosphorylation cascade is not yet clearly identified. It is likely that these three TCSs (VanRS, CroRS, Err06-Ehk06) may be part of a regulatory network since they all present a common phenotype towards vancomycin resistance.

It is still unknown which array of genes are controlled and which expression is dependent on each of these three TCSs. We previously determined the set of genes that are differentially transcribed under treatment with a therapeutic dose of vancomycin. It would be interesting to determine the stimulons of CroRS and TCS06 systems using vancomycin as a triggering signal and compare them in order to find the common features. This can be a case of overlapping stimulons when using vancomycin as the signal. Given the important functions that have been attributed to the already characterized enterococcal TCSs, it is likely that

some of the uncharacterized systems have interesting roles in antibiotic resistance.

TCSs responding to vancomycin are widely described in Gram-positive bacteria. However, as opposite to what is described for enterococci, for other Gram-positive bacteria vancomycin is not the only and exclusive signal and most bacteria have more than one TCS able to respond to vancomycin presence. For enterococci, VanRS is the only TCS described, so far, as responding to vancomycin and moreover no other signal has been described to trigger VanRS system. This study is the first where other enterococcal TCSs are reported as responding to vancomycin.

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Chapter 7

Induction of Resistance to Vancomycin in Susceptible *Enterococcus faecalis* Strains

Unpublished data

The experimental work presented on his chapter was performed by T. Ribeiro. The high throughput DNA sequencing was performed as a service by Tufts University Core Facility (Boston).

CONTENTS

ABSTRACT

In a previous study we have found that vancomycin susceptible enterococci carrying *vanA* and *vanB* genes were able to grow in the presence of vancomycin concentrations above their MIC value, if grown in increasing vancomycin concentrations. We now reproduced these experiments using strains with a known genetic background derived from *Enterococcus faecalis* V583. For control, we used also OG1-RF, JH2-2 and DSMZ 2570 which are all vancomycin susceptible strains and bear no genes related to vancomycin resistance. Serial transfer passages in two-fold increasing concentrations and in subinhibitory concentrations of vancomycin were performed. Only strains impaired in one gene relevant for vancomycin resistance or response were able to become high-level resistant, in both adaptation processes. Moreover, ten serial passages in drug-free media were not able to revert the acquired resistance phenotype. PFGE of adapted resistant cultures showed that no chromosomal rearrangements occurred during adaptation. High throughput sequencing of the V583Δ*vanB* mutant also revealed that the mechanism involved in adaptation does not rely on mutations. Whether this stable and reproducible phenotypic alteration is a phenotypic mutation or a long-term transcriptional change/adaptation is yet to be established.

INTRODUCTION

The propensity of different antibiotics to select for resistant bacteria in an initially susceptible population can be examined by repeated subculture of bacteria in subinhibitory concentrations. The subinhibitory antibiotic concentration is increased as the MIC increases. This procedure tests the ability of the organism to adapt to antibiotics in its environment, presumably in incremental steps, and ultimately become resistant to the maximum antibiotic levels achievable with standard dosing.

The general mechanism by which bacteria develop resistance through serial passages in presence of antibiotics is acquisition or accumulation of point mutations. However, several studies suggest that the observed phenomena of reduced susceptibility may be due to different mechanisms, and may be strain and antibiotic dependent. In a recent study by Schmidt *et al.* (2010) the authors succeeded in generating an *S. aureus* strain with reduced susceptibility to ramoplanin (Schmidt *et al.*, 2010). In this case the resistance mechanism was similar to that observed in VISA strains, with increased cell wall material and reduced autolytic enzyme activity. Like in VISA-type resistance, induction of resistance by serial passages in ramoplanin most likely arises from multiple mutations in many loci by a gradual adaptative process (Howden *et al.*, 2008, Neoh *et al.*, 2008, Cui *et al.*, 2009). Resistance to linezolid is frequently associated with mutations on 23S rRNA. However, in *S. epidermidis* development of resistance mechanisms to linezolid is not clear but it seem to be related with a decrease in drug uptake, due to changes in bacterial membrane permeability or due to an overexpression of an efflux system (Sierra *et al.*, 2009).

To our knowledge, in enterococci there are no described mutations that lead to glycopeptide resistance, most likely because in enterococci the resistance

mechanism involves a complicated and highly regulated genetic system rather than a panoply of phenotypic and morphological alterations, as in the case of *S. aureus* for which a single mutation could translate itself into a more immediate phenotypic alteration regarding vancomycin resistance. In fact in *S. aureus*, mutations in several genetic loci other than TCSs, such as *sigB* (Singh *et al.*, 2003), *trfAB* (Renzoni *et al.*, 2009), *tcaA* (Maki *et al.*, 2004), are known to contribute to glycopeptide resistance.

In recent years, several genetic alterations in two-component regulatory systems have been reported to be related to glycopeptide resistance phenotype, namely as point mutations in *vraSR* (Cui *et al.*, 2009) and *graSR* (Neoh *et al.*, 2008), and a defective *agr* function (Sakoulas *et al.*, 2002). However, these genetic changes have only been found in very limited number of clinical and laboratory isolates of *S. aureus* and never in enterococci. Furthermore, no mutations were found in *vraSR* among some pairs of vancomycin-susceptible and –resistant clinical isolates (Howden *et al.*, 2008). Hence, there are likely to be as yet unidentified loci and pathways linked to the glycopeptide resistance phenotype.

We have previously identified vancomycin susceptible enterococcal strains from different environments, carrying incomplete *van* operons (Ribeiro *et al.*, 2007). In an attempt to understand if the presence of incomplete *van* operons would confer any advantage under vancomycin selective pressure, we have subjected these environmental isolates to vancomycin selective pressure through serial passages in increasing vancomycin concentrations. In these experiments, strains bearing incomplete *van* operons were able to become resistant to vancomycin.

MATERIAL AND METHODS

MICROORGANISMS AND GROWTH CONDITIONS

For this study eleven enterococcal strains were selected (Table 1). Growth was done in Brain Heart Infusion (BHI) (Oxoid) and 15 µg/ml of tetracycline was added to the medium for growth of insertional mutants.

MUTANT CONSTRUCTION

The markerless *vanS* deletion mutant was constructed by double cross-over essentially as described previously (Brinster *et al*., 2007). Briefly, 5' and 3' flanking regions of the target gene were amplified from chromosomal DNA of V583Ery^S by PCR. The two PCR fragments were fused by PCR using the external primers and the resulting PCR fragment was cloned into the T/A cloning vector pGEM-T (Promega). The inserted PCR fragment was excised and subsequently cloned in pG+host9 plasmid (Maguin *et al.*, 1996), which was then electroporated in *E. faecalis* V583Ery^S. The *vanS* double-crossover mutant was selected as described by Brinster *et al.* (2007). Successful target mutations were identified by PCR screening and confirmed by sequencing. All primers used are listed in Table 1.

MIC DETERMINATION

Minimum inhibitory concentrations for vancomycin were determined by E-test (AB Biodisk) according to manufacturer´s instructions. *E. faecalis* DSMZ 2570 was used as a control strain.

Table 1. *E. faecalis* strains and primers used in this study.

Strains and Primers	Relevant properties/Sequence (5'-3')	Reference
Strain		
V583	Clinical isolate, Van^R	(Sahm *et al.*, 1989)
V583Ery^S	Derivative from V583 by deletion of EFA0007	A. Benachour
JH2-2	Fus^R Rif^R, plasmid free	(Jacob & Hobbs, 1974)
OG1-RF(TX4002)	Fus^R Rif^R, plasmid free	(Wirth *et al.*, 1986)
V583*croR::tet*	*err*05 insertion mutant of V583, Tet^R	(Hancock & Perego, 2004)
V583*vanR::tet*	*Err11* insertion mutant of V583, Tet^R	(Hancock & Perego, 2004)
V583*err06::tet*	*err*06 insertion mutant of V583, Tet^R	(Hancock & Perego, 2004)
SAVE 3	*vanS* deletion mutant of V583Ery^S	This study
SAVE 2	*vanB* deletion mutant of V583Ery^S	Chapter 4
JH2-2Δ*croR*	*err05* deletion mutant of JH2-2	(Le Breton *et al.*, 2003)
JH2-2Δ*rr06*	*err06* deletion mutant of JH2-2	(Le Breton *et al.*, 2003)
DSMZ 2570	Type Strain (Van^S)	DSMZ culture collection
DSMZ 12956	Type Strain (Van^R)	DSMZ culture collection
Primers		
vanS1	CCTCGACAAGTAGAATTCGTATC	This study
vanS2	CTTTGAAACTACAGGGAAACTAC	This study
vanS3	CACCAGCAGCAAAAGGATTTC	This study
vanS4	CTGACACTTCTGCCGGAG	This study
vanS5	GTTATACCTGTCGGTCAAAATC	This study
vanS6	GCTGGAAAACACCTCAGATG	This study

SERIAL PASSAGES IN VANCOMCYIN

Cells were grown overnight at 37ºC in drug-free BHI broth and then plated on BHI agar for overnight growth. Then, one colony was picked and inoculated in drug-free BHI broth. Passage through BHI containing doubling vancomycin concentrations was performed by daily inoculation with a 1:100 dilution from the previous overnight cultures at 37ºC. The lower vancomycin concentration was 0.125 µg/ml and the highest was 256 µg/ml. MIC values were determined after the strains were passed through the vancomycin concentrations of 0.5 µg/ml, 2 µg/ml, 8 µg/ml, 16 µg/ml, 32 µg/ml and 256 µg/ml. The last culture was also tested for stability of MIC value and cross resistance with other antibiotics by the

standard disk diffusion test. Stability of MIC values was tested after 10 consecutive subcultures in vancomycin-free media.

Serial passages in BHI media with a fixed subinhibitory concentration of vancomycin were also performed. Choice of the vancomycin concentration was based on the MIC value of each strain (Table 2): 4 µg/ml was used for SAVE 2 and V583*croR::tet*, and 1 µg/ml was used for V583Δ*vanS* (SAVE 3), V583*vanR::tet*, V583*err06::tet,* JH2-2, JH2-2Δ*croR*, JH2-2Δ*err06* and OG1RF. Although V583*croR::tet* and OG1RF strains have the same MIC the subinhibitory dose chosen for these two strains was different since at 4 µg/ml OG1RF strain grew poorly, reaching lower ODs when compared to V583*croR::tet*. After 10 passages in subinhibitory concentrations, the mutants were cultured on a second subinhibitory concentration. The second subinhibitory concentration was chosen according to the MIC value that each strain presented after the 10th passage on the first subinhibitory concentration, corresponding to approximately 1/3-1/4 of the MIC: 16 µg/ml was used for V583*croR::tet*, 4 µg/ml for JH2-2*ΔcroR* and 8 µg/ml was used for V583*err06::tet* and JH2-2*Δerr06*. MIC determination along the serial passages on subinhibitory concentrations was performed at the 3rd, 6th and 10th passage.

HIGH THROUGHPUT DNA SEQUENCING

Adapted SAVE 2 strain was sequenced using High throughput DNA sequencing, performed with an Illumina Genome Analyzer II with the single-paired end procedure (Tufts University Core Facility, Boston). Mapping and analysing of the reads was performed using the CLC Genomics Workbench software (CLC bio). Both sequencing and analysis were performed by the Tufts Core Facility, Boston.

CONFIRMATION OF MUTATIONS BY PCR AND SEQUENCING

Mutations found in adapted SAVE 2 strain were checked by PCR in the wild type strain V583Ery^S and in SAVE 2 before passage for confirmation. The *ddl* gene (EF0843) was also amplified and sequenced for SAVE 2, SAVE 3 and V583*vanR::tet* strains. Primers used were as presented in Table 1. PCR reactions were performed in a T-personal Combi thermocycler (Biometra) with PCR mastermix (Finnenzymes) and for each 50 µl PCR reaction mixture 250 ng of DNA were used with an annealing temperature of 55ºC. Primer synthesis and sequencing service were performed by STABVida.

PULSED-FIELD GEL ELECTROPHORESIS

Strains used in the induction assay at three different stages, namely before induction (BI), after induction (AI) with 256 µg/ml and after 10 daily passages in drug-free BHI (BHI), were typed by PFGE. Genomic DNA agarose discs and DNA digestion with *Sma*I were performed according to the method of Chung *et al.* (2000) except for lysis solution, which contained only RNase A and lysozyme (Chung *et al.*, 2000). Agarose discs were electrophoresed on a 1% (w/v) Seakem GTG agarose gel (FMC Bioproducts) in 0.5× TBE buffer low EDTA (50 mM Tris, 50 mM boric acid, 0.2 mM EDTA, pH 8.0) in a CHEF apparatus (Clamped Homogeneous Electric Fields) (BioRad) at 14ºC for 22 hours at 220V with pulse times of 5 s rising to 35 s during the electrophoresis period. Gels were stained by immersion in ethidium bromide (1 µg/ml) for 30 minutes and photographed with a UV light source.

ANTIMICROBIAL SUSCEPTIBILITY TEST

Antimicrobial susceptibility was determined using disc diffusion method according to CLSI (former NCCLS) (Clinical and Laboratory Standards Institute, 2000). Disk diffusion zone diameters were determined on Muller-Hinton Agar (Oxoid) and measured using a calliper. The antibiotics tested were selected in order to cover all the known chemical and functional groups of antibiotics: gentamycin 10 µg, chloramphenicol 30 µg, erythromycin 15 µg, nitrofurantoin 300 µg, tygecycline 15 µg, polymyxin B 300 IU, tetracycline 30 µg, mupirocin 5 µg, fusidic acid 10 µg, spiramycin 100 µg, cefoperazone 30 µg, sulphamethoxazole/trimethoprim (SxT) 25 µg, ofloxacin 5 µg, kanamycin 30 µg, lincomycin 15 µg, oxitetracyclin 30 µg, synercyd (quinupristin/dalfopristin) 15 µg, ampicillin 10 µg, amoxicillin 25 µg, linezolid 30 µg, ciprofloxacin 5 µg, tobramycin 10 µg, ceftriaxone 30 µg, penicillin G 10 IU, methicillin 5 µg, vancomycin 30 µg, bacitracin 10 µg, trimethoprim 1.25, enrofloxacin 5 µg, imipinem 10 µg, netilmycin 30 µg, colistin 10 µg, piperacillin 100 µg, rifampicin 5 µg. *E. faecalis* DSMZ 2570 was used as control, as recommended by CLSI (Clinical and Laboratory Standards Institute, 2000).

RESULTS AND DISCUSSION

PASSAGES IN TWO-FOLD INCREASING VANCOMYCIN CONCENTRATIONS

As shown in Table 2 all strains, except JH2-2, OG1RF and DSMZ 2570, were able to adapt and grow in high vancomycin concentrations until 256 µg/ml. The common features among the strains which were able to adapt is that they have at least one gene involved in response to vancomycin, deleted.

Table 2 – Vancomycin MIC values of *E. faecalis* strains during the two-fold increased serial passages in vancomycin.

Strain	Vancomycin concentration (µg/ml)[a]						
	0	**0.5**	**2**	**8**	**16**	**32**	**256**
V583	256	nd	nd	nd	nd	nd	nd
SAVE 2	6	4	4	**48**	**48**	**>256**	**>256**
SAVE 3	3	3	4	**48**	**48**	**>256**	**>256**
V583*vanR::tet*	3	3	4	**48**	**48**	**>256**	**>256**
V583*err06::tet*	1.5	12	12	**64**	**128**	**>256**	**>256**
V583*croR::tet*	4	4	6	**32**	**48**	**>256**	**>256**
JH2-2	3	4	4	ng	ng	ng	ng
JH2-2Δ*croR*	1.5	4	6	**32**	**128**	**>256**	**>256**
JH2-2Δ*err06*	1.5	12	12	**64**	**128**	**>256**	**>256**
OG1RF	4	4	4	ng	ng	ng	ng
DSMZ 2570	4	4	4	ng	ng	ng	ng

[a]values in bold correspond to resistant MIC values; nd, not done; ng, no visible growth.

In the case of SAVE 2, SAVE 3 and V583*vanR::tet*, at least one gene from *vanB* operon, involved in resistance to vancomycin, was missing. In the case of V583*err06::tet*, V583*croR::tet*, JH2-2Δ*croR* and JH2-2Δ*err06* at least one regulator, proven in chapter 6 to be involved in response to vancomycin, is absent. All strains turn to be phenotypically resistant after the passage with 8 µg/ml, suggesting that the observed MIC increase is not gradual but occurs at a certain concentration (Table 2). This phenomenon is illustrated in Figure 1.

err06 mutants, both in V583 and JH2-2, showed a significant increase in the MIC (from 1.5 to 12 µg/ml) right after passage through 0.5 µg/ml of vancomycin. Despite, the reproducibility of our results (4 independent experiments were performed), we cannot ascertain if this earlier increase, compared with the other strains tested, has any biological relevance or not.

In case of SAVE 2 mutant, one hypothesis for resistance development was that the *ddl* gene might have suffered a mutation that would alter its substrate specificity and started to incorporate D-Lac in the peptidoglycan precursors, as done by VanB ligase. However, the *ddl* gene was sequenced and presented no mutations. It is difficult to establish a hypothesis at this moment since, in our understanding, the fact that the phenotype is stable after 10 nonselective passages suggests a stable change in the chromosome, i. e., mutation or chromosome rearrangements.

Adapted and resistant SAVE 2 strain was thus sequenced. The sequencing revealed 6 SNPs (single nucleotide polymorphism) that translate themselves into amino acid changes. As those results were obtained by comparison with the published V583 genome and, due to the high genomic variability of strains, we decided to check if the mutations were already present in the V583 strain used to construct the SAVE 2 mutant. PCR reactions targeting the mutations found were performed using V583 DNA and the amplified fragments were sequenced. However, all mutations were also present in the V583 strain used for mutant construction, ruling out the possibility that any of these SNPs was responsible for the phenotype alteration observed.

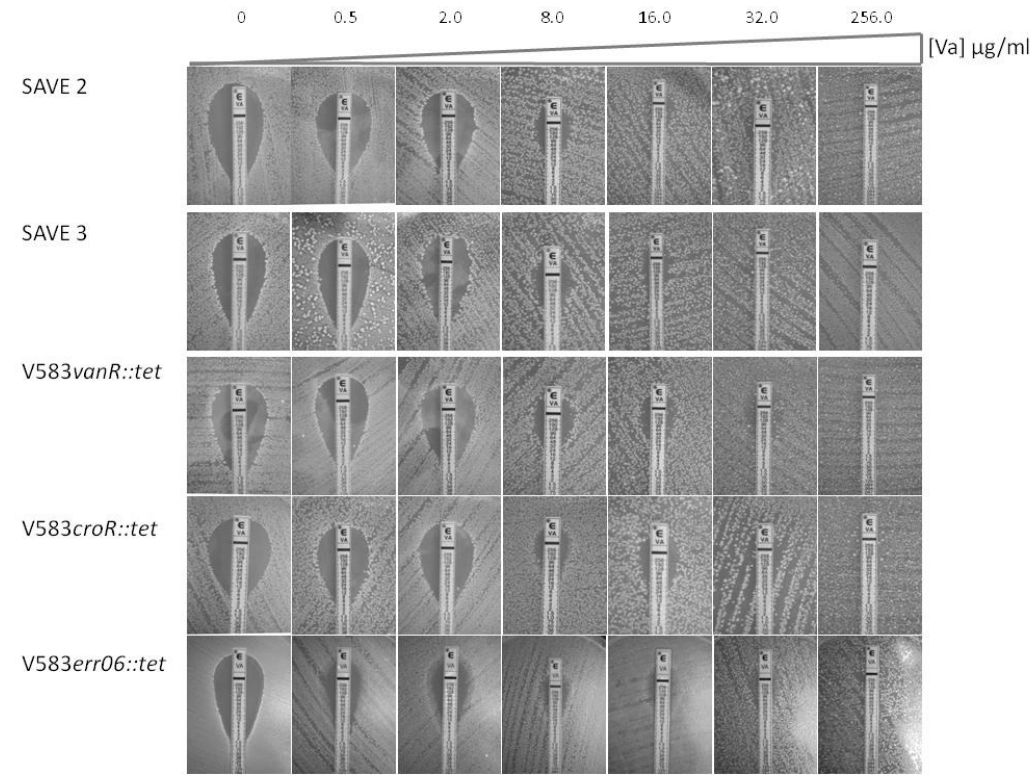

Figure 1. MIC determination by E-test along the passages in two-fold increased serial passages in vancomycin for V583 genetic background mutants.

Mwangi *et al.* (2007), using a similar approach, sequenced several *S. aureus* strains isolated from a patient undergoing chemotherapy with vancomycin and other antibiotics (Mwangi *et al.*, 2007). The authors observed that when comparing the genome of the last strain recovered from the patient with the first recovered strain, there were 35 point mutations on 31 different loci. From the identified mutations some have already been described as implicated on vancomycin reduced susceptibility in staphylococci, namely on *vraR* and on *agr* locus (Mwangi *et al.*, 2007). The fact that we did not find mutations that explain the increase of MIC in the enterococcal strains tested, once again, points out the

differences between the enterococcal and staphylococcal vancomycin resistance mechanisms.

In order to know if the adapted resistant strains were a product of chromosomal rearrangements of the original susceptible strains PFGE was done with DNA from strains, before induction (BI), after induction (AI) and after induction and after passages in drug-free media (BHI). It is not likely that we would observe a band pattern change if the phenotype alteration was caused by a single mutation, unless the single mutation was located in the restriction site of the enzyme used, which is not very probable. However, if the alteration was due to some rearrangement on the genome we would be able to see it. The band pattern remained unchanged after passages in increasing vancomycin concentrations and after the 10 nonselective passages. Exception was V583*vanR:tet* which, before the passages, lacked a high molecular weight band (Figure 2). The PFGE gels were repeated 5 times, and the restriction patterns were reproducible. All different mutants analyzed by macro-restriction presented the same PFGE profile. SAVE 2 and SAVE 3 are deletion mutants and V583*vanR::tet* and V583*croR::tet* are insertional mutants, meaning that none of the chromosomal changes enforced by the mutant construction procedure inserts or removes any restriction site, keeping the restriction profile of the V583 unaltered.

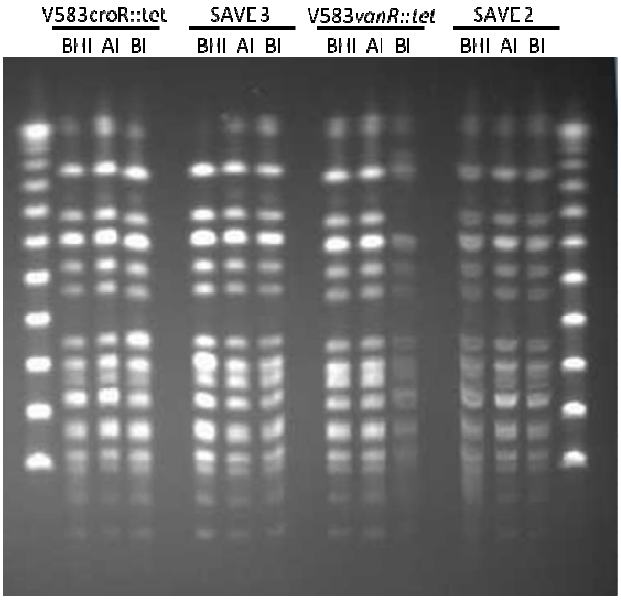

Figure 2. PFGE profile of V583 mutants before induction (BI), after induction (AI) and after 10 passages in nonselective media (BHI).

PFGE results showed the absence of chromosomal alteration as revealed by sequencing of the SAVE 2 strain. Therefore, mutations or chromosomal rearrangements seem unlikely to be the mechanism responsible for the stable phenotype observed after passage in increasing vancomycin concentrations.

The possibility of a transcriptomic change leading to a metabolic adjustment to the presence of the antibiotic is likely. However, we would expect that a transcriptomic change lasts while the pressure element is kept and once removed the metabolism of the cells comes back to its original pace. Another possibility is a phenotypic mutation, which is by definition an alteration on the phenotype that is somehow stabilized and kept for several generations to benefit the cells, although not individually subjected to inheritance and natural selection. The phenotypic mutations may constitute a potential future advantage to the cells progeny by subsequently buffering genomic mutations (Goldsmith & Tawfik, 2009).

Phenotypic mutations are supposed to vanish after enough generations. The matter is how many generations are needed and how dependent is that on the type of phenotypic mutation and nature of agent that causes the phenotypic mutation. In our case, if the phenotypic mutation is actually protecting the cellular progeny from subsequent genetic mutations is very likely that after 10 passages in drug-free media the phenotype is not yet lost.

Considering the fact that all mutant strains can be induced to grow in high vancomycin doses, we wondered if the mechanism underlying the observed phenotype was the same in different strains. It is known that in the absence of VanS, VanR can still function. Unphosphorylated VanR is still active, although with decreased activity compared to its phosphorylated form. VanR can undergo autophosphorylation by using acetyl-phosphate as a substrate or can be phosphorylated by a non-partner kinase chromosomally encoded (Holman *et al.*, 1994). Considering that CroRS and Err06-Ehk06 might have a role in response to vancomycin, their kinases are strong candidates to perform this cross-phosphorylation.

After the serial passages the induced mutants were screened for its susceptibility to other antibiotics by disk diffusion method. No relevant changes on antibiotic susceptibility were observed. The only change observed was an increased susceptibility to erythromycin and spiramycin in *vanB* and *vanS* mutants. Erythromycin was used as a selective antibiotic in the construction of both mutants. We are attributing this fact to some alteration occurring during mutant construction procedure.

PASSAGES IN SUBINHIBITORY VANCOMYCIN CONCENTRATIONS

In order to know if vancomycin was able to induce resistance if selection is made using a constant concentration, we passed the same strains using subinhibitory vancomycin concentrations. None of the *vanB* operon mutants increased its MIC value through this method. However, *croR::tet*, *err06::tet*, JH2-2Δ*croR* and JH2-2Δ*err06* were able to increase the MIC consistent with high level resistance, >256 µg/ml (Table 3).

The passages in subinhibitory concentrations were first done with a lower dose, according to the MIC of the strains but as the MIC increased the subinhibitory dose of vancomycin was also increased, to 16 µg/ml in the case of the *croR::tet*, 4 µg/ml for the JH2-2Δ*croR* mutant and to 8 µg/ml in the case of the *err06* mutants. The vancomycin subinhibitory concentration chosen to perform a second step-induction was different for the different mutants since their MIC at the 10th first step-induction passage was different.

Table 3. MIC values determined along passages in vancomycin subinhibitory doses.

		Strain			
Va dose (µg/ml)	**Passage Nr.**	**V583*croR::tet***	**JH2-2Δ*croR***	**V583*err06::tet***	**JH2-2Δ*err06***
1	3	-	4	12	6
	6	-	6	24	12
	10	-	12[b]	32[c]	32[c]
4	3	32	>256	-	-
	6	48	-	-	-
	10	48[a]	-	-	-
8	3	-	-	>256	>256
	6	-	-	-	-
	10	-	-	-	-
16	3	>256	-	-	-

Presented only the results for which strains a MIC increase was observed; [a]next passage with 16 µg/ml; [b]next passage with 4 µg/ml; [c]next passage with 8 µg/ml; -, not done.

Passages in subinhibitory concentrations of vancomycin were able to produce an increase in the MIC value of the culture. A further increment in the dose of exposure allowed, only for mutants in *croR* and *err06*, an increase in MIC consistent with high level resistance.

Err05-Ehk05 and Err06 were found to be directly induced by vancomycin (Chapter 6). These two systems are common to all *E. faecalis* strains (McBride *et al.*, 2007) and are likely relevant sensing systems in *E. faecalis*. The results obtained with induction with constant concentrations suggest that both regulators, *croR* and *err06*, play a role in this response which is independent of the presence of an intact VanRS system as demonstrated by the fact that in both V583 and JH2-2, *croR* and *err06* mutants behaved similarly.

It is possible that CroRS and Err06-Ehk06 are compensating for the absence of each other by increasing expression of the regulator still present and eventually acting as a regulator for the other TCS. This could be checked by both RT-PCR and by gel-shift assays for CroR binding to promoter of *err06* and vice-versa with Err06 and *croR* promoter.

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Chapter 8

Comparison of V583 with *vanB* and *vanR* Mutants by Transcriptional Analysis: *vanB* Operon Genes Are More Than Just Antibiotic Resistance Tools

Unpublished data

The author designed and performed all the experimental work presented in this chapter.

CONTENTS

ABSTRACT

Vancomycin resistance has become common and prevalent among enterococci, mostly in the clinical setting but also in the environment, jeopardizing the successful treatment of infections caused by Gram-positive bacteria. Previous data shows that although expression of vancomycin resistance was not found among a group of enterococcal strains from different environments, vancomycin resistance determinants are widely spread (Chapter 2).

To investigate the possibility of *vanB* operon influencing expression of additional genes, we used a transcriptional profiling approach to evaluate the genome wide alterations by an Affymetrix DNA microarray based on the *E. faecalis* strain V583 sequence. The transcriptional analysis was made comparing the *E. faecalis* strain V583 profile with its mutants on *vanB* (SAVE 2) and *vanR* genes (V583*vanR::tet*). Using a sensitivity threshold value of 3.0 and 2.0, respectively, the absence of *vanB* and *vanR* genes altered the transcription of a large number of genes. In the case of *vanB*, 309 genes on early-stationary phase and 205 on mid-exponential phase out of 3182 ORFs for *vanB* were differentially expressed. In the case of *vanR,* a total of 249 different genes were differentially transcribed (157 at mid-exponential phase and 137 at early-stationary phase) in the mutant as compared to the wild type. Among these, 25 are plasmid encoded, with 8 encoded on pTEF1 and 17 on pTEF2.

In both mutants, a high number of genes belonging to mobile genetic elements were differentially transcribed. However, the affected mobile genetic regions and the transcription direction were different for each mutant, indicating that it is a specificity response to the particular alteration induced on the cell by each mutation in particular. Both mutations seem to alter the transcription and likely the stability of the prophage regions.

The functional categories of genes that have more determinants being affected by the inactivation of *vanB* gene are energy metabolism, signal transduction, transport and binding proteins and the hypothetical and unknown function proteins. Several PTS systems involved on sugar uptake are downregulated. Genes involved in cell wall biosynthesis and sugar metabolism through glycolysis are also downregulated together with genes involved in the chorismate pathway. Simultaneously, we observed up-regulation of genes related to NamMN uptake, methionine, purine and pyrimidine biosynthesis.

In the absence of *vanR* many genes involved in cell envelope and energy metabolism, signal transduction and transport and binding proteins were downregulated, suggesting that these genes might be, directly or indirectly under *vanR* influence.

The newly potential Van-regulated targets include other factors, besides vancomycin resistance genes, with proteins implicated in several metabolic pathways being affected by these two genes. Our results suggest that the *vanB* operon genes might have a much wider role than previously described.

INTRODUCTION

Enterococcus faecalis is adapted to survive, persist and proliferate in a wide range of environments as different as the gastrointestinal tract, heart valves, water and soil (Mundt, 1986; Tannock, 2002).

Several organisms have developed sophisticated signaling systems for eliciting a variety of adaptive responses to their environment. Adaptive response systems is a class present in prokaryotes, lower eukaryotes, and plants which consist of at least two signal transduction proteins and are therefore referred to as two-component regulatory systems. Genomic sequencing of various microorganisms has revealed the presence of many two-component regulatory systems in every species. In *E. faecalis* V583, 17 sensor kinases and 18 response regulators were found (Paulsen *et al.*, 2003). Of the enterococcal two-component systems, only three have been characterized and assigned a role in the cell. CroRS is implicated in resistance to antimicrobial agents that target the cell wall, like β-lactams (Comenge *et al.*, 2003). It is known that the FsrCA is known to be activated by a quorum-sensing mechanism (Nakayama *et al.*, 2001) and has as role in biofilm formation and virulence (Hancock & Perego, 2004a) (Qin *et al.*, 2000). VanRS is by far the most well-known enterococcal two-component system and its role in the regulation of vancomycin resistance is well characterized (Arthur *et al.*, 1992, Arthur & Courvalin, 1993, Evers & Courvalin, 1996). However, the functions of the other systems remain unknown.

Transcriptomic approaches are powerful tools for the identification of groups of genes with similar expression patterns and for the establishment of putative regulatory relations. Using microarray technology, recent reports have described extensive changes in gene expression profiles when *E. faecalis* was challenged with different stress conditions (Chapter 4) (Aakra *et al.*, 2010, Aakra *et al.*, 2005,

Solheim *et al.*, 2007, Vebo *et al.*, 2009, Reyes-Jara *et al.*, 2010). A mutant on *fsrB* gene was studied also using a transcriptomic approach and the authors describe that *fsrB* influence a large number of genes belonging to different metabolic networks (Bourgogne *et al.*, 2006). These data show that regulator genes from two-component systems might have other cellular roles besides the ones directly attributed to its function as part of a TCS.

The sequencing of OG1RF strain revealed the presence of an ORF with 82% of similarity with $vanR_G$. $vanS_G$ and *vanY* homologues were also found (Bourgogne *et al.*, 2008). However, this OG1RF-unique two-component system does not seem to be a remnant form of a vancomycin resistance operon, but rather part of a still unknown regulatory pathway. Taking together, the fact that TCSs can regulate an extensive group of genes involved in different metabolic pathways and the description of a VanRS-like system, in an *E. faecalis* strain, that is not related to vancomycin resistance, gives strength to the belief that VanR might have a broader role on the cells, besides its well described role on vancomycin resistance.

We used custom made DNA microarray chips to profile the effect of *vanB* and *vanR* mutations on gene expression in the vancomycin resistant strain *E. faecalis* V583. Our results show that the *E. faecalis vanB* and *vanR* genes affect the expression of numerous genes in mid-exponential and early stationary growth phases.

MATERIALS AND METHODS

BACTERIAL STRAINS AND GROWTH CONDITIONS

This study was performed with *E. faecalis* V583::*tet*, V583*vanR::tet* (Hancock & Perego, 2004b), V583Ery^S and V583Ery^{S}Δ*vanB* (SAVE 2 from chapter 4). V583Ery^S is derived from V583 by deletion of *ermB* gene (EFA0007) (A. Benachour) and V583::*tet* is the V583 strain with p3TET inserted between e*hk*02 and the adjacent open reading frame encoding a putative lipoprotein (Hancock & Perego, 2004b). For the sake of simplification V583Ery^S and V583::*tet* will be referred from now on by V583. Strains were grown overnight in Brain Heart Infusion (BHI) medium (Oxoid), at 37°C. For microarray analysis, the overnight cultures were diluted 20 times and grown in BHI broth until an optical density of 0.4-0.45 at 600 nm was reached.

SAMPLING AND RNA EXTRACTION

Samples of each culture (5 ml) were collected 10 minutes (mid-exponential) and 30 minutes (early stationary) after cultures reached the OD 0.4-0.45. The double volume of RNA Protect Bacteria Reagent (QIAGEN) was added to cultures. The mixture was incubated for 5 minutes at room temperature, cells were centrifuged for 10 min at 3500 rpm at 4°C and pellets were kept at 4ºC until RNA extraction. Total RNA extraction was performed with RNeasy Mini columns (QIAGEN). RNA quality and quantity was determined by agarose gel electrophoresis and by measuring the absorbance at 260 and 280 nm, respectively. Total RNA from all samples was treated with RNase-free DNase I and incubated at 37ºC for 1 hour. Samples were further purified using RNeasy MinElute Cleanup kit (Qiagen) and quantified using the NanoDrop

Spectrophotometer (Nanodrop Technologies). 10 µg of each RNA sample was used for hybridization experiments.

ENTEROCOCCAL DNA CHIP DESIGN

The Affimetrix microarrays used were previously designed and described in McBride *et al*. (2007). Briefly, the microarray was designed to contain a total of 3582 probes including 3182 predicted ORFs from the chromosome of strain V583, additional pathogenicity island genes of strain MMH594, known to be absent in V583, and *E. faecalis* plasmid and antibiotic resistance genes or clusters from other *E. faecalis* strains for which nucleotide sequences had been reported, namely the *vanA* operon, *blaZ*, *bcr* operon, *vanG* operon, *vanE* operon, *tetM*, pRE25 plasmid/cat, as well as genes present in V583 endogenous plasmids pTEF1, pTEF2, and pTEF3. Additionally, microarrays included 111 Affymetrix designed eukaryotic and prokaryotic negative control probe sets. A full list of probe sets including genes represented and excluded from the microarray is available in the ArrayExpress public repository at http://www.ebi.ac.uk/ under accession number E-MEXP-1090. Details of the algorithm used in construction of custom Affymetrix GeneChips are available at the manufacturer's website (www.affymetrix.com/technology/index.affx).

DNA MICROARRAY DATA ANALYSIS

For each condition three batches of RNA were obtained in three separate growth experiments to provide true biological replicates. Each set of independent RNA samples was used to perform the microarray hybridizations. cDNA synthesis, fluorescent labelling, oligonucleotides array hybridization and preliminary data analysis was performed by the company Genome Explorations (Memphis, Tennesse).

Average relative fold changes were calculated from the average of the signal log ratio (SLR) from three separate experiments by using the following equations: for an SLR of ≥0, average relative fold change = 2^{SLR}; for an SLR of <0, average relative fold change =$-1\times2^{(-1\times SLR)}$ (Ng *et al.*, 2003).

REVERSE TRANSCRIPTASE PCR (RT-PCR)

To independently confirm the differential expression observed by DNA microarray experiments some genes were selected for analysis by semi-quantitative RT-PCR. The selected genes and primers used are listed in Table 1 and were synthesized and purchased from Eurofins (Ebersberg, Germany).

Total RNA preparation was performed exactly as described for the microarray assay. Residual DNA was removed using RNase-free DNase I (Invitrogen). cDNA was synthesized using Transcriptor High Fidelity cDNA Synthesis Sample (Roche) according to the manufacturer's instructions and using 4 µg of RNA as template.

Table 1. Primers used in this study to perform RT-PCR

Primer	Sequence (5'→3')
EF1811_fw	GCACTCCGAGAAACGGTTAG
EF1811_rev	GCTCTTGATTTCCCGCTGTA
EF2528_fw	GGTGGGTATGCTTCGGTAGA
EF2528_rev	TTCCATCAGCTCATTTGTTCC
EF2527_fw	TTGAACGCAATACTAAAGTAGCAA
EF2527_rev	AATCCAGCTTCTTCTCCATCC
EF1533_fw	GGCCAAGTTTTACGCAGAGA
EF1533_rev	GTATTGATGTGGGCCCCTTT
EF0568_fw	ATCGGGATTGCAGGTATGAG
EF0568_rev	ATCAAGGCCACGATAACGAC
EF0739_fw	GCATCATTTGTGTGAATTTAATTGCC
EF0739_rev	CCTAACAAAATATATGCAGCTACG
EF0746_fw	GCAACAAGCCAACACGCCAA
EF0746_rev	GCAGGAATTGAGAGCACTTGG
EF2777_fw	CCATTTATCACAAGAACGAGAGC
EF2777_rev	CCAGTGAAACAATCCAACTCAG

fw, forward; rev, reverse.

MIC DETERMINATION

Vancomycin MIC values for V583, V583Δ*vanB* and V583*vanR::tet* mutants was determined using vancomycin E-tests (AB Biodisk), according to manufacturer´s instructions.

RESULTS AND DISCUSSION

Using two mutants in genes essential for vancomycin resistance (*vanB* and $vanR_B$) we searched for genes putatively connected with *van* operon genes, in an attempt to unravel any possible gene network involving *vanB* operon. This could give us clues on the role of *vanB* operon in the cell besides the known role in vancomycin resistance.

The experimental design consisted in defining the transcriptomic profile of the two mutants at two different growth curve points, namely mid-exponential and early stationary. Cells were harvested at the mentioned sampling points, RNA was prepared and the number and nature of genes which underwent change in transcriptional pattern was evaluated using a custom made Affymetrix DNA microarray.

To our knowledge, the impact of the inactivation of these two genes at a global cellular level was never described before. The two mutants showed changes in properties like the vancomycin susceptibility (Chapter 4) (Hancock & Perego, 2004b), as expected and previously described, and the OD value at the late stationary phase. The mutants presented a reduction on the OD value reached at the late stationary phase, with a decrease on the OD value reached of about 14-19%, when compared with their wild-type strains (Figure 1).

All the strains were sampled 10 and 30 minutes after they reached an optical density of 0.4-0.45. These two sampling points were chosen to collect cultures at two moments of the growth curve, mid-exponential and early-stationary growth phases. However, as the wild-type strains grow more than the mutants the growth stages at which the wild-type strains were sampled do not correspond exactly to the growth stages of the mutants. Aware of the potential

biological differences that sampling at different stages might impose, we intend to be cautious during the analysis of the presented data.

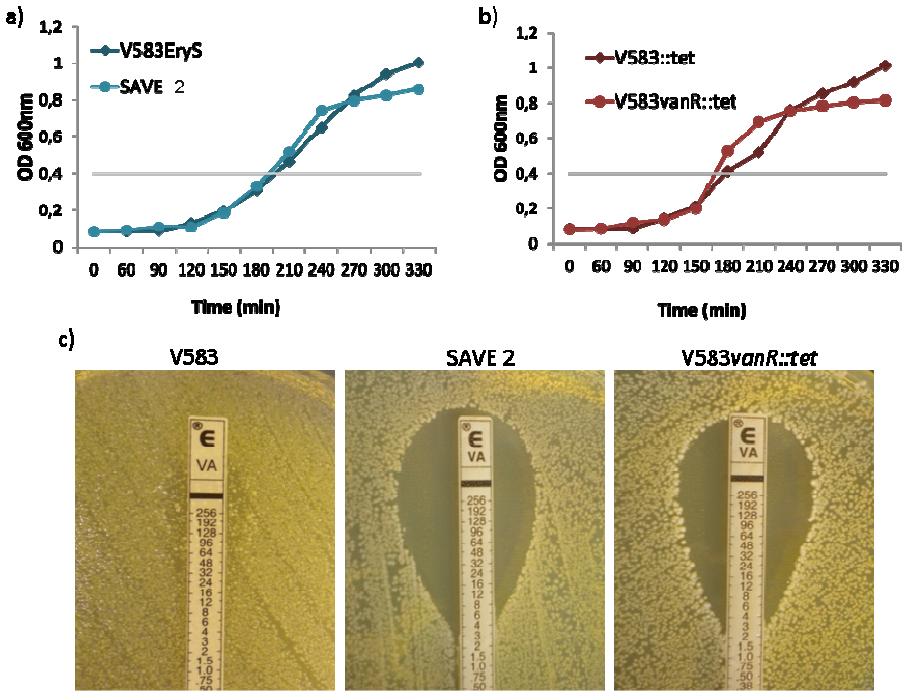

Figure 1. Phenotypic characterization of V583, SAVE 2 (V583Δ*vanB*) and V583*vanR::tet.* Growth curves were monitored at OD_{600}, a) V583Ery^S and SAVE 3; b) V583::*tet* and V583*vanR::tet*; (b) vancomycin MIC value was determined by E-test.

Analyzing the overall microarray results we observed that, in general, the fold-change values were lower for *vanR* mutant than for *vanB* mutant. Based on the differences observed on the fold-change values for both mutants we decide to assume a cutoff of 2.0 for the genes which are affected on *vanR* mutant and of 3.0 on the *vanB* mutant. If we had chosen for the analysis of the results obtained for the *vanR* mutant the same cutoff value (-3>|fold-change|>3) we used for the *vanB* mutant we would be reducing the number to about half. The goal was to find a good compromise between stringency of the criteria used and the relevance of

the information that a broader group of data can provide. Also, another study on the analysis of an *fsrB* mutant, also a two-component system regulator gene, adopted the same fold-change cutoff (Bourgogne *et al*., 2006).

Most likely, the genes that are differentially transcribed at both sampling moments constitute the core genes altered by the inactivation of these genes. In turn, genes that are only detected at one of the growth phases samples, are likely to be growth phase dependent.

vanB DELETION INDUCED CHANGES IN THE OVERALL TRANSCRIPTIONAL PROFILE IN V583

With a threshold value set at 3.0, 205 genes were affected at mid-exponential phase (41 up-regulated and 164 downregulated) out of the total of 3182 described open reading frames (ORFs) for *E. faecalis* V583. At early stationary phase the transcription of 309 genes was affected (52 up-regulated and 257 downregulated) (Table S1). With a threshold set at 5.0 the number of genes affected at mid-exponential and early stationary phases was reduced to 139 and 201 genes, respectively. These numbers reveal the high fold-change values observed, underlining the relevance of these results. Figure 2 illustrates the high fold-change values observed for the many of the genes that were differentially transcribed on the *vanB* mutant. It is observed a considerable overlap between genes differentially transcribed at both growth phases, with 178 genes common to both stages of the bacterial growth.

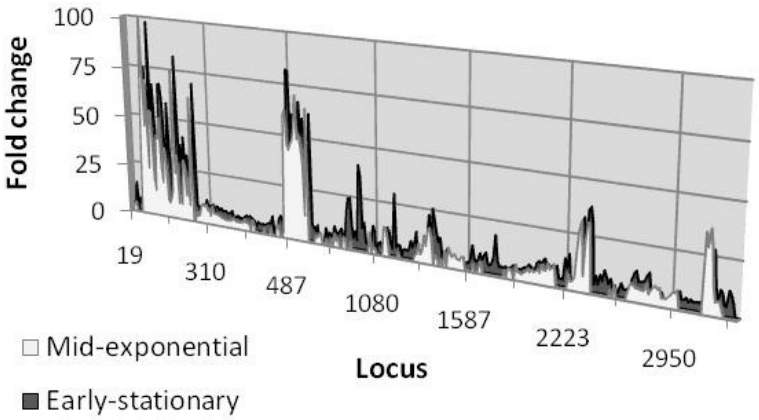

Figure 2. Representation of the fold change value for the genes differentially expressed in the absence of *vanB* gene.

A selected group of 8 genetic determinants showing differential transcription on the SAVE 2 mutant was chosen to test the validity of the microarray data using RT-PCR. The RT-PCR and microarrays results correlate well.

vanB DELETION INDUCES CHANGES IN THE EXPRESSION OF MOBILE GENETIC ELEMENTS (MGE) DETERMINANTS

Analysis of the results with *vanB* mutant revealed a high number of genes belonging to mobile and extra-chromosomal elements, namely integrated plasmids, phages and unidentified mobile genetic regions. The numbers reached 72% and 55% of the total number of genes differentially expressed in mid-exponential phase and early-stationary phase, respectively (Figure 3). MGE constitute about ¼ of the V583 genome sequence (Paulsen *et al*., 2003) and 175 (out of approximately 680 chromosomally MGE) were differentially transcribed in this study, corresponding to about 25% of all MGE genes.

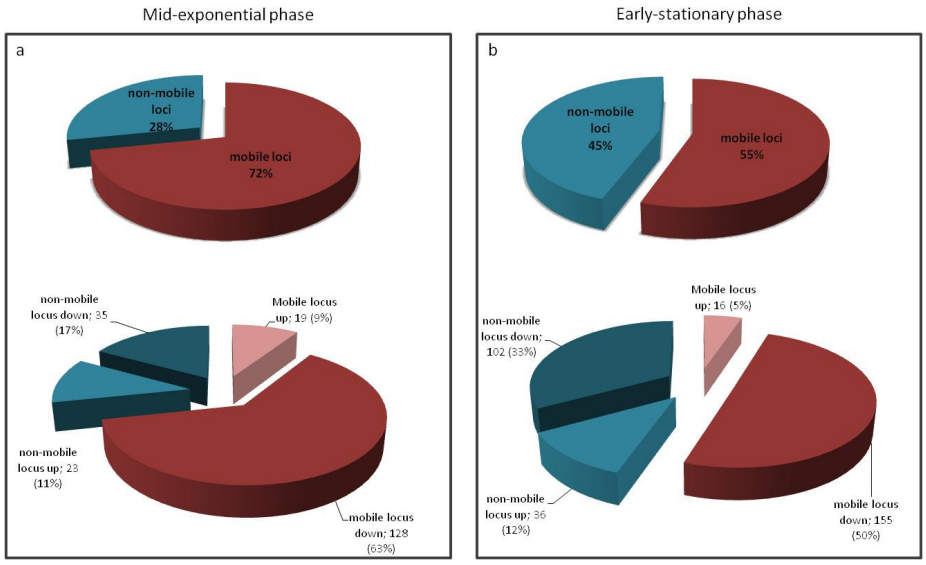

Figure 3. Numbers and percentages of genes differentially expressed plotted according to its mobile nature a) at mid-exponential phase and b) at early stationary phase.

The distribution and number of genes belonging to different mobile genetic regions described in V583 genome is presented in Figure 4. There is a clear tendency for genes located in MGE to be downregulated in *vanB* mutant (Figure 4), except for PAI genes (EF0479-EF0628) which were mostly upregulated. However, and according to Paulsen *et al.* (2003) the genes comprised between EF0506-EF0485 correspond to an integrated plasmid located within the PAI and appears to be derived from pTEF1-like plasmid. Thus, from the 18 PAI genes found to be differentially expressed, 14 are actually from this putative integrated pTEF1-like plasmid.

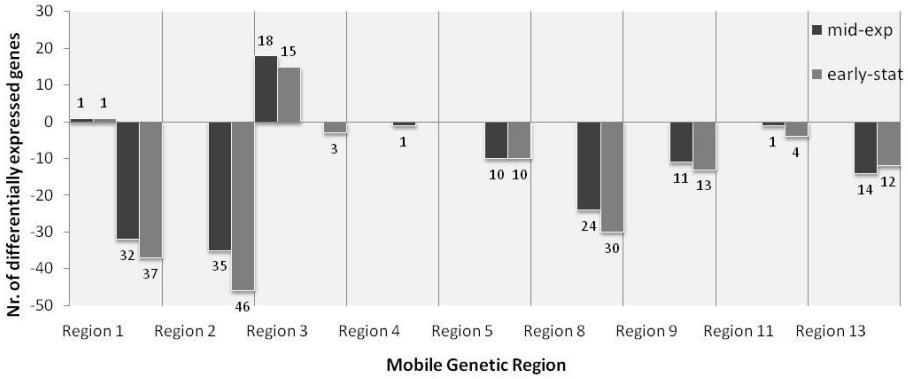

Figure 4. Distribution of genes differentially expressed in the *vanB* mutant within the mobile regions according to (McBride *et al.*, 2007).

E. faecalis V583 PAI encodes virulence determinants, namely enterococcal surface protein (Esp), cytolysin, and aggregation substance. However, it contains many components of unknown function which are hypothesized to facilitate survival and/or transmission in the health care setting (Shankar *et al.*, 2002; Paulsen *et al.*, 2003).

Genes belonging to region 4 (phage 02), region 6, region 7 (phage 04), region 10 (Tn/vanB) and region 12 (phage 06) were not affected by *vanB* absence. *E. faecalis* V583 carries seven chromosomally located prophages (phage 01 to phage07) (Paulsen *et al.*, 2003). These putative prophages are most closely related to phages from other low-GC Gram-positive bacteria. The integrated phage regions encode multiple homologs of *Streptococcus mitis* PblA and PblB, which have been implicated in binding human platelets, an important interaction in pathogenesis of infective endocarditis (Bensing *et al.*, 2001). Genes encoded within the MGE show a transcriptional tendency. Within the phage regions, for instance, 90% of the ORFs align with the direction of replication. Strong selective pressure for genes to be transcribed in the direction of replication

appears to be a common feature of the low-GC Gram-positive bacteria (Paulsen *et al*., 2003).

Within the firmicute division of Gram-positive bacteria, temperate bacteriophages are key vectors for the horizontal transfer of virulence genes. In *Staphylococcus aureus*, bacteriophages encode and mobilize an impressive array of immune evasion genes (van Wamel *et al.*, 2006; Tormo *et al.*, 2008) and Panton-Valentine leukocidin (Prevost *et al.*, 1995). Several bacteriophage-encoded virulence determinants also contribute to pathogenesis in group A *Streptococcus* (Banks *et al.*, 2003; Aziz *et al.*, 2004; Aziz *et al.*, 2005).

Not much information is available about prophages and their role in enterococcal genomes. From the enterococcal genomes recently made available (Bourgogne *et al*., 2008; Palmer *et al.*, 2010) it is possible to extract some information about the prevalence of prophages/prophage-like regions in *Enterococcus*. It seems that phage 02 is widely disseminated among *Enterococcus* spp. from different sources (clinical and environmental) and it was proposed to be part of *E. faecalis* core genome (McBride *et al*., 2007). Also, phage 04 and phage 06 are common among different strains. Phages 02, 04 and 06, the most prevalent among enterococcal strains, are the ones which genes were not differentially expressed in our study. Most likely these phages are not functionally related to *vanB* gene or *vanB* operon. In another transcriptional study, where V583 was exposed to chloramphenicol, the authors also observed the differential transcription of genes located on MGE (8% of total MGE genes) (Aakra *et al*., 2010). Genes from phages 02 and 04 were also not affected, however the authors did observe genes from phage 06 differentially expressed but it is not clear how many. The authors mention the upregulation of genes from phages 01, 06 and 07 and downregulation of PAI genes, exactly opposite from

what we observe in this study. Taken together, this might indicate that MGE are sensitive either to chromosomal alterations, such has deletion of a gene and to cellular perturbations imposed to the cells, such as the exposure to an antibiotic.

We can speculate that the most prevalent phages among different strains are more stable and not easily disturbed. However, at this point, and with the restricted number of transcriptomic studies it is not possible to establish a consistent hypothesis for the transcriptional destabilization pattern of prophage genes.

The actual knowledge on the role and metabolism of enterococcal phages is scarce. However, we can speculate that the observed downregulation of many of the genes within the prophages, that suffer transcriptomic alterations, might be associated with a destabilization of the genome created by the absence of *vanB* gene. It is conceivable that a chromosome destabilization trigger the entrance of prophages in a lytic or lysogenic state.

vanB **DELETION INDUCED CHANGES IN THE EXPRESSION OF NON-MGE DETERMINANTS**

In the *vanB* mutant we did not detect differential transcription of the other *vanB* operon genes, meaning that the deletion of *vanB* gene itself does not affect the transcription of the other vancomycin resistance determinants.

Regardless the presence of *vanB* ligase, which catalyzes the formation of the depsipeptide D-Ala-D-Lac that will be incorporated onto peptidoglycan, bacteria carries a chromosomally encoded *ddl* ligase gene that catalyzes the formation of D-Ala-D-Ala. This means that the normal peptidoglycan precursors are always being synthesized. However, if *vanB* is present, and because it is transcribed, it is likely that D-Ala-D-Lac is incorporated in the nascent peptidoglycan.

Many genes from different functional categories were differentially transcribed and the most affected by inactivation of *vanB* gene were energy metabolism, signal transduction, transport and binding proteins and hypothetical and unknown function proteins (Figure 6).

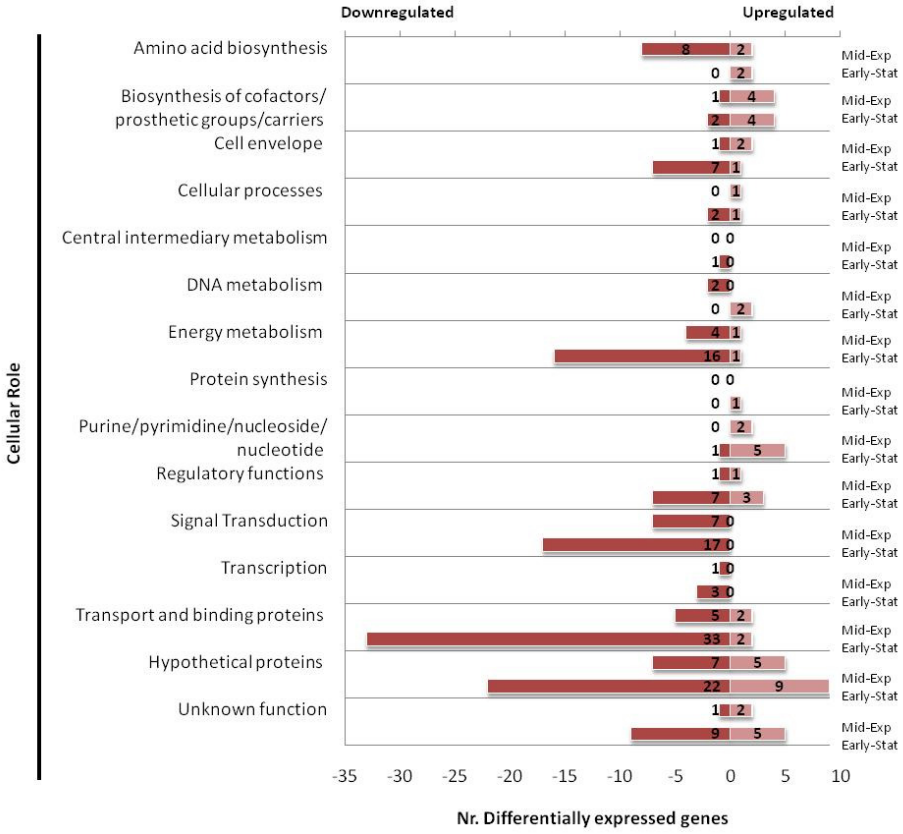

Figure 5. Number of differentially transcribed genes (up- and downregulated) at mid-exponential and early stationary phases, assembled according to their cellular role (based on http://cmr.jcvi.org). The genes with more than one cellular role were counted twice.

Overall genes were mainly downregulated and there are always more genes differentially expressed at early stationary phase than at mid-exponential phase, with clear exception of genes involved in amino acid biosynthesis. At mid-

exponential phase 8 out of 10 genes involved in amino acid biosynthesis were downregulated and at early-stationary phase only the same two genes are upregulated (EF0289 and EF0290). Figure 6 shows which genes are up- and downregulated and their role on the cellular metabolism.

Transcriptional changes appear to be operon specific, since within the same operon the genes always follow the same tendency, either up- or downregulated (Table S1).

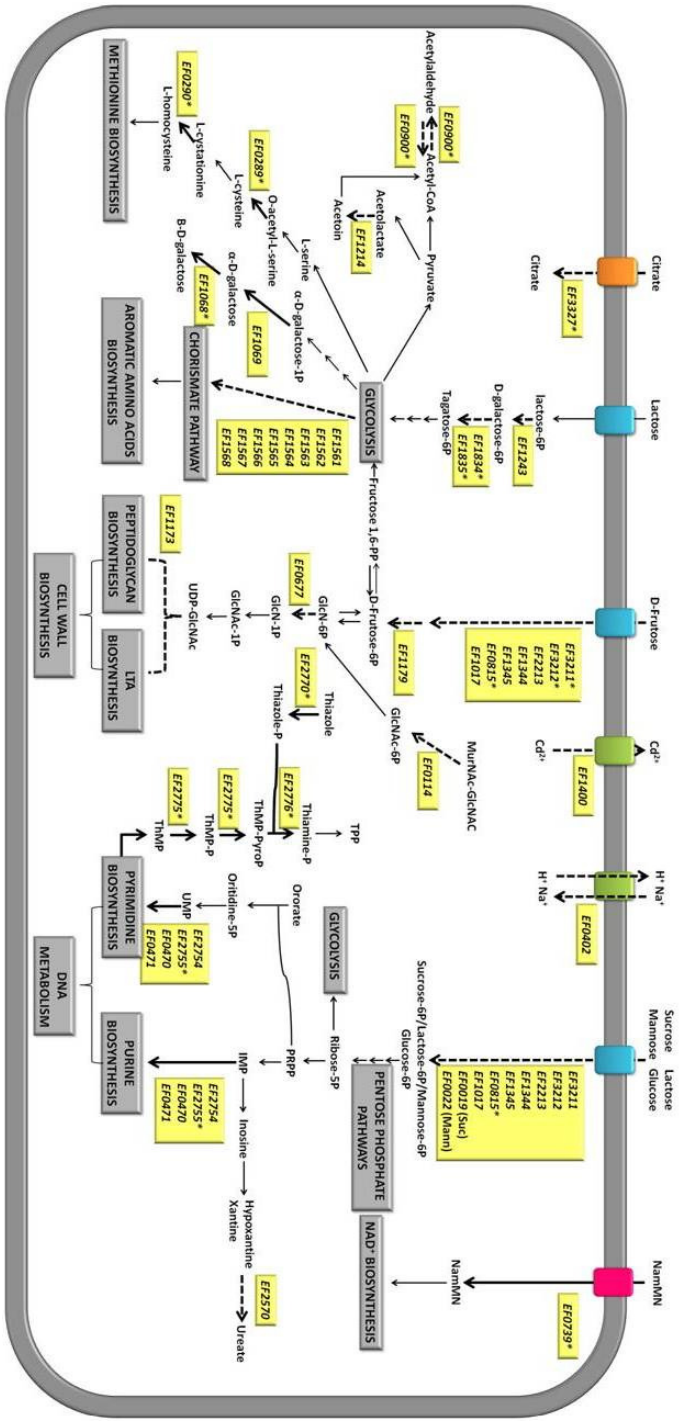

Figure 6. Metabolic pathways with genes differentially transcribed in *vanB* mutant. Reactions catalyzed by induced genes are indicated by solid bold arrows and those catalyzed by repressed genes are indicated by bold dashed arrows. Genes found to be differentially expressed are shaded in yellow. EF locus marked with a * were differentially expressed at both sampling points. GlcN, glucosamine; GlcNAc, N-acetylglucosamine; UDP-GlcNAc, undecaprenyl N-acetylglucosamine; MurNAc, N-acetyl muramic acid; LTA, lipotheicoic acid; UMP, uridine monophosphate; IMP, inosine monophosphate; PRPP, phosphoribosyl pyrophosphate; NAD^+, nicotinamide adenine dinucleotide; NamND, nicotinamide mononucleotide; ThMP, hydroxymethylpyrimidine; TPP, Thiamine pyrophosphate; thiazole, 4-methyl-5(β-hydroxyethyl)thiazole.

PTS (phosphoenolpyruvate phosphotransferase system transporter) sugar uptake and phosphorylation require the participation of a number of soluble and membrane-bound enzymes. These proteins catalyze the transfer of the phosphoryl moiety of the phosphoenolpyruvate to the sugar substrate. The overall reaction requires Mg^{2+}, and the products formed are sugar phosphate and pyruvate (Dills *et al.*, 1980). *E. faecalis* V583 genome contains 35 putative PTS systems, and pathways for exploitation of 15 different sugars have been predicted (Paulsen *et al*., 2003). Its genome also encodes several ABC transporters and other sugar uptake systems. PTS systems responsible for the uptake and phosphorylation of at least 5 different sugars were identified in our study and were all downregulated. In addition, at least two sugar ABC transporters (EF1344 and EF1345, which are not part of the same transcriptional unit according to biocyc software) were also downregulated. These results suggest that the uptake of sugars in particular is being shutdown in the *vanB* mutant (Figure 6). However, the uptake of nicotinamide mononucleotide (NamMN) is being induced. This uptake system is mediated by EF0739 which encodes a putative nicotinamide mononucleotide transporter PnuC. Although the biosynthesis *de novo* of NAD^+ uses precursors such as tryptophan and aspartic acid, the salvage metabolic pathway uses nicotinamide (Nam) and other analogous molecules like nicotinic acid (NA) and nicotinamide riboside (NR) (Zhu *et al.*, 1989). NAD^+ is used in abundance by the cell since it is a cofactor of many redox reactions, such as posttranslational modifications.

DNA ligases catalyze the formation of phosphodiester linkages between 5'-phosphoryl and 3'-hydroxyl groups in double-stranded DNA. These enzymes are essential for DNA replication and repair of damaged DNA (Wilkinson *et al.*, 2001). All bacteria encode at least one DNA ligase which uses NAD^+ as a coenzyme and

as the energy source for the reaction (Little *et al.*, 1967; Wilkinson *et al.*, 2001). V583 genome encodes a NAD^+-dependent DNA ligase (EF0722, *ligA*) which was not found to be differentially expressed in this study. The EF0739 nicotinamide transporter is upregulated at both points on the growth curve (3.06-fold change at mid-exponential and 5.41-fold change at early stationary), which might indicate the relevance of this transporter in response to absence of *vanB* gene.

We found genes belonging to purine and pyrimidine biosynthesis to be upregulated, namely EF0470 (*nrdF*) and EF0471 (*nrdE*), which encode the two subunits of the ribonucleoside-diphosphate reductase, and EF2754 (*nrdD*) and EF2455 (*nrdG*), which encode two ribonucleoside-triphosphate reductases. Ribonucleoside reductases catalyze the formation of deoxyribonucleotides from ribonucleotides. Considering the induction of determinants related to the NamMN uptake, methionine, purine and pyrimidine biosynthesis our results suggest that the cell is committed in maintain DNA replication and DNA protection and repair systems working properly. We observe upregulation of two genes involved in the methionine biosynthesis (EF0289 and EF0290, encoding a cysteine synthase and a cystathionine beta-lyase, respectively). The fact that methionine is the universal N-terminal amino acid of proteins as well as the use of its derivative *S*-adenosylmethionine (SAM) in a variety of methyltransferase reactions argue for the importance of methionine in the cellular metabolism.

The genes involved in cell wall biosynthesis and sugars metabolism through glycolysis are downregulated, as well as the genes involved in the chorismate pathway. Further work is need to formulate a robust hypothesis regarding other cellular roles of *vanB* ligase on the cell can be established however, this data indicate that *vanB* is likely to play a role other than just vancomycin resistance.

In *Saccharomyces cerevisiae* NAD^+ also has recently been implicated on the control of thiamine homeostasis (Li *et al*., 2010). The authors found that lowering the NAD^+ concentration the intracellular thiamine concentration is elevated due to increased thiamine biosynthesis and transport (Li *et al*., 2010). Thiamine phosphate derivatives are involved in many cellular processes. The best characterized form is thiamine pyrophosphate (TPP), a coenzyme in the catabolism of sugars and amino acids. In general TPP functions as a cofactor for key enzymes such as enzymes such as pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase, branched chained- 2-oxoacid dehydrogenase and transketolase (Pohl *et al*., 2004). Genes encoding thiamine biosynthesis enzymes in microorganisms are tightly regulated such that low environmental thiamine concentrations activate transcription and high concentrations are repressive. If there is sufficient thiamine present in the cell then the thiamine binds to the mRNA encoding genes required in the pathway preventing the translation of the enzymes. If there is no thiamine present then there is no inhibition, and the enzymes required for the biosynthesis are produced. We found the thiamine biosynthetic pathway upregulated with the four genes of the operon (EF2775-EF2776-EF2777-EF2778/*thiD-thiE-thiM-thiW*) being induced both at mid-exponential phase and early-stationary phase, with fold changes varying between 5.88 and 6.50 on mid-exponential phase and 8.41 and 11.39 at early-stationary phase. There might be a metabolic correlation between the induction of the NamMN transporter and the thiamine biosynthetic pathway. However, if there is, seems to be different from the one described on *S. cerevisiae* (Li *et al*., 2010).

Physiological processes, in virtually all organisms, require metal ions as cofactors, and cellular depletion of particular metals causes defects in numerous

biological functions. However, certain metal ions, such as cadmium, are harmful to organisms. We found a cadmium-translocating P-type ATPase being downregulated (EF1400). To our knowledge, there are no studies attributing a function for EF1400 and it is possible that this is not a cadmium-specific transporter but a transporter of a different(s) metallic cation(s). It is known that some bacteria are able to remove metal ions from the media in a detoxifying process (Momose & Iwahashi, 2001; Topcu & Bulat, 2010). In the conditions of our experiment cadmium is not present on the cells, so the activation of a detoxifying mechanism is in theory unnecessary and might represent an energetic burden to the cells. However, we may be witnessing not a direct effect of cadmium presence or any other metal that can be a substrate of this transporter, but an indirect effect by the fact that this extrusion mechanism being shut down likely to save energy for other cellular processes. At this point, these hypotheses are mere speculations.

vanR$_B$ MUTATION INDUCED CHANGES IN THE OVERALL TRANSCRIPTIONAL PROFILE OF V583

It is known that *vanR$_B$*, together with its cognate histidine kinase *vanS$_B$*, in presence of vancomycin regulate the *vanB* operon by binding to its own promoter P_{RB} and to the downstream promoter P_{YB} that regulates the expression of *vanY$_B$WH$_B$BX$_B$* genes.

In total, at both sampling time points, 249 different genes were found to be differentially expressed in the V583Δ*vanR$_B$* mutant when compared to V583, which corresponds to nearly 8% of the total number of ORFs in the V583 genome. 83 genes were significantly regulated at both sampling points. These

determinants are the ones that are likely *vanR*-regulated, since is likely that they are independent of the growth phase.

Inactivation of *vanR* gene had an overall effect in the cell since genes from several different cellular roles were affected. Table 2 lists the number of genes differentially expressed and organized by cellular role. The cellular role categories with more genes differentially transcribed are genes associated with functions on mobile and extrachromosomal elements, signal transduction, transport and binding and hypothetical/unknown function proteins. In addition, 25 genes located on V583 endogenous plasmids were differentially transcribed, 8 located on pTEF1 (EFA) and 17 located on pTEF2 (EFB). Moreover, all plasmid-located genes are downregulated and only detected at mid-exponential phase. No plasmid-located gene was found at early-stationary phase.

A complete list of all the genes differentially expressed is presented on Table S2 as supplemental material.

Table 2 – Number of genes differentially expressed in the *vanR* mutant compared with V583 wild type, grouped according to cellular role

Cellular role category	Nr. differentially expressed genes[a]			
	Mid-exp		Early-stat	
	Up	Down	Up	Down
Amino acid biosynthesis	0	1	0	0
Biosynthesis of cofactors/prosthetic groups/carriers	0	1	0	0
Cell envelope	0	7	4	1
Cellular processes	3	3	4	0
Central intermediary metabolism	1	1	0	0
DNA metabolism	1	0	4	0
Energy metabolism	0	5	1	0
Mobile and extrachromosomal element functions	2	13	11	7
Protein fate	0	0	1	0
Purine/pyrimidine/nucleoside/nucleotide	0	1	0	0
Regulatory functions	0	3	4	0
Signal Transduction	0	13	0	3
Transcription	0	1	0	0
Transport and binding protein	0	14	4	2
Hypothetical proteins	8	36	29	6
Unknown function	20	42	57	9
TOTAL	**35**	**141**	**119**	**28**
	176		**147**	

[a]Genes assigned to more than one cellular role were counted twice.

vanR$_B$ MUTANT INDUCED CHANGES IN THE EXPRESSION OF MGE DETERMINANTS

The origin and number of genes found to be differentially expressed are represented on Figure 7. In mid-exponential phase a total of 157 genes were detected and from these 89 are located on MGE and only 58 are located on the "native" chromosome. In early-stationary phase, 118 out of the 147 differentially expressed genes belong to the MGE described for V583 genome and only 19 genes are located on non-mobile regions of the genome.

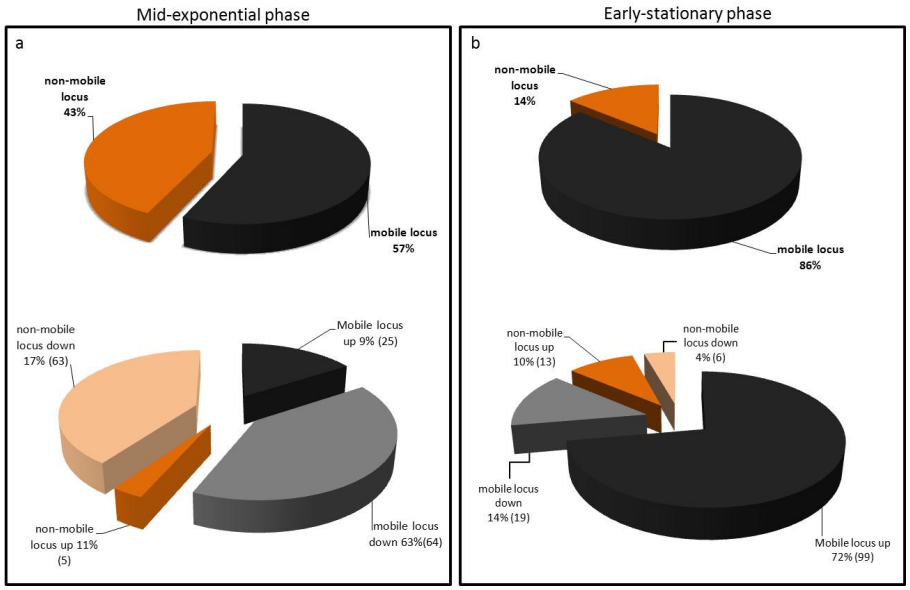

Figure 7. Numbers and percentages of the genes with differentially expression levels plotted according to its mobile nature; a) at mid-exponential phase and b) at early stationary phase.

There is not a preferential pattern of up- or downregulation among the genes belonging to different MGE (Figure 8). Overall, there is upregulation of genes from region 1, region 8 (phage 05) and region 11 (phage 06) and downregulation of genes from region 2 (phage 01) and region 03 (PAI). Regions 6, 12 (phage 06) and 13 (phage 07) have no representatives amongst the genes differentially expressed. The remaining regions have low number of genes being differentially transcribed. Within the same MGE the behavior of the genes is very consistent with all the genes adopting the same tendency (up- or downregulated).

However, depending on the growth phase at which the cultures were sampled there are major differences. For example, at mid-exponential phase determinants from region 5 (phage 03) were not found to be differentially expressed. On contrary, at early-stationary the genes from region 6, region 7

(phage 04), region 9 and region 12 (phage 06) were not affected by *vanR* inactivation.

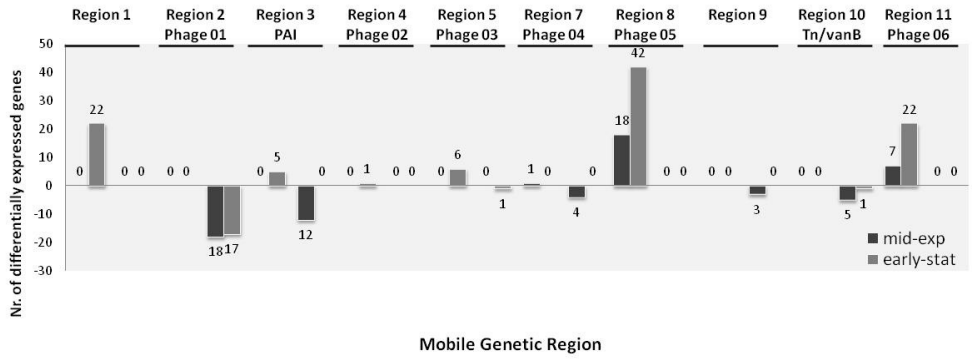

Figure 8. Distribution of the genes differentially expressed in the *vanR* mutant within the mobile regions according to McBride *et al.* (2007)(McBride *et al*., 2007).

According to Paulsen *et al.* (2003), the PAI (region 3) (EF0479-EF0628) contains an integrated plasmid (between EF0506-EF0485) that is likely a derivative from pTEF1-like plasmid. Curiously, the genes from this putative PAI-integrated plasmid are downregulated at mid-exponential phase but suffered no change at early-stationary phase. There is an operon (*kdpF-kdpA-kdpB-kdpC*) encoding a predicted ATP-driven transport of potassium that is upregulated (except *kdpF* which was not detected in this study) at early-stationary phase and presents no change at mid-exponential phase. Potassium (K^+) is essential for life in both prokaryotic and eukaryotic cells. K^+ plays a vital role in bacterial osmotic adaptation, pH regulation, gene expression and activation of cellular enzymes. Also, both as an extracellular signal and as an intracellular metabolic regulator, plays critical role in adaptation to stressful environments in bacteria [for a review see (Ballal *et al.*, 2007)]. Under extreme K^+ limitation or osmotic upshock, the high affinity Kdp-ATPase is expressed (Laimins *et al.*, 1981). We observe enhanced

transcription of EF0567 (*kdpA*)-EF0568 (*kdpB*)-EF0569 (*kdpC*) at early-stationary phase, however we did not detect differential transcription of EF0566 (*kdpF*) nor the regulatory system Ehk12-Err12 (EF0570-*kdpD* and EF0571-*kdpE*). The function of this two-component system is not clarified in *E. faecalis* but has likely the same function as the *E. coli* and *L. monocytogenes* Kdp regulator systems.

COMPARISON OF THE EFFECTS OF *vanB* AND *vanR* INACTIVATION ON EXPRESSION OF MGE

Although we cannot compare results from the two mutants, it is striking that the only common genes belong to MGE. Also, as we established the threshold values for the analysis of the results it became clear that the transcriptomic results from the inactivation of the two vancomycin resistance determinants were clearly different.

Under the conditions tested the inactivation of either *vanB* or *vanR* genes resulted in a major adjustment of the transcriptional profile of the cell. Particularly unanticipated was the number of genes belonging to MGE that were differentially transcribed in both experiments. In fact, there is a considerable overlap and 83 genes are common to both experiments, of which 74 are located in MGE (Figure 9). However, some differences regarding genes located in MGE were observed, namely concerning region 11 and phage 06.

V*anR* inactivation lead to differential expression of 29 genes of phage 06, but *van*B deletion had no effect on phage 06 genes. Aakra and co-workers (2010) also report the up-regulation of genes from phage 06 when V583 is exposed to chloramphenicol (Aakra *et al*., 2010). The same authors describe the downregulation of PAI genes (Aakra *et al*., 2010). The behavior of the PAI genes is another major difference that we observe between the inactivation of these two genes. While with *vanB* inactivation the PAI genes were upregulated, *vanR*

inactivation lead to downregulation of PAI genes. This might indicate that MGE response to different cellular perturbations is specific to each disturbing factor/gene.

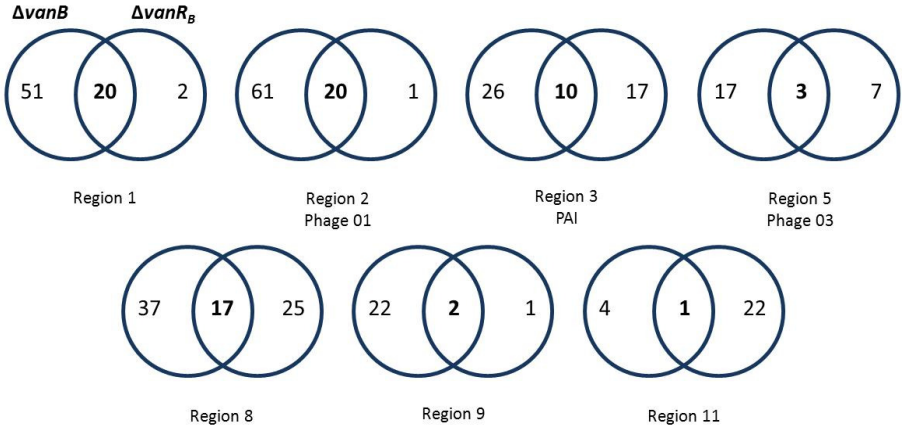

Figure 9. Diagram representing the overlapping of differentially expressed genes in the two experiments (inactivation of *vanB* genes and *vanR$_B$* gene). The numbers correspond to the total number of different genes differentially expressed in each experiment independently of the growth phase that they were detected. At the interception of each 2 circles is indicated the number of common genes in both experiments.

With so restricted knowledge of the prophage functions on the enterococcal genomes is not a goal to speculate about it here. However, our observations might be helpful in the future to unravel the role of these prophages and eventually indentify conditions that trigger their lytic and lysogenic cycles.

vanR$_B$ MUTATION INDUCED CHANGES IN THE EXPRESSION OF NON-MGE

Analyzing the group of genes not located on mobile genetic elements we can observe that the ones with higher fold-change values are hypothetical or unknown function proteins (Figure 10).

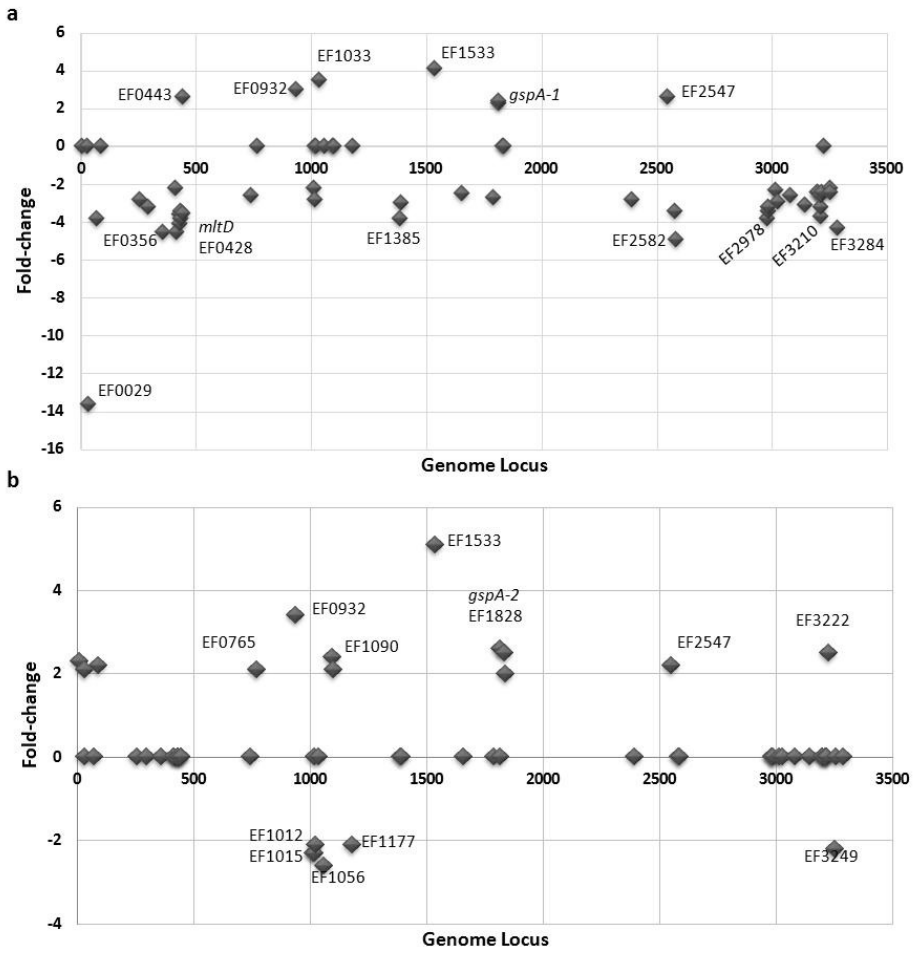

Figure 10. Representation of the non-MGE determinants found to be differentially expressed in *vanR* mutant. Determinants are represented according to their location on the V583 genome and in function of their fold-change value; a) mid-exponential phase; b) early stationary phase. EF2298 (*vanS*) is not represented.

At mid-exponential phase the ORF with higher fold-change is EF0029 which encodes an aminotransferase with unknown function, followed by EF0413 (*mltD*) encoding a mannitol-1-phosphate 5-dehydrogenase, EF1033 encoding a 6

aminohexanoate-cyclic-dimer hydrolase, EF1385 encode a putative molybdopterin-guanine dinucleotide biosynthesis protein A, EF1810 (*gspA-1*) encoding a general stress protein, EF2978 and EF3210 encoding PTS system components and EF3284 encoding a putative cystathionine gamma-synthase. EF0413 (*mltD*) is putatively transcribed from the same transcriptional unit than EF0411 (PTS system, mannitol-specific IIBC component) and EF0412 (*mltF*), involved on the transport and phosphorylation of carbohydrates. The determinants that suffered most significative transcriptional alterations encode for proteins that belong to different functional groups.

The transcriptional alteration of genes located on the V583 plasmids is just detected at mid-exponential phase. The fold-change for these genes is considerably high and are all downregulated. The majority of the plasmid-associated genes encode for hypothetical protein but among them there is downregulation of the aggregation substance (*asa1*), the surface protein (*prgC*) and a TraG family protein. Actually, *asa1* has the highest fold-change value with a transcriptional change of 10.9-fold. Aggregation substances enhance cellular aggregation or clumping facilitating plasmid transfer. Besides facilitating plasmids exchange potentially carrying virulence and antibiotic resistance traits, aggregation substances may increase adherence to intestinal and renal epithelial cells and to cardiac vegetations [for review see (Jett *et al.*, 1994)]. It thus is reasonable to speculate whether *asa1* transcription is *vanR* dependent or not and if the *vanR* mutant is affected on its conjugation potential and has a reduced virulence. However, further work is needed to elucidate the role of *vanR* in *E. faecalis* virulence aptitude.

pTEF1 and the pAD1 share extensive sequence similarity and the aggregation substance *asa1* encoded by pTEF1 is identical to the one of pAD1

(Paulsen et al., 2003). On the other hand pTEF2 share some regions of similarity with the sex pheromone plasmid pCF10, including identical copies of the conjugation genes *prgA-prgB-prgC*. According to Paulsen *et al*. (2003), *asa1* is a virulence genes predicted to be surface exposed and is an integrin since it contains a putative integrin-binding motif, while *prgC* is a signal peptide (Paulsen *et al*., 2003).

Also, at early-stationary phase the genes with the most significant fold-change values encode for hypothetical and unknown function proteins. The exceptions are EF1012 encoding a PTS system subunit, EF1811 (*gspA-2*) encoding a general stress protein and EF1828 encoding a glycerol uptake facilitator protein.

Comparing the results obtained for the *vanR* mutant with the results obtained on Chapter 4 for V583+Va10 µg/ml it is observed some overlap, with 33 genes common to both studies (Figure 11).

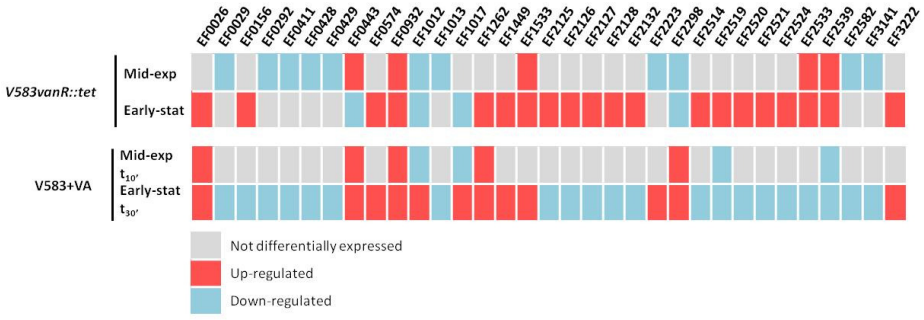

Figure 11. Comparison of the transcriptional tendency of the genes found to be common to *vanR* mutant and V583 exposed to 10 µg/ml of vancomycin (Chapter 4)**.**

The 10 min and the 30 minutes of vancomycin exposure correspond to the mid-exponential phase and to the early-stationary phase, respectively. Within this group of 33 genes we risk speculate that there are some genes which are regulated by *vanR*. Further studies, like gel shift mobility assays in presence and absence of induction by vancomycin, would be useful to confirm if *vanR* binds to the promoters of these *vanR*-regulated candidate genes. The genes we risk to point out as potentially regulated by *vanR* are the ones that present an opposite transcriptional behavior in both experiments. For example, if a gene is induced by vancomycin, assuming that the induction is *vanR*-dependent, in absence of *vanR* is expected to be down-regulated. Also, the strongest candidates are the ones that are differentially expressed at both sampling times, since are more likely related to vancomycin exposure and *vanR* absence than growth-related. We consider potential candidates for being *vanR*-dependent: EF2298 (*vanS*), EF2524 (sortase family protein), EF2533 (FtsK/SpoIIIE family protein, involved in cellular processes), and the hypothetical proteins (EF2514, EF2519, EF2520, EF2521 and EF2539). Also the hypothetical and unknown function proteins (EF0156, EF0443, EF2125, EF2126, EF2127 and EF2128), the PTS system components (EF1012 and EF1017) and EF2132 (recT protein, involved in DNA metabolism/mobile and extrachromosomal functions) are candidates however, they just presented differential expression at one of the sampling points.

In absence of *vanR* gene it is likely that other regulator genes are differentially expressed to overcome *vanR* absence. Also, regulators which transcription is *vanR*-dependent are potential candidates for members of secondary regulation pathways. The regulatory proteins that were affected by *vanR* inactivation throughout the different growth phases are listed in Table 3**.** In mid-exponential phase 4 genes coding for potential regulatory proteins showed

higher expression in the wild-type strain than in *vanR* mutant and were not detected on early-stationary phase.

There are two genes that were induced by *vanR* and encode for a two-component system, the Ehk02-Err02. The Ehk-Err02 two-component system shares similarity with the LytSR system in *Staphylococcus aureus* and the LytST system in *B. subtilis*. In *S. aureus*, LytSR system affects the rate of autolysis and penicillin tolerance (Brunskill & Bayles, 1996). LytSR was shown to regulate *lrgAB*, whose gene products modulate murein hydrolase activity and penicillin tolerance (Groicher *et al.*, 2000). LytSR system was found to sense decreases in $\Delta\psi$ (Patton *et al.*, 2006; Sharma-Kuinkel *et al.*, 2009) and consequently induce *lrgAB* promoter activity (Sharma-Kuinkel *et al*., 2009). *lrgAB* operon activity is directly correlated with *cidABC* operon which has been shown to be a regulator in the control of cell death and lysis (Groicher *et al*., 2000). Furthermore, the LytSR regulatory system was found to be necessary for normal biofilm development.

Table 3. Regulatory proteins influenced by *vanR$_B$* inactivation

Locus	Putative function	Fold-change	
		Mid-exp	Early-Stat
EF1423	transcriptional regulator, Cro/CI family	-	3.1
EF1886	transcriptional regulator, Cro/CI family	-2.7	-
EF2141	transcriptional regulator, Cro/CI family	-	2.2
EF2528	transcriptional regulator, Cro/CI family	-	3.1
EF2543	transcriptional regulator, putative	-	2.2
EF2981	sigma-54 dependent DNA-binding response regulator	-3.2	-
EF3196 (RR02)	response regulator	-2.4	-
EF3197 (HK02)	sensor histidine kinase	-2.4	-

RR, response regulator; HK, histidine kinase; Minus (-) indicates downregulation.

In *B. subtilis*, a comprehensive analysis of the two-component regulatory systems by use of DNA microarrays has revealed that LytST controls the expression of *ysbAB* (Kobayashi *et al.*, 2001). The YsbB protein is highly related to the *lrgB* gene product. Downstream of the *E. faecalis* HK-RR02 system is an operon with genes that share strong similarity to those of the *lrgAB* and *ysbAB* operons (EF3194-EF3193), suggesting the possibility that this two-component system is the *E. faecalis* orthologue of the Lyt system of *S. aureus* and *B. subtilis* (Hancock & Perego, 2002). However, no change in autolysis rate in a e*rr02* mutant was observed (Hancock & Perego, 2002).

Five regulator genes differentially expressed are located on MGE and four of them were induced on the *vanR* mutant, meaning that in presence of an intact *vanR* these genes are repressed. Whether the differential transcription of these regulator genes is directly related to *vanR* absence or indirectly related by some kind of chromosomal destabilization is yet to be established.

It is widely agreed that in bacteria the primary control of metabolism occurs at the transcription level. The wide range of determinants responding to inactivation of *vanB* and $vanR_B$ is a striking demonstration of the multitude and complexity of the transcription links that connect a large number of diverse cellular functions to a single gene involved on an alternative cell wall synthesis mechanism.

A comprehensive description of the alterations at the transcriptomic level induced by two independent *van*-genes mutations was the objective of this chapter. Aware that these data need confirmation, our goal at this point was to formulate hypothesis and raise some questions. The alterations in the *E. faecalis* transcriptome described in this study hopefully give a clue on how a single gene can be somehow involved in so many different cellular responses.

ACKNOWLEDGMENTS

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Supplemental Material

Table S1 – List of all genes found to be differentially expressed in a V583Δ*vanB* mutant. Genes are organized according to their chromosomal location/locus number. The gene name is listed when available for the V583 genome. Overexpressed genes have their fold change value in bold. Genes belonging to MGE are shaded in grey. Genes belonging to the same putative transcriptional unit are identified by a vertical bar.

Locus	Gene	Fold change		Description	Role
		Mid-exponential	Early-stationary		
EF0019		-	3.80	PTS system, IIB component	Transport and binding protein/Signal transduction
EF0022		-	3.42	PTS system, mannose-specific IID component	Transport and binding protein/Signal transduction
EF0054		-	14.25	Hypothetical protein	Hypothetical protein
EF0081		4.62	-	membrane protein, putative	Cell envelope
EF0095		4.62	6.25	lipoprotein, putative	Cell envelope
EF0097		-	4.41	regulatory protein pfoR, putative	Cellular processes
EF0114		-	4.00	glycosyl hydrolase, family 20	Cell envelope
EF0115		-	3.61	Endoribonuclease L-PSP, putative	Transcription
EF0124		-	11.39	Hypothetical protein	Hypothetical protein
EF0127		40.01	27.04	conserved hypothetical protein	
EF0128		70.56	74.82	Hypothetical protein	
EF0129		101.00	58.91	transcriptional regulator, Cro/CI family	
EF0130		45.56	24.26	Hypothetical protein	
EF0131		68.89	97.52	conserved domain protein	
EF0132		24.01	29.70	Hypothetical protein	

EF0133		53.66	66.42	Hypothetical protein		
EF0134		52.93	40.01	Hypothetical protein		
EF0135		19.14	31.36	conserved hypothetical protein		
EF0136		12.96	16.40	Hypothetical protein		
EF0137		41.60	66.83	nucleotidyltransferase domain protein		
EF0138		39.69	67.24	conserved domain protein		
EF0139		50.41	57.76	FtsKSpoIIIE family protein		
EF0140		25.00	33.06	conserved domain protein		
EF0141		17.43	16.20	Hypothetical protein		MGE, Region 1
EF0142		35.40	57.76	conserved hypothetical protein		
EF0143		29.98	30.53	transcriptional regulator, Cro/CI family		
EF0144		8.85	15.21	conserved domain protein		
EF0145		**7.56**	**8.12**	Hypothetical protein		
EF0146		10.24	20.03	surface exclusion protein, putative		
EF0149		13.88	20.48	aggregation substance, putative		
EF0150		75.69	81.90	membrane protein, putative		
EF0151		23.28	28.36	Hypothetical protein		
EF0152		32.21	37.21	Hypothetical protein		
EF0153		7.98	12.96	cell wall surface anchor family protein		
EF0154		12.08	41.60	conserved hypothetical protein		
EF0155		7.84	22.09	conserved hypothetical protein		
EF0158		30.53	32.49	conjugal transfer protein, putative		
EF0159		28.09	26.01	membrane protein, putative		
EF0160		12.60	17.43	conserved domain protein		
EF0162		21.39	28.36	Hypothetical protein		

EF0163		7.98	9.92	lipoprotein, putative		
EF0164		62.81	69.31	lipoprotein, putative		
EF0165		-	7.56	conserved hypothetical protein		
EF0166		-	7.02	site-specific recombinase, phage integrase family		
EF0289		**5.06**	**3.42**	cysteine synthase B, putative	Aminoacid biosynthesis	
EF0290	*metC*	**3.24**	**3.42**	cystathionine beta-lyase	Aminoacid biosynthesis	
EF0303		3.80	-	phage integrase		
EF0307		8.70	7.43	transcriptional regulator, Cro/CI family		
EF0308		9.77	11.06	conserved hypothetical protein		
EF0309		8.70	8.70	excisionase, putative		
EF0310		9.77	8.27	Hypothetical protein		
EF0311		7.43	8.41	Hypothetical protein		
EF0312		6.63	9.30	aspartate 1-decarboxylase domain protein		
EF0313		4.41	3.33	Hypothetical protein		
EF0314		9.46	8.70	Hypothetical protein		
EF0315		6.00	8.41	conserved hypothetical protein		
EF0316		5.76	6.89	Hypothetical protein		
EF0317		5.76	8.12	transcriptional regulator, Cro/CI family		
EF0318		5.18	7.02	conserved hypothetical protein		
EF0320		4.84	6.63	Hypothetical protein		
EF0321		4.31	7.16	Hypothetical protein		
EF0324		4.00	6.13	Hypothetical protein		
EF0326		3.61	6.25	conserved hypothetical protein		
EF0327		3.42	6.38	Hypothetical protein		
EF0328		3.61	7.70	conserved hypothetical protein		

EF0329	4.00	4.20	conserved hypothetical protein		
EF0330	3.61	4.20	SNF2 domain protein		
EF0332	5.52	5.64	conserved hypothetical protein	MGE, Region 2, Phage 01	
EF0333	4.31	5.41	conserved hypothetical protein TIGR01630		
EF0334	3.52	4.31	portal protein		
EF0335	4.10	4.95	minor head protein		
EF0337	3.52	4.84	Hypothetical protein		
EF0338	4.00	6.00	scaffold protein		
EF0339	3.52	6.63	major capsid protein, putative		
EF0340	5.64	6.13	Hypothetical protein		
EF0341	4.10	6.76	Hypothetical protein		
EF0342	4.00	5.88	Hypothetical protein		
EF0344	3.42	5.41	Hypothetical protein		
EF0345	-	4.62	conserved domain protein		
EF0346	5.88	-	Hypothetical protein		
EF0347	3.61	4.52	peptide methionine sulfoxide reductase domain protein		
EF0348	-	3.71	tail protein		
EF0349	-	3.52	tail protein, putative		
EF0350	-	4.00	conserved hypothetical protein		
EF0351	-	3.33	structural protein, putative		
EF0353	-	4.10	holin, putative		
EF0354	3.06	4.62	holin, putative		
EF0355	-	4.95	endolysin, putative		
EF0361	-	4.84	chitinase, family 2	Cell envelope	

EF0362		-	8.70	chitin binding protein, putative	Cell envelope
EF0402	*nhaC-1*	3.71	-	Na^+H^+ antiporter	Transport and binding protein
EF0405		-	3.71	hydrolase, haloacid dehalogenase-like family	Unknown function
EF0432		-	3.71	transcriptional regulator, AraC family	Regulatory functions
EF0455		-	7.70	PTS system, IIC component	Signal transduction
EF0468		6.25	9.61	LemA family protein	Unknown function
EF0469		4.84	7.70	conserved domain protein	Hypothetical protein
EF0470	*nrdF*	-	**3.90**	ribonucleoside-diphosphate reductase 2, beta subunit	Purines, pyrimidines, nucleosides, and nucleotides
EF0471	*nrdE*	-	**3.24**	ribonucleoside-diphosphate reductase 2, alphasubunit	Purines, pyrimidines, nucleosides, and nucleotides
EF0472	*nrdI*	**3.24**	**3.90**	nrdI protein	Purines, pyrimidines, nucleosides, and nucleotides
EF0486		**60.84**	**81.90**	Hypothetical protein	
EF0487		**63.20**	**81.45**	conserved domain protein	
EF0488		**46.24**	**44.22**	Hypothetical protein	
EF0489		**44.22**	**60.84**	conserved domain protein	
EF0490		**46.92**	**58.14**	cell wall surface anchor family protein	
EF0491		**49.00**	**46.92**	conserved domain protein	
EF0492		**54.76**	**46.58**	hypothetical protein	
EF0493		**70.98**	**67.24**	conserved hypothetical protein	
EF0494		**49.70**	**50.77**	conserved hypothetical protein	
EF0495		**52.20**	**59.29**	conserved domain protein	
EF0496		**46.24**	**47.27**	conserved hypothetical protein	
EF0497		**39.06**	**42.90**	conserved hypothetical protein	MGE, Region 3, PAI

EF0499	**12.96**	**12.43**	single-strand binding protein			
EF0500	**65.21**	**62.02**	conserved hypothetical protein			
EF0551	-	**7.70**	glycosyl hydrolase, family 31			
EF0552	-	**7.02**	PTS system, IIC component			
EF0553	-	**5.18**	PTS system, IID component			
EF0559	-	**8.12**	polysaccharide biosynthesis family protein			
EF0575	**3.71**	-	cationic ABC transporter, ATP-binding protein			
EF0577	**3.15**	-	adhesion lipoprotein			
EF0578	**5.18**	-	hellx-turn-helix protein, Iron dependent repressor family			
EF0579	**5.52**	-	transcriptional regulator			
EF0664	-	7.43	hypothetical protein	Hypothetical protein		
EF0677	-	3.24	phosphoglucomutase phosphomannomutase family protein	Energy metabolism		
EF0739	**3.06**	**5.42**	nicotinamide mononucleotide transporter PnuC, putative	Transport and binding protein		
EF0741	-	**3.90**	conserved domain protein	Hypothetical protein		
EF0743	-	4.31	hypothetical protein	Hypothetical protein		
EF0746	**3.52**	**9.15**	penicillin-binding protein, putative	Cell envelope		
EF0747	-	**5.41**	conserved hypothetical protein	Hypothetical protein		
EF0764	6.76	5.41	hypothetical protein	Hypothetical protein		
EF0765	3.06	7.16	hypothetical protein	Hypothetical protein		
EF0802	**4.41**	**8.70**	hypothetical protein	Hypothetical protein		
EF0812	-	3.90	glucoronyl hydrolase, putative	Energy metabolism		
EF0813	-	3.71	glycosyl hydrolase, family 35	Energy metabolism		

EF0815		5.88	17.43	PTS system, IIAB component	Transport and binding protein/Signal transduction
EF0816		4.95	24.01	PTS system, IIC component	Transport and binding protein/Signal transduction
EF0817		-	24.50	PTS system, IID component	Transport and binding protein/Signal transduction
EF0829		-	11.06	conserved hypothetical protein	Hypothetical protein
EF0900	*adhE*	4.10	4.95	aldehyde-alcohol dehydrogenase	Energy metabolism
EF0938		-	8.70	ABC transporter, ATP-binding TOBE domain protein	Transport and binding protein
EF0972		-	**4.20**	DNA repair exonuclease family protein	DNA metabolism
EF1012		-	40.96	PTS system, IIB component	Transport and binding protein/Signal transduction
EF1017		-	34.81	PTS system, IIB component	Transport and binding protein/Signal transduction
EF1018		-	10.73	PTS system, IIA component	Transport and binding protein/Signal transduction
EF1019		-	13.51	PTS system, IIC component	Transport and binding protein/Signal transduction
EF1025		-	4.31	CBS domain protein	Unknown function protein
EF1026		-	3.06	conserved hypothetical protein	Hypothetical protein
EF1033		-	4.95	6-aminohexanoate-cyclic-dimer hydrolase, putative	Central intermediary metabolism
EF1035		-	3.52	lipoprotein, putative	Cell envelope
EF1068	*galM*	10.24	12.60	aldose 1-epimerase	Energy metabolism
EF1069	*galK*	-	9.77	galactokinase	Energy metabolism
EF1080		3.24	-	ImpB/MucB/SamB family protein	DNA metabolism
EF1097		**3.06**	**4.52**	hypothetical protein	Hypothetical protein
EF1158		-	5.41	N4-(beta-N-acetylglucosaminyl)-L-asparaginase, putative	Energy metabolism
EF1173		-	3.15	glycosyl transferase, WecB/TagA/CpsF family	Cell envelope
EF1179	*cscK*	-	6.89	fructokinase	Energy metabolism

EF1214	*budA*	-	4.41	alpha-acetolactate decarboxylase	Energy metabolism
EF1225		14.06	12.96	thiamin biosynthesis ApbE, putative	Biosynthesis of cofactors, prosthetic groups, and carriers
EF1226		13.32	11.39	oxidoreductase, putative	Transport and binding protein/Signal transduction
EF1227		8.12	5.88	conserved hypothetical protein	Hypothetical protein
EF1231		-	**3.24**	conserved hypothetical protein	Hypothetical protein
EF1232		-	29.98	ABC transporter, permease protein	Transport and binding protein
EF1241		-	4.20	hypothetical protein	Hypothetical protein
EF1243		-	5.52	glycosyl hydrolase, family 1	Energy metabolism
EF1244		-	5.52	oxidoreductase, Gfo/Idh/MocA family	Unknown function protein
EF1278		3.24	-	hypothetical protein	MGE, Region 4, Phage 02
EF1302		-	**3.90**	transcriptional regulator, putative	Regulatory functions
EF1303		-	**4.00**	transcriptional regulator, LysR family	Regulatory functions
EF1322		-	3.42	conserved hypothetical protein	Hypothetical protein
EF1344		-	4.20	sugar ABC transporter, permease protein	Transport and binding protein
EF1345		-	8.56	sugar ABC transporter, sugar-binding protein	Transport and binding protein
EF1347		-	5.52	glycosyl hydrolase, family 13	Energy metabolism
EF1387		-	7.98	conserved hypothetical protein	Hypothetical protein
EF1393		-	13.32	molybdopterin cofactor biosynthesis protein A, putative	Biosynthesis of cofactors, prosthetic groups, and carriers
EF1400		3.24	-	cadmium-translocating P-type ATPase	Transport and binding protein
EF1412		3.90	-	conserved domain protein	Hypothetical protein
EF1413		**9.00**	-	msrC protein, putative	Cellular processes
EF1417		4.10	-	site-specific recombinase, phase Integrase family	
EF1418		5.52	3.06	hypothetical protein	

EF1423		13.32	10.40	transcriptional regulator, Cro/CI family	
EF1424		8.56	22.56	hypothetical protein	
EF1425		15.60	3.71	hypothetical protein	
EF1426		17.43	25.76	vrll protein, putative	MGE, Region 5, Phage 03
EF1428		11.56	20.03	hypothetical protein	
EF1429		-	14.63	hypothetical protein	
EF1430		4.95	10.24	conserved hypothetical protein	
EF1431		3.42	12.43	hypothetical protein	
EF1434		-	3.61	DnaD domain protein	
EF1489		3.33	-	hypothetical protein	
EF1518	-	-	**4.95**	conserved hypothetical protein	Hypothetical protein
EF1533	-	7.84	8.41	conserved hypothetical protein	Hypothetical protein
EF1535	-	-	5.88	conserved hypothetical protein	Hypothetical protein
EF1561	*aroE*	9.61	-	shikimate 5-dehydrogenase phosphor-2-dehydro-3-deoxyheptonate	Amino acid biosynthesis
EF1562	-	6.89	-	aldolase, putative	Amino acid biosynthesis
EF1563	*aroB*	5.41	-	3-dehydroquinate synthase	Amino acid biosynthesis
EF1564	*aroC*	7.02	-	chorismate synthase	Amino acid biosynthesis
EF1565	-	5.64	-	prephenate dehydrogenase	Amino acid biosynthesis
EF1566	*aroA*	4.84	-	3-phosphoshikimate 1-carboxyvinyltransferase	Amino acid biosynthesis
EF1567	*aroK*	4.52	-	shikimate kinase	Amino acid biosynthesis
EF1568	-	6.38	-	prepenate dehydratase	Amino acid biosynthesis
EF1586	*nox*	**6.25**	**3.15**	NADH oxidase	Energy metabolism
EF1587	-	-	**3.06**	Mut/nudix family protein	DNA metabolism
EF1591	-	-	4.95	transcriptional regulator, AraC family	Regulatory functions

EF1602	-	-	11.56	glycosyl hydrolase, family 13	Energy metabolism
EF1656	-	-	5.52	transcriptional regulator, LysR family	Regulatory functions
EF1686	-	4.00	4.10	hypothetical protein	Hypothetical protein
EF1707	-	-	5.29	glycosyl hydrolase, family 38	Energy metabolism
EF1708	-	-	8.41	conserved hypothetical protein	Hypothetical protein
EF1834	*lacB*	3.33	4.95	galactose-6-phosphate isomerase, LacB subunit	Energy metabolism
EF1835	*lacA*	5.88	7.70	galactose-6-phosphate isomerase, LacA subunit	Energy metabolism
EF1836	-	-	9.46	PTS system, IIA component, putative	Transport and binding protein/Signal transduction
EF1837	-	-	4.52	PTS system, IIB component, putative	Transport and binding protein/Signal transduction
EF1912	-	-	3.61	ROK family protein	Unknown function protein
EF1919	-	-	4.41	acetyltransferase, GNAT family	Unknown function protein
EF1920	-	-	7.70	C4-dicarboxylate anaerobic carrier IosIne-uridine preferring nucleoside	Transport and binding protein
EF1921	-	-	18.28	hydrolase	Purines, pyrimidines, nucleosides, and nucleotides
EF1955	-	-	6.76	sigma-54 dependent DNA-binding response regulator	Transcription/regulatory functions/signal transduction
EF2109		-	3.61	conserved domain protein	
EF2110		-	4.00	hypothetical protein	
EF2111		-	3.71	hypothetical protein	
EF2112		-	4.41	hypothetical protein	
EF2116		3.71	3.80	hypothetical protein	
EF2117		-	4.10	conserved hypothetical protein	
EF2118		4.95	4.62	conserved domain protein	
EF2119		4.84	5.52	hypothetical protein	

EF2120	3.24	5.88	conserved hypothetical protein		
EF2121	-	4.00	hypothetical protein		
EF2122	7.43	4.41	hypothetical protein		
EF2123	3.90	4.31	hypothetical protein		
EF2124	3.90	5.18	methyltransferase, putative		
EF2125	3.24	5.64	hypothetical protein		
EF2126	4.41	5.29	hypothetical protein	MGE, Region 8, Phage 05	
EF2127	4.84	7.29	hypothetical protein		
EF2128	6.76	6.38	conserved hypothetical protein		
EF2129	4.84	5.41	DNA replication protein, putative		
EF2130	6.38	4.84	DnaD domain protein		
EF2131	5.29	8.70	conserved hypothetical protein		
EF2132	6.13	9.77	recT protein, putative		
EF2133	6.89	9.00	hypothetical protein		
EF2134	5.41	9.61	hypothetical protein		
EF2135	9.46	11.39	hypothetical protein		
EF2136	7.02	9.00	hypothetical protein		
EF2137	7.02	9.00	hypothetical protein		
EF2138	10.89	6.89	transcriptional regulator, Cro/CI family		
EF2139	7.84	8.85	hypothetical protein		
EF2140	8.70	7.84	conserved hypothetical protein		
EF2141	10.56	9.00	transcriptional regulator, Cro/CI family		
EF2205	-	3.42	conserved hypothetical protein	Hypothetical protein	
EF2207	-	3.42	DNA-binding protein, Fis family	Regulatory functions	
EF2208	-	3.15	phenazine biosynthesis protein PhzF family	Cellular processes	

EF2213	-	-	13.14	PTS system, IIBC component	Transport and binding protein/Signal transduction
EF2223	-	-	8.70	ABC transporter, permease protein	Transport and binding protein
EF2236	-	-	15.41	conserved hypothetical protein	Hypothetical protein
EF2243		7.43	-	conserved hypothetical protein	
EF2244		4.00	-	conserved hypothetical protein	
EF2245		4.52	3.71	conserved hypothetical protein	
EF2247		6.38	5.52	transcriptional regulator	
EF2248		18.49	18.92	hypothetical protein	
EF2249		28.36	30.53	hypothetical protein	
EF2250		32.21	33.06	conserved domain protein	
EF2251		7.56	10.40	hypothetical protein	MGE, Region 9
EF2252		24.26	36.30	hypothetical protein	
EF2253		31.92	38.13	conserved hypothetical protein	
EF2254		31.36	30.53	conserved hypothetical protein	
EF2255		-	3.33	site-specific recombinase, phage integrase family	
EF2270		-	7.56	PTS system, IIC component	
EF2271		-	3.80	PTS system, IIB component	
EF2272		-	8.41	glucuronyl hydrolase, putative	
EF2440	-	-	5.64	celC-related protein	Unknown function protein
EF2441	-	-	10.73	conserved hypothetical protein	Hypothetical protein
EF2442	-	-	7.98	phosphate transporter family protein	Transport and binding protein
EF2525		-	4.95	cell wall surface anchor family protein	
EF2530		-	7.43	hypothetical protein	MGE, Region 11
EF2539		3.52	3.06	hypothetical protein	
EF2541		-	4.84	hypothetical protein	

EF2546	-	6.13	-	site-specific recombinase, phage integrase family	DNA metabolism
EF2568	-	-	3.15	aminotransferase, class V	Unknown function protein
EF2570	-	-	6.25	aldehyde oxidoreductase, putative	Unknown function protein
EF2713	-	-	3.90	cell wall surface anchor family protein	Cell envelope
EF2734	-	-	**3.61**	oxidoreductase, Gfo/Idh/MocA family	Unknown function protein
EF2754	*nrdD*	-	**4.31**	anaerobic ribonucleoside-triphosphate reductase	Purines, pyrimidines, nucleosides, and nucleotides
EF2755	*nrdG*	**3.06**	**4.00**	anaerobic ribonucleoside-triphosphate reductase activating protein	Purines, pyrimidines, nucleosides, and nucleotides
EF2767	**-**	**6.76**	**8.85**	transcriptional regulator	Regulatory function protein
EF2768	**-**	**8.27**	**11.73**	conserved hypothetical protein	Hypothetical protein
EF2769	**-**	**7.29**	**13.14**	ABC transporter, ATP-binding protein	Transport and binding protein
EF2770	**-**	**6.25**	**8.27**	conserved hypothetical protein	Hypothetical protein
EF2775	*thiD*	**6.50**	**8.41**	phosphomethylpyrimidine kinase	Biosynthesis of cofactors, prosthetic groups, and carriers
EF2776	*thiE*	**6.00**	**8.56**	thiamine-phosphate pyrophosphorylase	Biosynthesis of cofactors, prosthetic groups, and carriers
EF2777	*thiM*	**5.88**	**9.15**	hydroxyethylthiazole kinase, putative	Biosynthesis of cofactors, prosthetic groups, and carriers
EF2778	*thiW*	**5.88**	**11.39**	conserved hypothetical protein	Biosynthesis of cofactors, prosthetic groups, and carriers Transport and binding protein
EF2896	**-**	**9.46**	**13.32**	hypothetical protein	Hypothetical protein
EF2939		4.62	4.95	cold-shock domain family protein	
EF2940		6.63	5.64	hypothetical protein	
EF2941		8.12	6.38	hypothetical protein	

EF2942		8.85	5.76	hypothetical protein		
EF2943		7.56	5.64	hypothetical protein		
EF2944		8.12	5.18	hypothetical protein		
EF2946		3.80	-	hypothetical protein	MGE, Region 13, Phage 07	
EF2947		4.10	-	conserved hypothetical protein		
EF2948		3.90	3.42	DNA primase domain protein		
EF2949		5.06	4.41	hypothetical protein		
EF2950		6.00	3.33	hypothetical protein		
EF2951		7.56	6.38	conserved hypothetical protein		
EF2952		7.29	6.38	hypothetical protein		
EF2953		8.70	7.29	hypothetical protein		
EF2964	-	-	3.80	putative transport protein, SgaT family	Transport and binding protein	
EF2965	-	-	3.90	conserved hypothetical protein	Hypothetical protein	
EF2966	-	-	4.95	transcriptional antiterminator, bglG family	Regulatory functions	
EF3033	-	-	3.24	PTS system, IIA component	Transport and binding protein/Signal transduction	
EF3060	-	-	**4.62**	secreted lipase, putative	Unknown function protein	
EF3107	-	-	3.71	peptide ABC transporter, permease protein	Transport and binding protein	
EF3108	-	-	3.61	peptide ABC transporter, permease protein	Transport and binding protein	
EF3109	-	-	3.90	peptide ABC transporter, ATP-binding protein	Transport and binding protein	
EF3110	-	-	4.00	peptide ABC transporter, ATP binding protein	Transport and binding protein	
EF3164	*msrB*	-	**3.06**	PilB family protein	Unknown function protein	
EF3200	-	-	4.31	ABC transporter, ATP-binding protein	Transport and binding protein/Signal transduction	
EF3210	-	36.00	31.08	PTS system, IIA component, putative	Transport and binding protein/Signal transduction	
EF3211	-	29.43	30.25	PTS system, IIB component	Transport and binding protein/Signal transduction	
EF3212	-	37.82	27.83	PTS system, IIC component	Transport and binding protein/Signal transduction	

EF3213	-	29.43	27.83	PTS system, IID component	Transport and binding protein/Signal transduction
EF3214	-	12.96	12.60	ATP-dependent helicase, DEAH-box family, putative	Transcription
EF3216	-	6.25	4.62	transcriptional regulator, putative	Regulatory functions
EF3244	-	**7.16**	**11.06**	hypothetical protein	Hypothetical protein
EF3245	-	**6.63**	**9.92**	cell-envelope associated acid phosphatase	Unknown function protein
EF3311	*gldA*	**3.15**	**4.95**	glucose-inhibited division protein A	Unknown function protein
EF3312	*trmE*	-	**4.10**	tRNA modification GTPase TrmE	Cellular processes/Protein synthesis
EF3325	-	-	11.22	sodium ion-translocating decarboxylase, biotin carboxyl carrier protein	Transport and binding protein/Energy metabolism
EF3326	-	-	9.46	conserved hypothetical protein	Hypothetical protein
EF3327	-	-	6.00	citrate transporter	Transport and binding protein

Table S2 - List of all genes found to be differentially expressed in a V583*vanR:tet* mutant. Genes are organized according to their chromosomal location/locus number. The gene name is listed when available for the V583 genome. Overexpressed genes have their fold change value in bold. Genes belonging to MGE are shaded in grey. Genes belonging to the same putative transcriptional unit are identified by a vertical bar.

Locus	Gene	Fold-change		Description	Role
		Mid-exp	Early-stat		
EF0005	*gyrB*	-	**2.3**	DNA gyrase, B subunit	DNA metabolism
EF0026	-	-	**2.1**	conserved hypothetical protein	Hypothetical proteins
EF0029	-	13.6	-	aminotransferase, class II	unknown function
EF0068	-	3.8	-	hypothetical protein	unknown function
EF0085	-	-	**2.2**	conserved domain protein	Hypothetical proteins
EF0131	-	-	**2.7**	conserved domain protein	
EF0132	-	-	**2.4**	hypothetical protein	
EF0133	-	-	**2.2**	hypothetical protein	
EF0134	-	-	**2.9**	hypothetical protein	
EF0136	-	-	**2.9**	hypothetical protein	
EF0138	-	-	**2.9**	conserved domain protein	
EF0139	-	-	**2.4**	FtsK/SpoIIIE family protein	
EF0140	-	-	**2.5**	conserved domain protein	
EF0141	-	-	**2.3**	hypothetical protein	
EF0142	-	-	**2.2**	conserved hypothetical protein	Region 1
EF0144	-	-	**2.3**	conserved domain protein	
EF0151	-	-	**2.1**	hypothetical protein	
EF0152	-	-	**2.3**	hypothetical protein	

EF0153	-	-	**2.2**	cell wall surface anchor family protein	
EF0154	-	-	**2.4**	conserved hypothetical protein	
EF0155	-	-	**2.2**	conserved hypothetical protein	
EF0156	-	-	**2.7**	conserved hypothetical protein	
EF0158	-	-	**2.4**	conjugal transfer protein, putative	
EF0161	-	-	**2.3**	hypothetical protein	
EF0162	-	-	**2.3**	hypothetical protein	
EF0165	-	-	**2.6**	conserved hypothetical protein	
EF0166	-	-	**2.6**	site-specific recombinase, phage integrase family	
EF0253	-	2.8	-	aldehyde dehydrogenase	Energy metabolism
EF0292	-	3.2	-	PTS system, IIC component	Signal transduction/Transport and binding proteins
EF0332	-	2.2	-2.1	conserved hypothetical protein	
EF0333	-	2.4	2.1	conserved hypothetical protein TIGR01630	
EF0334	-	2.3	-	portal protein	
EF0335	-	2.7	2.1	minor head protein	
EF0337	-	2.7	2.1	hypothetical protein	
EF0339	-	2.2	-	major capsid protein, putative	
EF0340	-	2.0	2.2	hypothetical protein	
EF0341	-	2.2	2.2	hypothetical protein	
EF0342	-	-	2.3	hypothetical protein	
EF0344	-	2.2	-	hypothetical protein	
EF0345	-	2.3	2.1	conserved domain protein	Region 2, Phage 1
EF0346	-	3.3	-	hypothetical protein	
EF0347	-	2.8	2.3	peptide methionine sulfoxide reductase domain protein	

EF0348	-	-	2.1	tail protein	
EF0349	-	-	2.1	tail protein, putative	
EF0350	-	2.3	2.2	conserved hypothetical protein	
EF0351	-	2.3	2.2	structural protein, putative	
EF0352	-	2.6	2.0	hypothetical protein	
EF0353	-	3.1	2.3	holin, putative	
EF0354	-	3.1	2.6	holin, putative	
EF0355	-	2.6	2.0	endolysin, putative	
EF0356	-	4.5	-	hypothetical protein	unknown function
EF0411	-	2.2	-	PTS system, mannitol-specfic IIBC components	Signal transduction/Transport and binding proteins
EF0413	*mltD*	4.5	-	mannitol-1-phosphate 5-dehydrogenase	Energy metabolism
EF0428	-	4.1	-	conserved hypothetical protein	Hypothetical proteins
EF0429	-	3.6	-	TRAP dicarboxylate transporter, DctP subunit	Transport and binding proteins
EF0430	-	3.4	-	TRAP dicarboxylate transporter, DctQ subunit	Transport and binding proteins
EF0431	-	3.8	-	TRAP dicarboxylate transporter, DctM subunit	Transport and binding proteins
EF0441	-	3.5	-	hypothetical protein	unknown function
EF0443	-	**2.6**	-	LysM domain protein	unknown function
EF0486	-	8.3	-	hypothetical protein	
EF0487	-	7.1	-	conserved domain protein	
EF0488	-	2.3	-	hypothetical protein	
EF0489	-	1.8	-	conserved domain protein	
EF0493	-	4.1	-	conserved hypothetical protein	
EF0494	-	3.2	-	conserved hypothetical protein	
EF0496	-	3.6	-	conserved hypothetical protein	

EF0497	-	3.4	-	conserved hypothetical protein	
EF0500	-	5.4	-	conserved hypothetical protein	Region 3, PAI
EF0540	-	3.5	-	N-acetylmannosamine-6-phosphate epimerase, putative	
EF0551	-	2.8	-	glycosyl hydrolase, family 31	
EF0567	*kdpA*	-	**3.0**	potassium-transporting ATPase, subunit A	
EF0568	*kdpB*	-	**3.0**	potassium-transporting ATPase, subunit B	
EF0569	*kdpC*	-	**3.0**	potassium-transporting ATPase, subunit C	
EF0574	-	-	**2.2**	hypothetical protein	
EF0612	-	2.8	-	hypothetical protein	
EF0613	-	-	**2.4**	hypothetical protein	
EF0738	-	2.6	-	hypothetical protein	unknown function
EF0765	-	-	**2.1**	hypothetical protein	unknown function
EF0932	-	**3.0**	**3.4**	hypothetical protein	unknown function
EF1012	-	2.2	2.3	PTS system, IIB component	Signal transduction/Transport and binding proteins
EF1013	-	2.8	-	PTS system, IIC component	Signal transduction/Transport and binding proteins
EF1015	-	-	2.3	hypothetical protein	unknown function
EF1017	-	-	2.1	PTS system, IIB component	Signal transduction/Transport and binding proteins
EF1033	-	**3.5**	-	6-aminohexanoate-cyclic-dimer hydrolase, putative	Central intermediary metabolism
EF1056	-	-	2.6	hypothetical protein	unknown function
EF1090	-	-	**2.4**	hypothetical protein	unknown function
EF1096	-	-	**2.1**	conserved hypothetical protein	Hypothetical proteins
EF1177	-	-	2.1	hypothetical protein	unknown function
EF1262	-	-	**2.9**	hypothetical protein	Region 4
EF1385	-	3.8	-	molybdopterin-guanine dinucleotide	Biosynthesis of cofactors, prosthetic groups, and

					biosynthesis protein A, putative	carriers
EF1389	-	3.0	-		NAD-depe-nt formate dehydrogenase, beta subunit, putative	Energy metabolism
EF1423	-	-	**3.1**		transcriptional regulator, Cro/CI family	
EF1424	-	-	**2.9**		hypothetical protein	
EF1426	-	-	**2.5**		vrlI protein, putative	
EF1427	-	-	**4.3**		hypothetical protein	Region 5, Phage 03
EF1436	-	-	**2.1**		hypothetical protein	
EF1449	-	-	**2.6**		conserved hypothetical protein	
EF1459	-	-	2.2		conserved hypothetical protein	
EF1533	-	**4.1**	**5.1**		conserved hypothetical protein	Hypothetical proteins
EF1652	-	2.5	-		DNA processing protein DprA, putative	Cellular processes
EF1787	*purE*	2.7	-		phosphoribosylaminoimidazole carboxylase, catalytic subunit	Purines, pyrimidines, nucleosides, and nucleotides
EF1810	*gspA-1*	**2.3**	-		general stress protein A	Cellular processes
EF1811	*gspA-2*	**2.4**	**2.6**		general stress protein A	Cellular processes
EF1828	-	-	**2.5**		glycerol uptake facilitator protein, putative	Transport and binding proteins
EF1835	*lacA*	-	**2.0**		galactose-6-phosphate isomerase, LacA subunit	Energy metabolism
EF1849	-	4.0	-		conserved hypothetical protein	
EF1850	-	3.1	-		conserved hypothetical protein	
EF1875	-	3.6	-		conserved hypothetical protein	Region 6
EF1882	-	3.2	-		conserved hypothetical protein	
EF1886	-	2.7	-		transcriptional regulator, Cro/CI family	
EF1993	-	5.7	-		Holin	
EF2001	-	3.2	-		minor structural protein	

EF2004	-	**4.3**	-	conserved domain protein	Region 7, Phage 04
EF2028	-	2.5	-	hypothetical protein	
EF2034	-	3.0	-	hypothetical protein	
EF2084	-	-	**2.2**	hypothetical protein	
EF2087	-	-	**2.3**	Holin	
EF2088	-	-	**2.6**	hypothetical protein	
EF2089	-	-	**2.6**	hypothetical protein	
EF2090	-	-	**2.5**	hypothetical protein	
EF2091	-	-	**2.9**	hypothetical protein	
EF2092	-	-	**2.9**	hypothetical protein	
EF2093	-	-	**3.2**	endolysin domain protein	
EF2094	-	-	**3.5**	hypothetical protein	
EF2095	-	-	**3.4**	hypothetical protein	
EF2096	-	**2.5**	**3.7**	tail protein	
EF2097	-	**2.8**	**3.3**	hypothetical protein	
EF2098	-	-	**2.8**	hypothetical protein	
EF2099	-	**2.6**	**3.4**	hypothetical protein	
EF2100	-	**2.7**	**3.1**	hypothetical protein	
EF2101	-	**2.8**	**3.2**	hypothetical protein	
EF2102	-	**2.9**	**3.0**	hypothetical protein	
EF2103	-	**2.6**	**3.3**	hypothetical protein	
EF2104	-	**2.6**	**3.0**	hypothetical protein	Region 8, Phage 05
EF2105	-	**3.1**	**3.5**	hypothetical protein	
EF2106	-	**2.7**	**2.9**	conserved domain protein	
EF2107	-	**3.2**	**3.1**	hypothetical protein	

EF2108	-	**2.7**	**3.0**	hypothetical protein	
EF2109	-	**2.8**	**3.1**	conserved domain protein	
EF2110	-	**2.9**	**3.1**	hypothetical protein	
EF2111	-	**3.2**	**3.4**	hypothetical protein	
EF2112	-	**3.0**	**2.9**	hypothetical protein	
EF2113	-	**3.2**	**3.0**	conserved hypothetical protein	
EF2114	-	**3.1**	**3.6**	adenine methyltransferase, putative	
EF2120	-	-	**2.1**	conserved hypothetical protein	
EF2122	-	-	**2.7**	hypothetical protein	
EF2124	-	-	**2.2**	methyltransferase, putative	
EF2125	-	-	**2.1**	hypothetical protein	
EF2126	-	-	**2.2**	hypothetical protein	
EF2127	-	-	**2.2**	hypothetical protein	
EF2128	-	-	**2.2**	conserved hypothetical protein	
EF2130	-	-	**2.2**	DnaD domain protein	
EF2131	-	-	**2.5**	conserved hypothetical protein	
EF2132	-	-	**2.1**	recT protein, putative	
EF2139	-	-	**2.1**	hypothetical protein	
EF2140	-	-	**2.2**	conserved hypothetical protein	
EF2141	-	-	**2.2**	transcriptional regulator, Cro/CI family	
EF2223	-	3.0	-	ABC transporter, permease protein	Region 9
EF2251	-	3.2	-	hypothetical protein	
EF2288	-	5.1	-	hypothetical protein	
EF2298	*vanS*$_B$	48.9	29.9	sensor histidine kinase VanS$_B$	
EF2319	-	2.0	-	hypothetical protein	Region 10, Tn*VanB*

EF2321	-	2.1	-	hypothetical protein	
EF2322	-	4.1	-	conserved domain protein	
EF2345	-	3.4	-	conserved hypothetical protein	Region 9
EF2389	-	2.8	-	hypothetical protein	unknown function
EF2513	-	-	**2.6**	lipoprotein, putative	
EF2514	-	-	**2.7**	hypothetical protein	
EF2515	-	-	**2.7**	conserved domain protein	
EF2516	-	-	**2.1**	membrane protein, putative	
EF2517	-	-	**3.0**	conjugal transfer protein, putative	
EF2519	-	-	**2.5**	conserved hypothetical protein	
EF2520	-	-	**2.4**	conserved hypothetical protein	
EF2521	-	-	**2.4**	conserved hypothetical protein	
EF2522	-	-	**3.1**	hypothetical protein	
EF2523	-	-	**2.9**	hypothetical protein	Region 11
EF2524	-	-	**2.5**	sortase family protein	
EF2526	-	-	**2.6**	hypothetical protein	
EF2527	-	**4.5**	**4.0**	conserved domain protein	
EF2528	-	-	**3.1**	transcriptional regulator, Cro/CI family	
EF2529	-	-	**2.8**	conserved hypothetical protein	
EF2533	-	**3.0**	**2.5**	FtsK/SpoIIIE family protein	
EF2534	-	**2.7**	**2.6**	conserved hypothetical protein	
EF2536	-	**4.1**	**3.3**	conserved hypothetical protein	
EF2537	-	**4.6**	**3.4**	hypothetical protein	
EF2538	-	**3.9**	**3.1**	hypothetical protein	
EF2539	-	**4.1**	**3.4**	hypothetical protein	

EF2543	-	-	**2.2**	transcriptional regulator, putative	
EF2547	-	**2.6**	**2.2**	hypothetical protein	unknown function
EF2579	-	3.4	-	diaminopropionate ammonia-lyase, putative	Energy metabolism
EF2582	-	4.9	-	chlorohydrolase family protein	unknown function
EF2802	-	3.7	-	Endolysin	
EF2805	-	12.7	-	conserved hypothetical protein	
EF2806	-	4.5	-	hypothetical protein	
EF2810	-	2.6	-	conserved hypothetical protein	
EF2814	-	3.9	-	hypothetical protein	
EF2817	-	2.9	-	conserved hypothetical protein	
EF2818	-	3.8	**2.4**	minor structural protein, putative	
EF2819	-	16.2	-	hypothetical protein	Region 12, Phage 06
EF2823	-	3.1	-	terminase, large subunit, putative	
EF2825	-	5.7	-	conserved hypothetical protein	
EF2826	-	3.3	-	hypothetical protein	
EF2842	-	3.5	-	hypothetical protein	
EF2843	-	4.0	-	hypothetical protein	
EF2844	-	3.5	-	hypothetical protein	
EF2845	-	3.8	-	hypothetical protein	
EF2849	-	2.4	-	conserved hypothetical protein	
EF2978	-	3.8	-	PTS system, IIC component	
EF2980	-	3.4	-	PTS system, IIA component	Signal transduction/Transport and binding proteins
EF2981	-	3.2	-	sigma-54 depe-nt DNA-binding response regulator	Transcription/ Regulatory functions/ Signal transduction
EF3012	-	2.3	-	membrane protein, putative	Cell envelope

EF3024	-	2.9	-	hypothetical protein	unknown function
EF3076	-	2.6	-	cell wall surface anchor family protein	Cell envelope
EF3141	-	3.1	-	D-isomer specific 2-hydroxyacid dehydrogenase family protein	unknown function
EF3196	-	2.4	-	response regulator	Regulatory functions
EF3197	-	2.4	-	sensor histidine kinase	Signal transduction
EF3210	-	3.7	-	PTS system, IIA component, putative	Signal transduction/Transport and binding proteins
EF3211	-	3.2	-	PTS system, IIB component	Signal transduction/Transport and binding proteins
EF3212	-	2.6	-	PTS system, IIC component	Signal transduction/Transport and binding proteins
EF3213	-	2.4	-	PTS system, IID component	Signal transduction/Transport and binding proteins
EF3222	-	-	**2.5**	hypothetical protein	unknown function
EF3249	-	2.2	2.2	conserved hypothetical protein	Hypothetical proteins
EF3253	-	2.4	-	cell wall surface anchor family protein	Cell envelope
EF3284	-	4.3	-	cystathionine gamma-synthase, putative	Amino acid biosynthesis
EFA0025	-	4.0	-	hypothetical protein	unknown function
EFA0028	-	5.1	-	conserved domain protein	Hypothetical proteins
EFA0030	-	3.4	-	conserved hypothetical protein	Hypothetical proteins
EFA0031	-	5.4	-	hypothetical protein	unknown function
EFA0047	*asa1*	10.9	-	aggregation substance Asa1	Cell envelope/ Cellular processes
EFA0048	-	3.7	-	conserved hypothetical protein	Hypothetical proteins
EFA0050	-	4.6	-	hypothetical protein	unknown function
EFA0051	-	8.1	-	hypothetical protein	unknown function
EFB0012	*prgC*	3.5	-	surface protein PrgC	Cell envelope/ Cellular processes
EFB0013	-	3.6	-	conserved hypothetical protein	Hypothetical proteins
EFB0014	-	6.2	-	conserved hypothetical protein	Hypothetical proteins
EFB0015	-	3.5	-	hypothetical protein	Hypothetical proteins

EFB0017	-	3.6	-	conserved hypothetical protein	Hypothetical proteins
EFB0018	-	3.6	-	hypothetical protein	unknown function
EFB0019	-	4.2	-	conserved domain protein	Hypothetical proteins
EFB0021	-	3.4	-	conserved hypothetical protein	Hypothetical proteins
EFB0022	-	4.5	-	hypothetical protein	unknown function
EFB0024	-	5.7	-	hypothetical protein	Hypothetical proteins
EFB0025	-	4.9	-	TraG family protein	Mobile and extrachromosomal element functions
EFB0026	-	3.4	-	conserved domain protein	Hypothetical proteins
EFB0029	-	3.3	-	conserved hypothetical protein	Hypothetical proteins
EFB0035	-	4.0	-	hypothetical protein	unknown function
EFB0036	-	2.9	-	hypothetical protein	unknown function
EFB0037	-	3.5	-	hypothetical protein	unknown function
EFB0056	-	2.3	-	hypothetical protein	unknown function

Chapter 9

General Discussion and

Future Perspectives

Unlike *S. aureus*, enterococci are not primary pathogens. Rather, they are normal components of the human bowel flora and are not problematic for healthy people, except as occasional agents of urinary tract infections. However, the genus has proven adept at causing opportunistic infections in hospital patients. These are mostly caused by *E. faecalis*, though infections caused by *E. faecium* raise greatest clinical concern because of a greater multidrug resistance in this species (Murray, 1990).

The species of most concern have not changed in the last two decades but they have become more resistant. This directly implies less therapeutic options for critically ill patients that are infected with these opportunistic pathogens. Vancomycin-resistant enterococci are widespread, but are a greater problem in the US than in Europe. Many of the strains that cause hospital outbreaks in different countries represent expansion of a single successful clonal lineage by direct spread or repeated selection in multiple places. In addition, the horizontal spread of glycopeptide resistance genes is superimposed on this background. The biggest concern is the transfer of vancomycin resistance from enterococci to MRSA. Sporadic cases have already been documented in the US (Sievert *et al.*, 2008; Tenover, 2008; Zhu *et al.*, 2008) but it is of utmost concern if this vancomycin resistance transfer events become common and frequent and raising the possibility that Van^R-MRSA might supplant the other established health care MRSA clones.

Enterococci harbouring the most common vancomycin resistance operons, *vanA* or *vanB*, are high-level resistant or present variable levels of resistance to vancomycin, respectively. Lack of detection and control can result in endemic occurrence of the organism. So, it is of great clinical, social and economical importance that enterococcal strains are very well characterized and

closely monitored in order to draw a clear picture of the vancomycin resistance scenario. Consequently, the microbiology laboratory must be part of the first line of defense against the spread of VRE.

In Europe, opposite to what happens in the United States for enterococci and in Europe for MRSA, VRE colonization is not restricted to the hospital setting. It is fundamental to know the dimension of vancomycin resistance, dissemination of resistance determinants and an accurate detection of VRE is mandatory to prevent strain dissemination. The work described in the first part of this thesis was focused on molecular and microbiological approaches for the identification of vancomycin resistance determinants among enterococcal dairy food and clinical human and veterinary isolates.

Detection of vancomycin resistance determinants in Portugal

Several studies have described fundamental differences in the epidemiology of vancomycin resistance in the United States and Europe (Goossens, 1998; Martone, 1998; Schouten *et al.*, 2000). In Europe the initial VRE scenario consisted of polyclonal enterococcal population with a high diversity of Tn*1546* types in the community and with scarce presence on the nosocomial setting (Jensen *et al.*, 1998; Schouten *et al.*, 2001; Garcia-Migura *et al.*, 2007). This epidemiological pattern seems to have changed in the last few years. Five European countries have reported prevalence rates of VRE in hospitals above 20%. Frequent reports of nosocomial VRE outbreaks in most countries and a variable presence of these bacteria in community settings have been described (Leavis *et al.*, 2006a). In chapter 2, we describe the screening of vancomycin resistance determinants among strains from different biological origins (human clinical, veterinary clinical – dog pets, dairy products – cheese and milk). Portugal

is currently one of the European countries with the highest VRE prevalence rate of invasive isolates in the nosocomial setting, has a frequent recovery rate in animals, healthy humans, and the environment, as seen in previous surveillance studies (Novais *et al.*, 2005a; Novais *et al.*, 2005b; Novais *et al.*, 2005c; Poeta *et al.*, 2005; Novais *et al.*, 2006). In our study it was determined that, within the sample screened and, although only one *E. faecium* VRE strain from a clinical sample was detected, *vanA* and *vanB* genes were found in a significative number of strains. *vanA* was detected in 40% of the clinical isolates and in 35% of the dairy food isolates whereas *vanB* was detected in 18% of the clinical isolates and in only 6.3% of the dairy food isolates. The incongruence between phenotype and genotype was explained by the fact that none of the isolates screened carried a complete Tn*1546*-like element. PFGE analysis of the *vanA*-carrying strains suggests that, in general, the isolates are not genetically related and that the dissemination of *vanA* gene is more likely to have been through transposable elements, like Tn*1546*-like elements, rather than by clonal dissemination. However, in a study by Novais *et al.* (2008) the authors point out that both clonal dissemination and horizontal transfer of particular genetic elements seem to play a relevant role in the recent wide dissemination of VRE in Portugal (Novais *et al.*, 2008). Moreover, common PFGE profiles among vancomycin resistant *E. faecalis* isolates from human origin and from cheese were found (Bertrand *et al.*, 2000).

The isolates studied in this work were isolated in the late 80s. Although this fact may, on one hand, justify the low frequency of VRE detection since vancomycin resistance has increased more recently, on the other hand hints that even before vancomycin resistance became a serious clinical concern the vancomycin resistance determinants were already spread among strains from clinical origin and, even more disturbing, among dairy food isolates. Enterococci

are found in a variety of food products, including milk and cheese produced in the south of Europe as non-starter lactic acid bacteria (Flahaut *et al.*, 1997; Barros, 1998; Lopes *et al.*, 1999). In many of these cheeses enterococci play a key role in the maturation and final organoleptic characteristics of the food product and constitute the main micropopulation in the final product (Franz *et al.*, 1999). Moreover, in Portugal cheese is part of the daily diet in rural areas as well as fashionable food products in urban centers. There are no studies on screening of VRE on dairy food isolates in Portugal. The available data on food isolates are restricted to samples isolated from poultry carcasses (Novais *et al*., 2005a; Novais *et al*., 2008) where the percentage of detected VRE is very high (>40%). Although there are studies indicating that vancomycin resistance determinants are spread from animals to humans (Simonsen *et al.*, 1998; Klare *et al.*, 1999) there are no unequivocal data and no demonstrated correlation between ingestion of food products containing enterococci and enterococci responsible for infections in humans (Phillips *et al.*, 2004). This is still a question in debate and it is crucial to know how vancomycin resistance determinants are spread in different food products. Also, we detected vancomycin resistance determinants in clinical isolates from pets and in a previous study these same isolates were found to be highly multiresistant to the same antibiotics of human clinical isolates (de Fatima Silva Lopes *et al.*, 2005), a fact that is of some concern due to the intimacy of pets with humans. However, in our study no genetic relatedness was found between human clinical and veterinary clinical isolates or between dairy isolates and human clinical isolates.

The spread of vancomycin resistance, therefore, may not be confined to the spread of resistant strains. Clearly, further work is required to investigate and characterize the mobile element carrying vancomycin genes in strains for which

only the detection of some of the vancomycin determinants from the Tn*1546*-like element was possible. Techniques such as primer walking or an optimized inverse PCR might be used. Further work will hopefully provide new knowledge to gain more detailed insight and to prevent spread of infections caused by vancomycin resistant enterococci.

As previously mentioned, Portugal is one of the European countries with the highest rate of reported VRE in the nosocomial setting but only a very limited number of studies on the molecular characterization of VRE isolated from portuguese hospitals have been published, and only focusing on the two enterococcal species most commonly found in hospitals. In chapter 3 we performed the molecular characterization of four vancomycin resistant *E. faecium* isolated from a hospital in the center of Portugal (Leiria). Although the prevalence of VRE in this region of the country is unknown, we were informed that VRE detection by the hospital microbiology laboratory was uncommon.

From the four VREfm strains identified by the hospital routine screening, at the arrival to our laboratory only three presented a MIC value compatible with the high-level vancomycin phenotype reported to us, although all strains were positive for *vanA* gene. The three confirmed VREfm strains were genetically related as proven by PFGE profile analysis, belong to ST18, ampicillin and ciprofloxacin resistant and carried *esp* gene, which makes us classify them as belonging to the global hospital-adapted clonal lineage CC17. IS*Ef1* insertion sequence was detected in the *vanX-vanY* intergenic region in these strains. IS*Ef1* insertion sequence (IS*30* family), together with IS*1216* (IS*6* family), is one of the most frequently detected insertion sequences within Tn*1546* (Novais *et al*., 2008). IS*Ef1* was first identified within Tn*1546* among clinical Portuguese strains (Novais

et al., 2004), but these Tn*1546* variants might be currently dispersed among European hospitals as they have recently been identified in enterococcal strains from a hospital outbreak in Germany (Werner *et al.*, 2008). Transposon polymorphisms have been demonstrated for the first time by Handwerger *et al*. (1995) (Handwerger & Skoble, 1995). More recently, several more studies on the determination of genetic polymorphisms in Tn*1546* have been published (Jensen *et al*., 1998; Woodford *et al.*, 1998; Novais *et al.*, 2008; Sung *et al.*, 2008; Lopez *et al.*, 2010). A common finding of these later studies was that although the coding potential for resistance remains generally unaffected, transposon polymorphisms could be detected quite easily. The presence of additional insertion elements in intergenic positions or deletions at the transposon termini is encountered frequently. The characterization of Tn*1546* polymorphism allows tracking of transposon types, identification of source-specific transposons and, from this knowledge, several epidemiologic scenarios can be envisaged. Epidemic VRE can be identified and in the UK, for instance, there are examples of strains that seem to have travelled long distances (Morrison *et al.*, 1995). Certain VRE are capable of highly efficient bacterial transfer from patient to patient in the hospital environment (Pegues *et al.*, 1997), as was the case of Leiria Hospital VREfm strains. On the other hand, long-term colonization with a single type of VRE in a single individual has also been observed (Bonten *et al.*, 1998). In contrast, in certain hospital settings, highly promiscuous transposon types have been described. Instead of epidemic spread of a strain, a specific Tn*1546* type is encountered in various VRE genomes. In conclusion, the spread of vancomycin resistance is facilitated by the epidemic capacity of both strains and transposons. In combination with the large number of environmental and animal reservoirs that have already been identified, at least in Europe, this means

that the clinical threat posed by VRE may not be easily overcome in the near future.

The *E. faecium* ST17 lineage is predicted to be the founder of the designated complex-17 (Willems *et al.*, 2005). ST17 evolved through genetic diversification, including mutations and recombination, and became the most successful hospital-adapted subpopulation which has spread globally (USA, Europe, Asia, Australia, Africa and South America). CC17 seemed to be responsible for the worldwide emergence of nosocomial VRE, as most of the infection related and outbreak strains belong to this complex. CC17 isolates possess a wide group of features that makes them successful conquers of the nosocomial environment. In addition to being ampicillin resistant they are also characterized by high levels of quinolone resistance (Torell *et al.*, 2003; Coque *et al.*, 2005; Leavis *et al.*, 2006b) due to point mutations in *gyrA* and *parC*, in the quinolone resistance determining region (Leavis *et al*., 2006b). It is interesting that *E. faecium* isolates that harboured amino acid substitutions in the DNA mismatch repair proteins MutS and MutL belong to CC17 (Willems *et al.*, 2003), which may suggest that CC17 isolates are more prone to undergo mutations. In addition to resistant traits, the majority of CC17 isolates are characterized by the presence of a putative pathogenicity island of at least 14 kb (Leavis *et al.*, 2004) carrying the *esp* gene (Willems *et al.*, 2001; Coque *et al.*, 2002; Leavis *et al.*, 2003, Willems *et al*., 2005). On the putative *E. faecium* PAI, the genetic region contained seven ORFs, representing putative genes implicated in virulence (*nox* and muramidase), regulation of transcription (a sigma-like factor and *araC*), antibiotic resistance (a multidrug resistance efflux pump) and potentially mobilization of the putative PAI (a hypothetical bacteriophage protein). This pathogenicity island is completely different from the *esp*-containing PAI previously disclosed in *E. faecalis* (van

Schaik *et al.*, 2010). Furthermore, *hyl*, the putative virulence gene encoding a hyaluronidase (Rice *et al.*, 2003), was enriched in CC17 isolates (Klare *et al.*, 2005; Oh *et al.*, 2005). All these findings suggest that hospital-adaptation of CC17 has been a multi-step process involving the sequential acquisition of adaptative mechanisms that provide CC17 with selective advantages, thus enabling the acquisition of even more adaptations. From our data on clinical VREfm, we learned that also in Portugal enterococcal infections in hospitalized patients are caused by *E. faecium* CC17 isolates. Since CC17, representing multidrug resistant isolates, already succeeded in acquiring resistance and virulence traits, development of resistance to recently introduced antibiotics seems inevitable. Furthermore, linkage of antibiotic resistance genes with post segregational killing systems, as demonstrated for vancomycin resistance genes (Sorum *et al.*, 2006; Dahl *et al.*, 2007, Moritz & Hergenrother, 2007, Sletvold *et al.*, 2007,), predicts that reduction in antibiotic use may not result in reduced numbers of resistant enterococci. New strategies need to be developed to combat this multiresistant bacterium in hospitals. Identifying genes that might contribute to survival and spread in hospitals is mandatory. Unraveling how the bacterium can use CC17 specific genes, most likely in concert with core genes, to colonize the human host and invade susceptible subjects is the necessary next step. The development of selective therapies directed at CC17, such as vaccines, phages or selective antibodies, seems a promising approach to combat the bacterium.

Infections commonly caused by enterococci include urinary tract infections, endocarditis, bacteremia, catheter-related infections, wound infections, and intra-abdominal and pelvic infections. Many infecting strains originate from the patient's intestinal flora. From here, they can spread and cause urinary tract infection,

intra-abdominal infection, and surgical wound infection. Enterococcal wound infections often manifest as part of a mixed infection (Giacometti *et al.*, 2000). In chapter 3 we provide the first report of two cases of surgical wound infections due to *E. hirae*. The majority of enterococcal strains isolated from human specimens belong to the species *E. faecalis* and *E. faecium*. Other enterococcal species are rarely isolated. The *E. hirae* species is known to cause infection in a range of young farmed species and psittacine birds (Devriese *et al.*, 1991; Devriese *et al.*, 1992), rats (Etheridge *et al.*, 1988), kittens (Lapointe *et al.*, 2000) but is very rare in humans. The first description of *E. hirae* infection in humans, by Gilad *et al.* (1998), reported a case of septicemia in a patient with end-stage renal disease undergoing hemodialysis (Gilad *et al.*, 1998). More recently, Poyart *et al.* (2002) reported a case of patient with native valve endocarditis due to *E. hirae* (Poyart *et al.*, 2002). In our study, as well as in the other two reported cases, the *E. hirae* strains responsible for the infections are still susceptible to the commonly used antibiotics for treatment of enterococcal infections. However, close monitoring of the spectrum of etiological agents in certain hospital setting is crucial for early detection of cases of uncommon strains causing infections that might have acquired antibiotic resistance determinants, thus jeopardizing the available therapeutics.

In the second part of this Doctoral work we carried out a more extensive study on the effects of vancomycin exposure and how *E. faecalis* copes metabolically with this exposure.

Identification of new players and their roles in response to vancomycin

In hospitals, 190 million doses of antibiotics are administered each day. Among non-hospitalized patients, more than 133 million courses of antibiotics are prescribed by doctors each year, and many of these prescriptions are unnecessary.

Ideally, and by principle, the choice of a certain therapeutic protocol relies on the susceptibility of the etiological agent causing infection to the available antibiotics and on the pharmacokinetics of the drug of choice. The principle is in its essence correct but is vital to be aware that when one antibiotic (or a combination of antibiotics) is administered to a patient it will not only have an effect on the target microorganism but affects the whole patient microbiota. Also, during the course of treatment, at times, the antibiotic reaches concentrations in blood, serum and tissues that are below the therapeutic concentration.

In chapter 4, we compared the expression levels of *E. faecalis* V583 under exposure to a subinhibitory vancomycin concentration using a whole genome-based approach. This study was the first to evaluate the effect of vancomycin exposure to a subinhibitory dose on the expression profile of *E. faecalis*. Other studies report the effects of vancomycin exposure on other genus such as *B. subtilis* (Cao & Helmann, 2002; Wecke *et al.*, 2006), *S. aureus* (Kuroda *et al.*, 2003; McAleese *et al.*, 2006; McCallum *et al.*, 2006), *S. pneumoniae* (Haas *et al.*, 2005) and *M. tuberculosis* (Provvedi *et al.*, 2009). Apart from all the differences in the design of the experiments, it is unanimous that vancomycin exposure affects globally the transcriptomic profile of a strain and that exposure to subinhibitory concentrations potentiate the number of genes with altered transcription. This study brought new insights and contributed to the general knowledge on *E. faecalis* response to vancomycin. Our data is fundamental to help establishing a

vancomycin stimulon for *E. faecalis* which would constitute a valuable tool for antibacterial drug development with the recognition of new targets.

However, from this study there are questions that remain unanswered and that would be interesting to further investigate. Establishing the connection between exposure to subinhibitory vancomycin concentrations and virulence, or persistence in the host, would provide additional support for prudent vancomycin use. Previous studies have suggested that antimicrobial agents can affect the expression of virulence genes in bacteria (Yim *et al.*, 2006). Thus, it would be interesting to determine whether the increased transcription of putative virulence-associated genes is related with an increased ability of vancomycin-treated enterococci to invade host cells.

Another interesting outcome of this study was the identification of an ORF (EF2292) annotated as a putative hypothetical protein that presented the same levels of induction as the specific vancomycin resistance genes from the *vanB* operon. EF2292 is located on the *E. faecalis* V583 chromosome upstream of the $vanX_B$ gene. In chapter 5 we provide a new scheme for the composition of the *vanB* operon in *E. faecalis* which includes the EF2292 ORF, renamed in this work as *vanV*. We state that VanV does not contribute to *E. faecalis* V583 vancomycin resistant phenotype. It is most likely an accessory protein, such as VanW, but its contribution for the overall function of the operon, if there is one, as well as its role in the cell remain to be clarified.

From the microarray data obtained in chapter 4 we got new information on regulatory genes putatively involved, directly or indirectly, in vancomycin response. Two TCSs were induced in the presence of vancomycin (TCS05 – CroRS, and TCS06) none never before unequivocally related to vancomycin

resistance. However, and since TCSs are, by definition, the prototypical stimulus-response coupling mechanism we decided to further investigate the role of these two TCSs in response to vancomycin exposure. In chapter 6, we performed RT-PCR experiments using *croR* and *err06* mutants in V583 and JH2-2 genetic backgrounds and measured the induction of those genes and of *vanB* operon genes after exposure to vancomycin. We confirmed that both CroRS and Err06-Ehk06 respond directly to vancomycin, regardless of the presence of vancomycin resistance gene cluster. We hypothesize that CroRS is likely sensing perturbations induced by vancomycin on the bacterial cell-wall and its induction probably triggers or provides the signals for vancomycin induction of both VanRS and TCS06. These results contribute for clarifying the cellular role of these two TCSs. However, and despite the involvement of CroRS in the response to other cell-wall active antibiotics has been described and TCS06 has been previously associated with response to oxidative stress, the mechanisms are not yet fully understood. Our work gives new insights on the functions of these two TCSs.

In the 80s, when vancomycin was re-introduced in the clinical practice, it was not predictable that resistance would arise so promptly. Since vancomycin does not get inside the bacterial cell but targets cell-wall precursors, it was generally assumed that vancomycin-resistant organisms would be difficult to generate. However, shortly after vancomycin re-introduction the first vancomycin resistant *Enterococcus* was isolated (Leclercq *et al.*, 1989). Although it is not fully understood how such a unique and complex resistance mechanism came together in enterococci, the fact is that, from the bacteria point of view, it is a very successful resistance mechanism.

In chapter 7 we describe the development of vancomycin resistance phenotype when susceptible enterococci are submitted to serial passages in the

presence of the antibiotic. Several *in vitro* and *in vivo* studies in *Staphylococcus* spp. have documented the stepwise development of resistance either to vancomycin or teicoplanin (Kaatz *et al.*, 1990; Biavasco *et al.*, 1991; Shlaes *et al.*, 1993; Hiramatsu, 1998; Howe *et al.*, 1999; Sieradzki *et al.*, 1999; Kuroda *et al.*, 2000). In general, these studies indicate that the induction of resistance occurs but at a low frequency, bacteria reach a low-level of resistance. Moreover, the selection of resistance subpopulations is more successful for teicoplanin. Our study raises new questions regarding the development of vancomycin resistance by antibiotic induction, since the enterococcal strains we tested are fundamentally different from the staphylococci strains used on the above mentioned studies. Staphylococci do not possess a specific resistance mechanism for glycopeptides. We only observed development of resistance in strains which harbouring some vancomycin resistance mechanism, either *van* genes or the *croRS* and *err06-ehk06* (chapter 6), had one of the genes involved in the resistance mechanisms disrupted. Since we were not successful in inducing vancomycin resistance in enterococcal strains with no vancomycin resistance-related genes, it is likely that the induction of resistance in susceptible enterococci and staphylococci occurs by different mechanisms and involves different cellular tools. However, the induction of vancomycin resistance in strains harbouring *van*-resistance-related genes is very successful and the strains develop stable MICs compatible with high-level resistance. Considering the stability of the phenotype and the absence of detection of chromosomal mutations or rearrangements, the hypothesis of transcriptomic alterations or stabilization of phenotypic mutations become reinforced. Further work is needed to clarify the mechanisms beneath the induction of resistance in these strains

Despite the glycopeptide-specific differences, teicoplanin- and vancomycin-resistant mutants resulting from *in vivo* or *in vitro* exposure of staphylococci to the antibiotics have some biochemical and morphological changes in common, in particular significant cell wall thickening, increased penicillin-binding protein 2 production, and an increased binding capacity for glycopeptides by peptidoglycan (Shlaes *et al*., 1993; Hiramatsu, 1998; Sieradzki *et al*., 1999; Kuroda *et al*., 2000).

In order to obtain further knowledge on the induction mechanism it would be interesting to perform microarray experiments and compare the transcriptomic profile of one of our adapted strains before and after passages in the presence of vancomycin. This would clarify what happens at the transcription level. From the transcriptional data we could possibly identify potential proteins that might be produced, repressed or stabilized in order to stabilize the resistant phenotype. Also, a longer experiment submitting the strains, after induction in presence of vancomycin, to more passages in non-selective medium would provide more information regarding the stability of the alteration. It would be of utmost importance to perform induction experiments using an *in vivo* model to understand if it occurs in the host and at what extent.

The fact that vancomycin resistance genes are stable and kept on the chromosome of host bacteria (as described in chapter 2) even in the absence of selective pressure, might be an indication that genes belonging to vancomycin resistance operons might have a role on other cellular processes. In chapter 8 we performed microarrays using two mutants in genes essential for vancomycin resistance (*vanB* and *vanR*) and we searched for genes putatively connected to *van* operon genes, in an attempt to unravel any possible gene network involving *vanB* operon. We observed that a wide number of genes are affected by the

absence of these genes, corroborating our theory of extra-vancomycin resistance functions for these genes.

Many genes located on mobile genetic elements were affected by the absence of *vanB* and *vanR* genes. In the light of the current knowledge it is difficult to elaborate a unifying hypothesis for this observation. One may speculate that the inactivation of these genes cause a chromosome destabilization and that the mobile regions, namely the prophages, sense this destabilization and alter their expression in order to initiate a lysogenic phase, and keep integrated on the genome. PCR with specific primers for the sites of integration of the phages could be performed, providing information if the phage is integrated within the chromosome or if it was excised. This information would be very useful to unravel the role of phages on the stability of enterococcal genomes. The data presented in chapter 8 constitute valuable information to explain the role of prophage genes in the bacterial metabolism. However, we are still a long way from a complete understanding of how *vanB* and *vanR* genes influence the transcription of functional unrelated genes. Further work is needed to clarify whether the effects of *vanB* and *vanR* genes on the metabolic pathways and genes found in this study are direct or indirect. For instance, performing gel shift assays using VanR protein to test to which promoters the VanR protein binds to would give valued information on the genes directly regulated by this protein.

The results from this Doctoral work have answered some questions but many new ones have been raised. The results obtained can be applied to better understand the stress response mechanism in *E. faecalis*. As such, we believe that the analysis of the global response to vancomycin exposure carried out in the course of this project may be extrapolated to other cell-wall active antibiotics and

contribute to the design of new anti-bacterial agents active and effective against Gram-positive bacteria.

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APPENDIX

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Granular Layer in the Periplasmic Space of Gram-Positive Bacteria and Fine Structures of *Enterococcus gallinarum* and *Streptococcus gordonii* Septa Revealed by Cryo-Electron Microscopy of Vitreous Sections

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High-resolution structural information on optimally preserved bacterial cells can be obtained with cryo-electron microscopy of vitreous sections. With the help of this technique, the existence of a periplasmic space between the plasma membrane and the thick peptidoglycan layer of the gram-positive bacteria *Bacillus subtilis* and *Staphylococcus aureus* was recently shown. This raises questions about the mode of polymerization of peptidoglycan. In the present study, we report the structure of the cell envelope of three gram-positive bacteria (*B. subtilis*, *Streptococcus gordonii*, and *Enterococcus gallinarum*). In the three cases, a previously undescribed granular layer adjacent to the plasma membrane is found in the periplasmic space. In order to better understand how nascent peptidoglycan is incorporated into the mature peptidoglycan, we investigated cellular regions known to represent the sites of cell wall production. Each of these sites possesses a specific structure. We propose a hypothetic model of peptidoglycan polymerization that accommodates these differences: peptidoglycan precursors could be exported from the cytoplasm to the periplasmic space, where they could diffuse until they would interact with the interface between the granular layer and the thick peptidoglycan layer. They could then polymerize with mature peptidoglycan. We report cytoplasmic structures at the *E. gallinarum* septum that could be interpreted as cytoskeletal elements driving cell division (FtsZ ring). Although immunoelectron microscopy and fluorescence microscopy studies have demonstrated the septal and cytoplasmic localization of FtsZ, direct visualization of in situ FtsZ filaments has not been obtained in any electron microscopy study of fixed and dehydrated bacteria.

Gram-positive bacteria possess a cell envelope composed of a plasma membrane and a thick network of peptidoglycan, secondary acids, and proteins, which maintains cell shape and provides resistance to osmotic pressure (39). Electron microscopy studies performed on fixed (either chemically or by freezing) and dehydrated specimens favored the widely accepted model of the cell envelope consisting of the peptidoglycan network in direct contact with the plasma membrane (4). Nevertheless, the existence of a periplasmic space separated from the cytoplasm and the outer medium by a plasma membrane and a thick cell wall has been suggested by previous biochemical studies (3, 14, 25). Recent data obtained with cryo-electron microscopy of vitreous sections (CEMOVIS), a method that allows the observation of biological specimens in the closest-to-native state, challenged the classical model (23, 24). CEMOVIS consists of cooling the specimen at high pressure so rapidly that water cannot crystallize and instead becomes vitreous. Water stays liquid, but its viscosity dramatically increases (10). It is then possible to make thin sections at low temperatures and observe them using a cryo-electron microscope (1). Consequently, water does not need to be removed from the sample, and molecular aggregation is suppressed (11). Moreover, CEMOVIS is performed in the absence of any stain so that contrast in the image is proportional to density variations in the sample. Using this technique, Matias and Beveridge previously demonstrated that *Bacillus subtilis* 168 and *Staphylococcus aureus* possess a periplasmic space (termed the inner wall zone [IWZ]) lying between the plasma membrane and the mature peptidoglycan (termed the outer wall zone [OWZ]) in which molecules should be able to freely diffuse (23, 24). Those authors proposed that the low-density periplasmic space seen with CEMOVIS corresponds to the electron-dense innermost layer observed in stained sections of freeze-substituted gram-positive bacteria (15). Accordingly, they suggested that the periplasmic space is filled with a low-density material that has a high affinity for heavy metal stains (23).

The general pathway of peptidoglycan synthesis is as follows: (i) generation of nucleotide sugar-linked precursors UDP-*N*-acetylmuramyl-pentapeptide and UDP-*N*-acetylglucosamine in the cytoplasm, (ii) dimerization and translocation through the plasma membrane, and (iii) cross-linking of the periplasmic precursors with mature peptidoglycan via transglycosylation and transpeptidation reactions. This last step is performed

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by a set of membrane-bound proteins, termed the penicillin-binding proteins (PBPs) (37, 40). Several studies have shown that translocated peptidoglycan precursors and PBPs are confined to specific areas of the bacterial cell (8, 27, 31, 32, 36). In particular, most gram-positive bacteria have them concentrated at the septum, indicating that polymerization of the mature peptidoglycan happens at least partly in this area (37). The septum is a cellular structure consisting of a ring of invaginating cell envelope. In the vast majority of bacterial species, its development, eventually leading to cell division, is orchestrated by FtsZ, a protein homologous to eukaryotic tubulin (22, 44). FtsZ is expected to form a ring beneath the septal plasma membrane, although such structures have never been observed by any electron microscopy study (5, 45).

Because PBPs are bound to the plasma membrane, it is generally considered that polymerization takes place at the contact between the plasma membrane and the mature peptidoglycan. Evidence for the presence of a wide periplasmic space (22.3 nm in *B. subtilis* and 15.8 nm in *S. aureus*) raises questions about the spatial organization and the mechanism of assembly of peptidoglycan. In their recent publication, Matias and Beveridge reported that at least part of the septum of *S. aureus* possesses a periplasmic space (24). The deeper part of the septum where the plasma membrane is curved, which we define as the constriction ring, was not investigated at high resolution. Whether mature peptidoglycan is in contact with the plasma membrane at this place thus remained an open question, which we have investigated.

We report the structure of the cell envelope of three gram-positive bacteria (*B. subtilis*, *Enterococcus gallinarum*, and *Streptococcus gordonii*) revealed by CEMOVIS. In the three cases, we observed a previously undescribed granular layer (GL) adjacent to the plasma membrane in the periplasmic space. The structure of peptidoglycan assembly sites in the coccus species (*E. gallinarum* and *S. gordonii*) suggests a common mechanism of peptidoglycan cross-linking. We propose that nascent peptidoglycan diffuses into the periplasmic space and is cross-linked by components of the granular layer to form mature peptidoglycan. Finally, CEMOVIS revealed cytoplasmic structures associated with the constriction ring, which could be interpreted as being FtsZ complexes.

MATERIALS AND METHODS

Culture conditions. *B. subtilis* 168 and *B. subtilis* W23 were cultured in TS medium [1.4% K_2HPO_4, 0.6% KH_2PO_4, 0.2% $(NH_4)_2SO_4$, 0.1% Na_3-citrate · $2H_2O$, 0.02% $MgSO_4 \cdot 7H_2O$, 5 μM $MnSO_4$, 0.5% glucose, 0.5% Na-glutamate, and 0.001% yeast extract prepared in bidistilled water] (41) at 37°C with aeration. Cells were harvested after the end of the exponential growth phase (optical density at 600 nm [OD_{600}] of about 2.0). *S. gordonii* Challis (33) and *E. gallinarum* DSMZ 20628 (DSMZ, Braunschweig, Germany) cells were cultured in brain heart infusion (BHI) broth (Oxoid Ltd., Hampshire, United Kingdom) at 37°C without aeration. *S. gordonii* cells were harvested in the middle of the exponential growth phase (OD_{620} of about 0.5). *E. gallinarum* cells were harvested after the end of the exponential growth phase (OD_{600} of about 1.6). In control experiments, *S. gordonii* cells were grown in BHI broth supplemented with 10% glycerol (10% glycerol–BHI) and harvested in the middle of the exponential growth phase (OD_{620} of about 0.5).

Vitrification and cryosectioning. *B. subtilis* and *S. gordonii* cells were washed twice in phosphate-buffered saline (PBS) supplemented with 20% dextran (20% dextran–PBS) (average mass, 40 kDa; Sigma-Aldrich, Buchs, Switzerland). *E. gallinarum* cells were washed twice in water supplemented with 20% dextran. Cells were then introduced into copper tubes and high-pressure frozen with an EM PACT high-pressure freezer (Leica, Vienna, Austria). The tubes were trimmed at −140°C in an FCS Ultracut S cryomicrotome (Leica). Ultrathin sections were produced with a 45° diamond cryo-knife (Diatome, Biel, Switzerland) at a nominal thickness of 50 nm and at a nominal cutting feed of 1 mm/s.

For control experiments, *S. gordonii* cells were washed twice in 10 mM HEPES (pH 7.0) supplemented with 10% glycerol (10% glycerol–HEPES). After the last centrifugation, the excess medium was absorbed with filter paper. The concentrated pellet was introduced into a sandwich aluminum carrier (sample cavity depth, 200 μm; Bal-Tec, Balzers, Liechtenstein). Empty space was filled with 1-hexadecene. Cells were then vitrified using an HPM 010 high-pressure freezer (Bal-Tec). At −140°C, the cells were mounted onto a specimen holder covered with cryoglue (mixture of ethanol-isopropanol [ratio, 1:3]) (34). Blocks were trimmed, and ultrathin sections were produced at −160°C.

Cryo-electron microscopy. Sections were transferred onto carbon-covered 1,000-mesh copper grids (Agar Scientific, Essex, United Kingdom). Grids were transferred to a Gatan cryoholder (Gatan, Warrendale, PA) kept at a temperature below −170°C and inserted into a CM100 cryo-electron microscope (Philips, Eindhoven, The Netherlands) equipped with an LaB6 cathode. The accelerating voltage was either 80 or 100 kV. Specimens were irradiated with a low electron dose. Electron diffraction was used to check whether water was vitreous or crystalline. Crystalline sections were discarded. Images were recorded with a TemCam-F224HD charge-coupled-device camera (Tietz Video and Image Processing Systems, Munich, Germany) at magnifications ranging from ×1,350 to ×22,500. No image processing other than that described in the figure legends was performed.

Dimension measurements. Pixel size and post magnification factor were calibrated by using a two-dimensional catalase crystal (Agar Scientific). Dimensions were measured using images recorded at a magnification of ×22,500. At this magnification, the post magnification factor is 1.69 and the pixel size is 0.63 nm. The horizontal resolution is therefore approximately 1.3 nm. During cryosectioning, material was compressed along the cutting direction. We could not precisely determine the average compression rate because the studied specimens were not perfectly spherical. By analogy to our previous study, where we used similar cutting conditions, we estimate the average compression rate to be 0.2 to 0.3 (46). Nevertheless, it has been shown that compression does not affect dimensions measured perpendicularly to the cutting direction (11). Dimensions were measured accordingly with ImageJ software (NIH, Bethesda, MD). Average-density profiles were recorded along rectangular selections with ImageJ. The width of selection rectangles is specified in the figure legends.

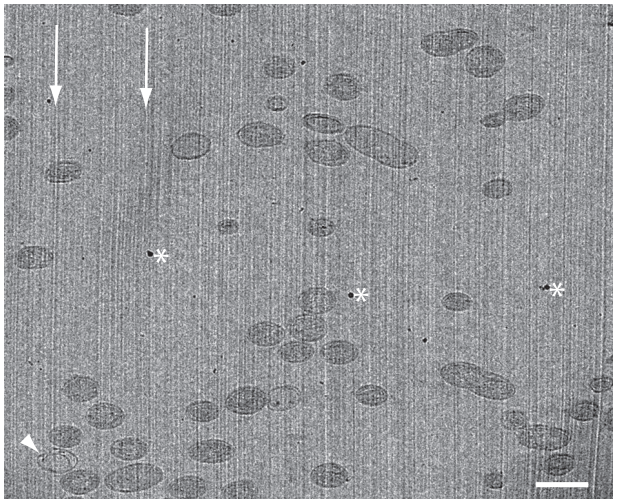

FIG. 1. Low-magnification view of a cryosection of *S. gordonii*. Arrows, knife marks; asterisks, ice contamination; arrowhead, lysed cell. Bar, 1 μm.

RESULTS

Aspect of vitreous sections. A typical section of *S. gordonii* cells vitrified in 20% dextran–PBS is shown in Fig. 1. It is devoid of crevasses, chatter, and cracks, which are the most severe artifacts induced by cryosectioning and which complicate image in-

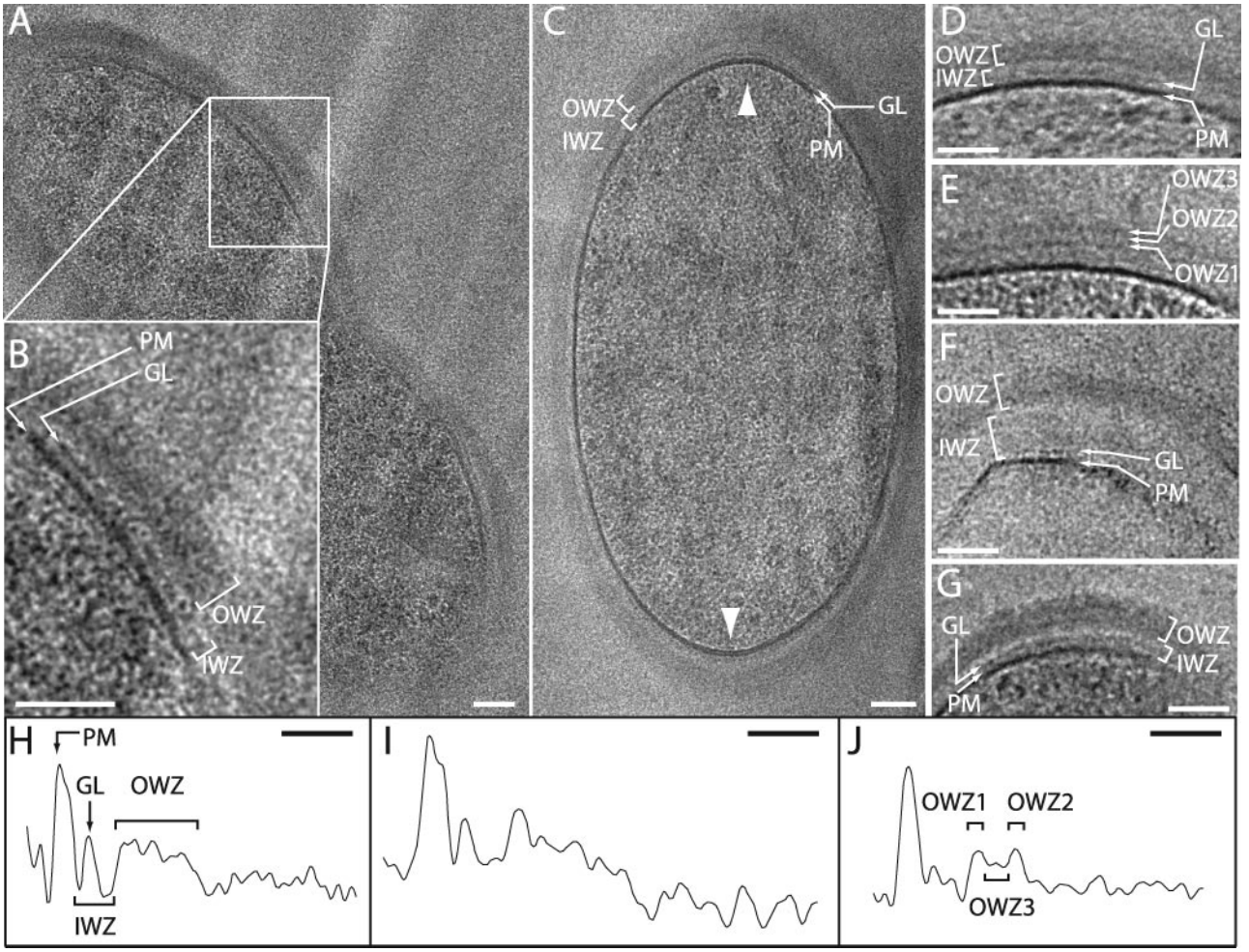

FIG. 2. Structure of the cell envelope of three gram-positive species. (A) *S. gordonii*. (B) Higher magnification of the square in A. (C) *B. subtilis* W23. (D and E) *E. gallinarum*. (F) *S. gordonii* lysed cell. (G) *S. gordonii* cell grown and vitrified in the presence of 10% glycerol. (H to J) Density profiles acquired perpendicularly to the cutting direction through the cell envelope of *S. gordonii* (H), *B. subtilis* W23 (I), and *E. gallinarum* (J). They were not acquired on the images shown in A to E. GL, granular layer; IWZ, inner wall zone; OWZ, outer wall zone; PM, plasma membrane. Arrowheads, which are perpendicular to the cutting direction, show the regions where dimensions of the uncompressed layers of the cell envelope can be accurately measured. B, D, E, F, and G were denoised by Gaussian filtering (radius, 1 pixel). Density profiles in H, I, and J have been obtained from images denoised by Gaussian filtering (radius, 1.5 pixels) and have been averaged over a width of 60 pixels (H), 33 pixels (I), and 69 pixels (J). Bars, 50 nm (A to G) and 20 nm (H to J).

terpretation because they are not homogenous in space. Only homogenous deformation, namely, compression along the cutting direction and knife marks, cannot be avoided. A low level of ice contaminations lies on the surface of the section.

Granular layer in the periplasmic space. The structures of the envelopes of three representative species of gram-positive bacteria (*S. gordonii*, *B. subtilis*, and *E. gallinarum*) are shown in Fig. 2. Images are two-dimensional projections of three-dimensional sections, and for geometrical reasons, the envelope is clearly visible, and membranes look sharp only when they are aligned with the electron beam (see reference 46 for a schematic explanation). Therefore, an apparent discontinuity of a membrane (e.g., bottom of Fig. 2A) is most likely due to a change in the orientation of the plasma membrane. This statement is supported by stereo-pair images (data not shown). As described previously by Matias and Beveridge, who used CEMOVIS, the envelope consists of a plasma membrane, a low-density IWZ identified as a periplasmic space, and a high-density OWZ corresponding to the network of cell wall polymers (23, 24). In addition, a previously unreported dense GL in the IWZ is visible in most of our images and in each of the investigated species. It is more contrasted and therefore denser in *S. gordonii* (Fig. 2A and B) and *B. subtilis* (strains W23 [Fig. 2C] and 168 [data not shown]) than in *E. gallinarum* (Fig. 2D and E). Images were recorded with defocus values ranging from −1.5 μm to −5.7 μm, which corresponds to the first minima of the contrast transfer function in the range from 2.3 nm to 4.7 nm. A GL is visible over this whole range, indicating that it is not an optical artifact. The GL is 3 to 4 nm thick and is 8 to 9 nm distant from the plasma membrane. Measurements of thickness and the positions of the envelope structures are summarized in Table 1. In *S. gordonii* cultures, a small proportion of lysed cells are present (Fig. 1, arrowhead). Similarly to observations in osmotically lysed cells described previously by

TABLE 1. Dimensions of cell envelope structures

Organism and structure	GL position (nm) ± SD[a]	Mean thickness (nm) ± SD (no. of measurements)[b]						
		GL	IWZ	OWZ	Cell envelope[c]	OWZ1	OWZ2	OWZ3
S. gordonii								
Normal cells	8.3 ± 0.5 (10)	4.1 ± 0.9 (9)	16.0 ± 1.0 (10)	26.4 ± 3.6 (10)	42.3 ± 3.4 (10)	NA	NA	NA
Lysed cells[d]	7.7 ± 0.7 (8)*	3.7 ± 0.4 (8)#	NA	31.8 ± 4.4 (19)**	NA	NA	NA	NA
B. subtilis								
Strain W23	9.1 ± 0.8 (27)	3.8 ± 0.6 (11)	19.8 ± 1.7 (29)	33.0 ± 5.4 (29)	52.7 ± 5.7 (29)	NA	NA	NA
Strain 168	8.8 ± 1.0 (7)	3.3 ± 0.4 (7)	19.6 ± 2.7 (10)	29.8 ± 3.3 (10)	49.3 ± 4.6 (10)	NA	NA	NA
E. gallinarum								
Normal cell envelope	9.0 ± 1.2 (17)	3.4 ± 0.8 (15)	18.4 ± 1.9 (20)	18.7 ± 2.8 (20)	37.1 ± 3.4 (20)	4.3 ± 0.6 (17)	6.6 ± 2.7 (17)	8.0 ± 3.0 (17)
Septal cell envelope[e]	9.8 ± 0.5 (6)#	3.5 ± 0.6 (6)#	16.8 ± 1.4 (14)**	23.4 ± 2.0 (7)**	56.9 ± 2.6 (7)	NA	NA	NA

[a] Measured from the center of mass of the plasma membrane to the center of mass of the GL.
[b] Values in nm are presented as mean values ± standard deviations (with numbers of measurements in parentheses). They have been measured using images with a pixel size of 0.63 nm. NA, not applicable.
[c] In the septum, cell envelope thickness corresponds to the distance between the two plasma membranes.
[d] *t* test was performed between corresponding structures of normal and lysed cells. #, no statistical difference; *, $P < 0.05$; **, $P < 0.01$.
[e] *t* test was performed, between the normal cell envelope and the septal envelope.

Matias and Beveridge, the IWZ thickness is larger and highly variable in every single lysed cell, suggesting that the plasma membrane and OWZ are not bound anymore (Fig. 2F) (23, 24). Also, in agreement with the observations reported previously by those authors, the OWZ is thicker in lysed cells than in normal cells (Table 1). This increase is consistent with lysis-induced release of osmotic pressure acting on the OWZ, allowing the relaxation of cell wall polymers (23, 24). In some of the lysed cells, a GL is parallel to the plasma membrane (Fig. 2F). The GL is slightly closer to the plasma membrane in lysed cells than in normal cells, but its thickness and granular aspect are identical in both kinds of cell (Table 1).

Dextran does not induce the granular layer. Dextran was used as a cryoprotectant because it produces a minimal osmotic effect due to its high molecular weight, and it provides good cryosectioning properties (2). In order to rule out the possibility that the previously undescribed GL is an artifact due to the sudden addition of dextran to the extracellular fluid, *S. gordonii* cells were grown in medium containing 10% glycerol as described previously (23, 24) and high-pressure frozen in a buffered solution containing the same amount of this cryoprotectant. *S. gordonii* cells grew similarly with or without glycerol. The only difference was a slight decrease in the growth rate (doubling time, 37 min without glycerol; doubling time, 48 min with glycerol). Cryosections of good quality were rarely obtained when glycerol was used instead of dextran. However, in the regions devoid of the severe artifacts that we could occasionally obtain, the cell envelope is the same as that in *S. gordonii* cells cryoprotected with 20% dextran (Fig. 2G).

Structure of the outer wall zone. *B. subtilis* 168 IWZ and OWZ thicknesses are similar to previously published values (Table 1) (23). The density of the OWZ fades from the inner face to the outer face in *B. subtilis* (168 and W23) (Fig. 2I) and, less steeply, in *S. gordonii* (Fig. 2H). The structure of the *E. gallinarum* OWZ is different: it is subdivided into three layers of different densities (Fig. 2J). This feature is best visible in Fig. 2E. The first layer (OWZ1) is close to the inner face, the second layer (OWZ2) corresponds to the central portion, and the third layer (OWZ3) is close to the outer face of the OWZ. The central portion is less dense than the first and the third portions. The thickness of OWZ1 is smaller and more constant among individuals than the thicknesses of OWZ2 and OWZ3 (Table 1).

Structure of the *E. gallinarum* septum. Is the structure of the cell envelope different in cellular domains involved in peptidoglycan cross-linking? In *E. gallinarum*, peptidoglycan precursors are translocated at the septum (T. Ribeiro, M. Ruivo, and F. Lopes, unpublished data). A cell with septa at two different steps of formation is shown in Fig. 3A. High-magnification views of septa of this cell and other cells are shown in the rest of Fig. 3. The OWZ enters the septum already at the initial step of its formation (Fig. 3B) and develops during constriction of the septum (Fig. 3C, D, and E). The GL and IWZ also enter the septum (best seen in Fig. 3D and E). The OWZ is in contact with the plasma membrane at the constriction ring, and no space remains in between for the IWZ and GL (Fig. 3, arrowheads). In some septa, OWZ density is uniform (Fig. 3D); in others, the central layer of the OWZ is less dense than the layers closer to the surfaces (Fig. 3A and E). The septal IWZ is thinner than the regular IWZ by 9.5% ($P < 0.01$). In contrast, the septal OWZ is thicker than the regular OWZ by 25.1% ($P < 0.01$).

Structure of the *S. gordonii* septum. In *Streptococcus pneumoniae*, PBPs responsible for the cross-linking of peptidoglycan are concentrated at the equatorial ring and at the septum (26, 27). We investigated their structure in *S. gordonii*, a close nonpathogenic relative of *S. pneumoniae* (18). *S. pneumoniae* and *S. gordonii* are ovoid (43). The equatorial ring is identified by an increased curvature of the plasma membrane and is approximately halfway between the septum and the cell pole (6, 43). The GL, IWZ, and OWZ are not different at the equatorial ring (Fig. 4A). Contrary to the situation in *E. gallinarum* and similarly to *S. pneumoniae*, the cell envelope is not deeply invaginated at the level of the *S. gordonii* septum (Fig. 4) (38, 43). Accordingly, the septal OWZ is most probably split in two simultaneously with the addition of new components on its inner surface. The GL does not penetrate the septum but is slightly concave (Fig. 4B and C). The OWZ is also slightly

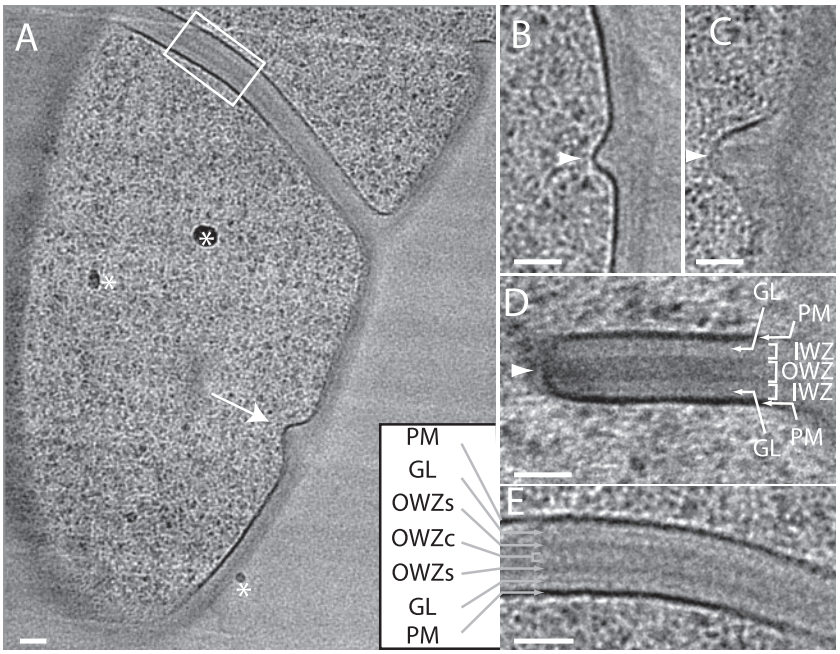

FIG. 3. *E. gallinarum* septum. (A) Dividing coccus. Arrow, newly forming septum. (B to D) Progressive formation of septum. Arrowhead, constriction ring. (E) Higher magnification of the rectangular area in A. Asterisks, ice contamination. See the legend to Fig. 2 for definitions of abbreviations. OWZs, layer closer to the surface of the OWZ; OWZc, central layer of the OWZ. A model of the *E. gallinarum* septum is shown in Fig. 6. Every image has been denoised by Gaussian filtering (radius, 1 pixel). Bars, 50 nm.

concave, but it does not cross the GL and is excluded from the septum. Only the IWZ penetrates the septum, where its density is higher.

Cytoplasmic structures at the constriction ring of *E. gallinarum*. At the level of the constriction ring of the *E. gallinarum* septum, dense elements are found below the plasma membrane. In some cases, they have the aspect of concentrated dots, without higher-order organization (Fig. 5A). In others, the dots are assembled in one or two layers parallel to the plasma membrane (Fig. 5B, C, and D, arrowheads). The density profile of such a structure is shown in Fig. 5G. The dots are 3 to 5 nm in diameter. The distance between the plasma membrane and the first cytoplasmic layer as well as the distance between the first and the second cytoplasmic layers is between 7 and 12 nm. Inside the layers, the distance between the center of mass of the dots lies between 5 and 7 nm. Finally, in some images, weakly contrasted cytoplasmic filaments are situated between the two edges of the septum (Fig. 5E and F). Figure 5G shows a density profile recorded across four filaments of Fig. 5E. These are 3 to 6 nm in diameter, i.e., similar to the diameter of the dots.

DISCUSSION

In every species investigated in the present study, the previously reported IWZ and OWZ were observed. This confirms the new model of the structure of the gram-positive bacterial cell envelope (23). Besides differences in IWZ and OWZ thicknesses, subtle differences in mass distribution within the OWZ exist between species. In *B. subtilis*, the density of the OWZ decreases from the inner to the outer face, which is consistent with a centrifuge turnover of the cell wall (4, 20). According to this model, nascent polymers are inserted on the inner face of the cell wall, where they are not stretched. Due to a continuous addition of new material, they are gradually displaced towards the exterior of the wall. During the first step of this displacement, the polymers become stretched. Consequently, they provide resistance against osmotic pressure but also become more prone to cleavage by autolysins. This leads to reduced crosslinking and eventually to the release of peptidoglycan fragments in the extracellular medium. This mechanism, in combination with the fact that the cell wall is inert (i.e., not synthesized or degraded) at cell poles, is thought to be at the origin of the rod shape (19, 20). In *S. gordonii*, the OWZ also fades but in a less pronounced manner, reflecting the probably reduced centrifuge turnover due to the incorporation of wall polymers in very restricted sites. A similar difference between *B. subtilis* and *S. aureus* was previously noticed and interpreted likewise by Matias and Beveridge (23). Indeed, in *S. aureus*, the cell wall is polymerized exclusively at the septum (31). In *E. gallinarum*, whose peptidoglycan polymers are also mostly assembled at the septum (Ribeiro et al., unpublished), the center of the OWZ is less dense than its surfaces, suggesting yet another mode for the regulation of wall polymer degradation.

We report a dense GL located in the IWZ, which was not seen previously. The GL is observed over a wide range of defocus values, including close to focus (Fig. 2C). This demonstrates that the GL is not an optical artifact of phase contrast. To confirm that the GL was not induced by osmotic shock due to the sudden change in dextran concentrations, we adapted the protocol used previously by Matias and Beveridge (23, 24): *S. gordonii* cells were grown in 10% glycerol–BHI and

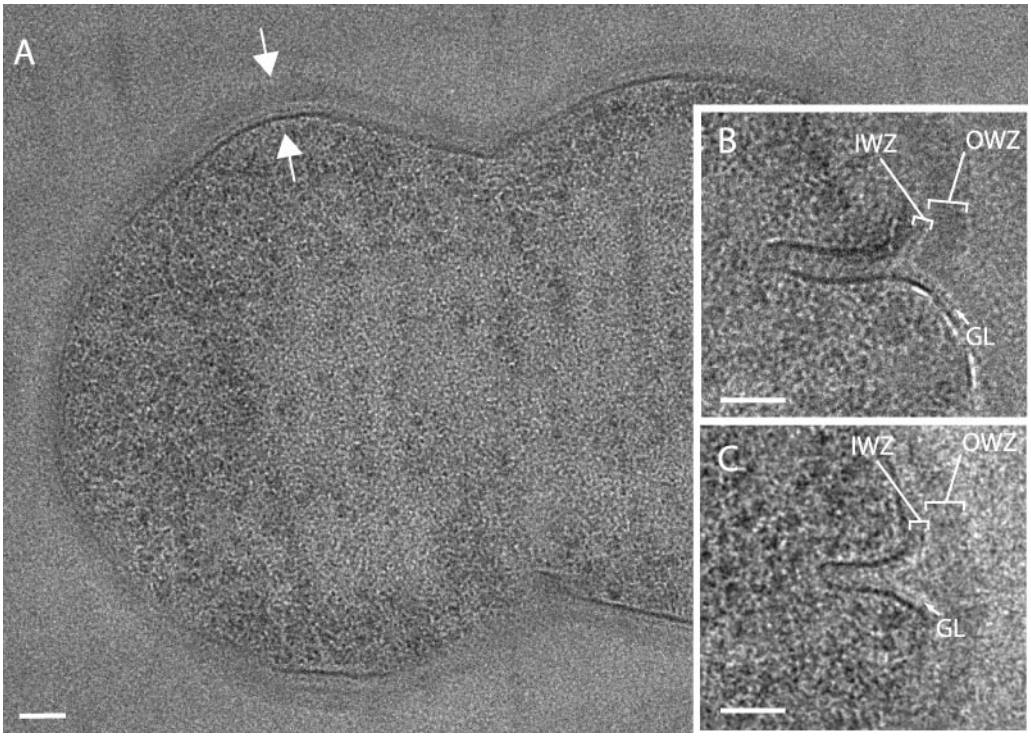

FIG. 4. *S. gordonii* equatorial ring and septum. (A) Dividing coccus. A well-defined equatorial ring is shown between arrows. (B and C) Septa of other cocci. See the legend to Fig. 2 and the text for definitions of the abbreviations. A model of the *S. gordonii* septum is shown in Fig. 6. Bars, 50 nm.

vitrified in 10% glycerol–HEPES. The GL is also visible under these conditions, indicating that the GL is certainly a native feature of the cell envelope. The reason why the GL was not discovered by Matias and Beveridge (23, 24) is perhaps related to the use of glycerol, which prevents the routine production of crevasse-free sections. Lysed cells of *S. gordonii* reveal that the GL is retained by the plasma membrane, suggesting that GL components are anchored in the plasma membrane (Fig. 2F). The only exception is found at the septum of (nonlysed) *S. gordonii*, where the GL is aligned not with the plasma membrane but with the OWZ (Fig. 4B and C and 6B). A possible interpretation is that under normal conditions, the GL bridges the plasma membrane to the mature peptidoglycan; under special conditions, one of the connections can be broken.

In order to better understand how nascent polymers are incorporated into the OWZ, we investigated areas of cell wall assembly. In *B. subtilis*, these areas have been identified as the septum and in a helical structure along the rod (7, 36). With CEMOVIS, it was not possible to routinely observe *B. subtilis* assembly sites because on the one hand, rod cells align longitudinally along the long axis of the freezing tube when they are introduced into the tube (23). Consequently, only cross-sections of the cells and almost no longitudinal sections are obtained, making the observation of septa in rod cells virtually impossible. On the other hand, the helical pattern of assembly is not well defined morphologically.

Nevertheless, CEMOVIS revealed the close-to-native structure of peptidoglycan assembly sites of *E. gallinarum* in the stationary state (septum) and of *S. gordonii* in the exponential state (septum and equatorial ring), whose shape is closer to a sphere, allowing the random orientation of cells in the freezing tube. The structure of the cell envelope at the *S. gordonii* equatorial ring is not different than that in the rest of the cell. The structures of the *E. gallinarum* and *S. gordonii* septa are modeled in Fig. 6 according to the observations shown in Fig. 3 and 4. It is unlikely that these two cartoons represent two successive steps of one septum maturation process, since we have never observed the typical wide OWZ-containing septum of *E. gallinarum* in *S. gordonii* or the characteristic narrow OWZ-free septum of *S. gordonii* in *E. gallinarum*. The structures of the three peptidoglycan assembly sites studied are not identical but can be interpreted with a common mechanism. In the three cases, the OWZ does not cross the GL. The OWZ is therefore always exposed to the outer face of the GL. Moreover, the GL is always in the vicinity of the OWZ. Together, these data suggest that after translocation through the plasma membrane in the periplasmic space (IWZ), peptidoglycan precursors could diffuse relatively freely without polymerizing until they interact with the interface between the GL and the OWZ. Precursors could then polymerize with the OWZ. In the case of the *S. gordonii* septum, the high density of the septal IWZ situated below the GL might represent a high concentration of unpolymerized precursors (Fig. 4B and C). In the *E. gallinarum* septum, the OWZ reaches the plasma membrane in the constriction ring (Fig. 6A). At this site, peptidoglycan precursors are exposed to the outer face of the GL immediately after translocation. On the basis of these considerations, the GL could contain PBPs, the enzymes responsible for peptidoglycan cross-linking. The X-ray structure indicates that PBPs protrude 9 to 13 nm into the periplasmic space, which is con-

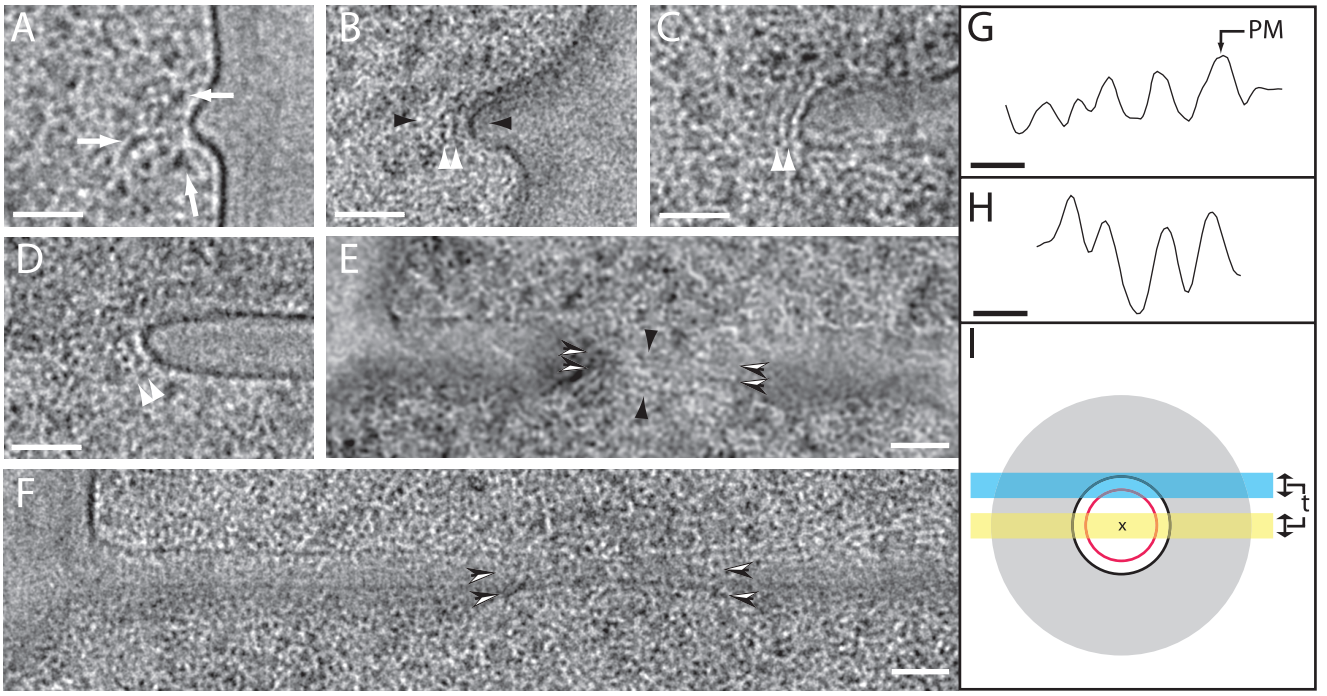

FIG. 5. *E. gallinarum* cytoplasmic structures associated with the constriction ring. (A to D) Arrows indicate concentrated dots without higher-order organization; white arrowheads indicate dots assembled in parallel layers. (E and F) Black and white arrowheads show some of the weakly contrasted lines. (G) Density profile of the area comprised between black arrowheads in B. PM, plasma membrane. (H) Density profile of the area comprised between black arrowheads in E. The left extremity of the profile corresponds to the upper arrowhead. (I) Schematic representation of the bacterial septal plane. X corresponds to the center of the septal ring. The OWZ is represented in gray; the plasma membrane in the constriction ring is shown in black. The cytoplasmic filament ring is in red. A section of thickness, t, across the middle of the septal ring, corresponding to A to D, is drawn in yellow. A section of the same thickness cut tangentially to the septal ring, corresponding to E and F, is represented in blue. Images have been denoised by Gaussian filtering with a radius of 1 pixel in A to D and F and a radius of 2.5 pixels in E. Density profiles have been recorded on Gaussian-filtered images with a radius of 1 pixel in G and 1.5 pixels in H. They have been averaged over a width of 11 pixels in I and 21 pixels in H. Bars, 50 nm (A to F) and 10 nm (G and H).

sistent with the distance between the centers of mass of the GL and of the plasma membrane (21, 29). PBPs are not distributed homogenously over the plasma membrane; different members of the PBP family are concentrated in specific areas (8, 26, 27, 32). The GL is, however, homogenously distributed over the whole cell (except in the situations described above). It is therefore most probable that the GL is not only composed of the various PBPs. Other molecules interacting with the plasma membrane, such as lipoproteins and lipoteichoic acids, could compose the GL. It may also contain molecules that are not bound to the plasma membrane, as suggested by the structure of the GL at the *S. gordonii* septum (Fig. 6B). Interestingly, electron microscopy experiments using labeled penicillin in *S. aureus* cells indicated that a fraction of the PBPs may not be bound to the plasma membrane (30). Since vitreous sections cannot be immunolabeled, targeted gene deletions or selective

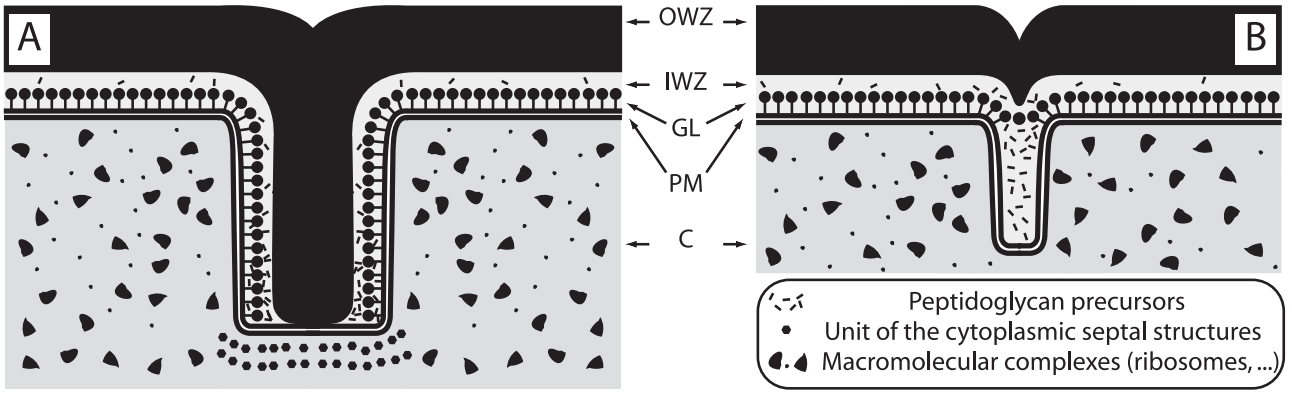

FIG. 6. Model septa of (A) *E. gallinarum* and (B) *S. gordonii*. The position of peptidoglycan precursors is hypothetic and based on considerations explained in the Discussion. C, cytoplasm. For definitions of other abbreviations, see the legend to Fig. 2 and the text.

chemical extractions might contribute to the characterization of the molecular composition of the GL.

The layered structure of the gram-positive envelope raises questions. It is well accepted that the plasma membrane is the diffusion barrier between the cell interior and the outer world. It is subjected to the effect of the osmotic pressure, but it does not have the mechanical strength to hold it. The peptidoglycan layer of the cell wall (OWZ) probably confers the mechanical resistance (39). However, the low-density zone of the IWZ, in which we propose that envelope precursors can diffuse, separates the plasma membrane from the OWZ. How is the force that is generated at the plasma membrane reported to the OWZ through the IWZ? One possibility, proposed previously by Matias and Beveridge, is that rigid molecules keep the OWZ distant from the plasma membrane by forming a scaffold in between (24). Alternatively, pressure could be transferred from the plasma membrane to the base of the OWZ by a gel composed of osmotically active polymers freely floating in the IWZ. Their osmotic effect would preclude that the IWZ volume decreases beyond a threshold value reached when the periplasmic osmotic pressure equals the cytoplasmic osmotic pressure. The osmotically active polymers should be large enough not to diffuse through the plasma membrane or through the OWZ, and they should be flexible enough (short persistence length) to be osmotically active at relatively low concentrations, which would be consistent with the low density of the IWZ (9). Regarding this hypothesis, it is noteworthy that the cytoplasm and periplasm of gram-negative bacteria are isosmotic (42). Obviously, the cell envelope still has many facets to reveal until its structure and functions are fully understood.

CEMOVIS revealed cytoplasmic structures at the constriction ring of the *E. gallinarum* septum. They are strongly contrasted dots (Fig. 5A to D) or weakly contrasted lines (E and F) of 3 to 6 nm in diameter. The signal is far above the noise level, as is demonstrated by two successive images recorded in the same area (data not shown). Dots of similar sizes are found everywhere in the cell but not at this high concentration nor with this layered organization. Weak lines were not found anywhere other than in the septal area. These structures are thus specific for the septum. In *S. gordonii*, the occurrence of such structures was rarely observed.

The contrasted dots are present in images where the plasma membrane in the constriction ring (i.e., the bottom of the septum) is sharp or relatively sharp, indicating that these sections cross the center of the septum (shown in yellow in Fig. 5I). The weak lines are found in images where the plasma membrane is not defined in the constriction ring, implying that these sections are cut tangentially to the constriction ring (shown in blue in Fig. 5I). Note that membranes and cell wall layers that are close to parallel to the section plane are expected to slightly darken the corresponding area of the image but are not expected to produce any sharp structure (46). The blurred aspect of the lateral invaginated membranes indicates that they are slightly tilted, but this tilt cannot account for the blurred aspect of the membrane in the constriction ring. Together, these data are consistent with a ring of cytoplasmic filaments localized near the plasma membrane (red ring in Fig. 5I). Filaments are indeed seen with CEMOVIS as contrasted dots in top views and as weak lines in longitudinal views (12, 35). From this assumption, we speculate that the observed structure may represent polymerized FtsZ proteins. FtsZ is a prokaryotic cytoskeletal protein that is homologous to eukaryotic tubulin and is thought to be organized as a cytoplasmic ring of filaments beneath the plasma membrane (5). It is essential for septation and serves as a scaffold for proteins involved in the division process (22, 37). Although immunoelectron microscopy and fluorescence microscopy studies have proven its septal localization and suggested a ring organization, FtsZ filaments have never been observed in thin sections of fixed and dehydrated bacteria (45). Most probably, FtsZ collapses during dehydration steps and becomes indiscernible from the plasma membrane. In spite of that, an electron microscopy study of isolated FtsZ has revealed that it can polymerize in vitro as (i) straight single protofilaments, (ii) two-dimensional sheets of protofilaments, or (iii) curved protofilaments (13). The cytoplasmic dots concentrated near the constriction ring observed with CEMOVIS (Fig. 5A, arrows) can be interpreted as cross-sectioned protofilaments; the layers of dots parallel to the plasma membrane may represent cross-sectioned two-dimensional sheets of protofilaments (Fig. 5B, C, and D). Supporting this hypothesis is the agreement of the 5.3-nm distance between protofilaments in vitro and the 5- to 7-nm distance between dots within a single layer in close-to-native cells (13). A three-dimensional representation of the cytoplasmic structures is necessary to confirm our interpretation (by determining the curvature of the filaments and their relationship with the plasma membrane, for example), and the developing technique of cryo-electron tomography of vitreous sections should make it possible to solve the structures in the next few years (16, 17, 28). Obtaining tomograms of vitreous sections at 3-nm resolution will certainly require the thinnest possible sections. Therefore, the probability of having a complete septal ring in a section will be very low. Moreover, it might be very difficult to detect such a configuration, because sections must be screened at a low magnification to minimize the electron dose. Such a cell slice might lack highly contrasted characteristic features visible at low magnifications. However, tomograms of sections containing an important part of septal ring, as shown in Fig. 5E and F, should be sufficient to measure the curvature of the filaments and their relationship with the plasma membrane. If this prediction comes true, it will be possible to gain structural insights into the cell division macromolecular machinery at work, and this will most likely help to solve its mechanism.

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Vancomycin-susceptible dairy and clinical enterococcal isolates carry *vanA* and *vanB* genes

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Abstract

A total of 109 enterococcal isolates from dairy food products and from human and dog infections, isolated in Portugal, and 26 type and reference strains of the genus *Enterococcus* were screened for vancomycin resistance. MIC values, both for vancomycin and teicoplanin, were determined. The genetic relatedness of isolates carrying either *vanA* and/or *vanB* was determined using Pulsed Field Gel Electrophoresis. For *vanA* carrying isolates, transposon Tn*1546* was partially mapped using PCR. None of the 59 dairy isolates was resistant to vancomycin. Among the 50 clinical isolates, only one, carrying *vanB*, behaved as resistant, with a MIC value of 256 μg/mL. The type and reference strains used were susceptible both to vancomycin and teicoplanin. *vanA* was found in 37% of the dairy isolates and 40% of the clinical isolates. *vanB* was only detected in 18% of the clinical, both human and dog, isolates. PCR partial mapping of Tn*1546* revealed 23 different patterns among 42 isolates. Some patterns were shared between dairy and clinical isolates. Using Pulsed Field Gel Electrophoresis six groups of isolates were found to be genetically undistinguishable and grouping was found to be geographically and location specific/related. No genetic relatedness was found between isolates from dairy, human and veterinary sources. These results show that an incomplete and/or unfunctional Tn*1546* element may explain the absence of resistant behaviour in the studied isolates, even when *vanA* gene is present. Moreover, the work reported shows that both clinical (human and animal) and dairy isolates have been in contact with VanA genotype of resistance and suggest that dissemination of *vanA* gene has been through transposable elements, like Tn*1546*, and not by clonal dissemination of a resistant strain. Therefore, a national strategy should be implemented to survey both vancomycin resistance and its genetic dissemination.

Keywords: Dairy enterococci; Clinical enterococci; Vancomycin; Antibiotic resistance; Tn*1546*

1. Introduction

Enterococci constitute part of the natural gut microflora in mammals (Devriese et al., 1992). They are also found in a variety of food products, namely milk and cheese produced in the south of Europe (Flahaut et al., 1997; Barros, 1998; Lopes et al., 1999). In some of these cheeses enterococci play a key role in the maturation and final organoleptic characteristics of the food product and constitute the main microbial population in the final product (Franz et al., 1999). For many years enterococci have been considered harmless to man. However, in the last decade, they have become the second most frequently reported cause of surgical wound infections and nosocomial urinary tract infections and the third most frequently reported cause of bactereamia (Klein et al., 1998). Moreover, treatment of these infections has become more and more difficult due to the increasing number of antibiotic resistant enterococci.

The optimal therapy for endocarditis, one of the most serious enterococcal infections, involves a combination of a cell-wall active agent and an aminoglycoside, usually gentamicin (Malani et al., 2002). With the appearance of high-level gentamicin resistance the only antibiotic available for a successful treatment of enterococcal infections is vancomycin, a glycopeptide. However, over the past decade, there has been a rapid increase in vancomycin resistant enterococci (VRE).

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Colonization and infection by VRE have been reported in hospitalized patients and in the community, and has also been associated with environmental sources and meat products. Glycopeptide resistance in enterococci is generally acquired and is encoded in genetic mobile elements, like transposons. This is the case of VanA, VanB, VanD, VanE and VanG phenotypes. It can also be encoded in a non-transferable chromosomal determinant, as in VanC phenotype (Tendolkar et al., 2003). VanA and VanB are the most globally widespread and prevalent phenotypes. The prototype *vanA* element is Tn*1546*, a 10.8-kb transposon that carries a cluster of nine genes, encoding nine different proteins, which mediates high-level inducible resistance to both vancomycin and teicoplanin. The nine polypeptides can be divided in four functional groups: transposition (ORF 1 and 2), regulation (VanR and VanS), resistance to glycopeptides (VanH, VanA and VanX) and synthesis of peptidoglycan (VanY and VanZ). The VanB cluster encodes inducible resistance to vancomycin, but not to teicoplanin and is disseminated by large transposons such as Tn*1547* and Tn*5382* (Tendolkar et al., 2003). Tn*1547* is homologous to Tn*1546* and most of the VanB genes share a large percentage of homology to the VanA cluster genes. In Europe, VanA has been found in VRE isolated in the community, from sewage, animal faeces and raw meat, which suggests that these environments can act as reservoirs of these microorganisms (Simjee et al., 2002). In fact, the emergence of VRE has been related to the use of antimicrobial agents both in human and veterinary medicine and as growth promoters in animal feed. Although enterococci isolated from traditional Portuguese cheeses have not been subjected to the selective pressure of the use of glycopeptides, it would be of the utmost importance to ascertain the dissemination of vancomycin resistance in this environment. Therefore, the aim of this study was to survey vancomycin resistance among a group of dairy and clinical isolates and to ascertain the possible relatedness of the VRE isolates. *vanA* and *vanB* genes were chosen since they are the most widely found among VRE. Type and reference strains of the genus *Enterococcus* were included in this study since they are the representatives of the genus, but are never included in published works on this subject.

2. Materials and methods

2.1. Microorganisms

A total of 135 (109 wild types plus 26 control and reference) enterococci were used in this study. Fifty-nine strains were isolated from Portuguese dairy products (ewe's cheese and milk) collected from four different Registered Designation of Origin (RDO) designated as A, B, C and D, as described by Lopes et al. (1999). Thirty-nine isolates were obtained from human infections in several Portuguese hospitals. Eleven dog-infecting isolates were obtained from Lisbon Veterinary Faculty. The dairy isolates were identified by phenotypic and molecular methods as 25 *Enterococcus faecalis*, 6 *E. faecium*, 24 *E. durans*, 1 *E. raffinosus*, 6 *E. hirae*, 1 *E. dispar* and 33 *Enterococcus* spp. (Alves et al., 2004). The same methods applied to the clinical isolates identified 31 as *E. faecalis*, 8 as *E. faecium*, 1 as *E. solitarius*, 1 as *E. durans* and 9 as *Enterococcus* spp. Twenty-six type and reference strains of the genus *Enterococcus* were also studied and are described in Table 3. The more recently described species *E. pallens*, *E. gilvus* (Tyrrell et al., 2002), *E. phoeniculicola* (Law-Brown and Meyers, 2003), *E. italicus* (Grazia Fortina et al., 2004) and *E. hermaniensis* (Koort et al., 2004) were not included in this study. *E. faecalis* ATCC 29212 was used as a control strain in the *E*-test, for MIC determination. Vancomycin MIC values for this control strain coincided always with the expected ones. *E. faecium* BM 4147 and *E. faecalis* DSMZ 12956 were used as positive control strains for *vanA* and *vanB* PCR reactions, respectively.

2.2. MIC determination

Minimum inhibitory concentrations for vancomycin and teicoplanin were determined using the *E*-test (Biodisk, Solna, Sweden), according to manufacturer's instructions. Each plate was inoculated with a suspension of the microorganism equivalent to 2 in the MacFarland turbidity standard. Vancomycin and teicoplanin were purchased from Sigma (Steinheim, Germany). *E. faecalis* ATCC 29212 was used as a control strain. Strains were classified as resistant (MIC $\geq$ 32 μg/mL), intermediate (MIC $\geq$ 8 μg/mL and MIC $\leq$ 16 μg/mL) and susceptible (MIC $\leq$ 4 μg/mL).

2.3. Preparation of DNA

Total DNA was extracted from cells according to the method of Pitcher et al. (1989).

Table 1
Sequence of each primer (forward, f and reverse, r) used to amplify the nine genes/intergenic regions (IR) searched for using PCR and respective expected amplicon sizes

Gene / IR	Sequence[a]	Size of amplicon
vanA	f 5′-GGGAAAACGACAATTGC-3′ r 5′-GTACAATGCGGCCGTTA-3′	732 bp
vanB	f 5′-ATGGGAAGCCGATAGTC-3′ r 5′-GATTTCGTTCCTCGACC-3′	635 bp
vanR	f 5′-AGCGATAAAATACTTATTGTGGA-3′ r 5′-TCGGAGCTAACCACATTC-3′	533 bp
vanS	f 5′-AACGACTATTCCAAACTAGAAC-3′ r 5′-GCTGGAAGCTCTACCCTAAA-3′	1049 bp
SH	f 5′-ACGTTTAGGGTAGAGCTTCC-3′ r 5′-CTCATCCTGCTCACATCCATAAACA-3′	313 bp
vanH	f 5′-ATCGGCATTACTGTTTATGGAT-3′ r 5′-TTACGTCATGCTCCTCTGAG-3′	1016 bp
vanX	f 5′-ACTTGGGATAATTTCACCGG-3′ r 5′-TGCGATTTTGCGCTTCATTG-3′	404 bp
XY	f 5′-GCATATAGCCTCGAATGG-3′ r 5′-TCCTGGAAAACAGTGCTTCATTAA-3′	594 bp
vanY	f 5′-ACTTAGGTTATGACTACGTTAAT-3′ r 5′-TCGTCAAGCTTGATCCTA-3′	486 bp
YZ	f 5′-GTTTCCCGGATCAACACATACTA-3′ r 5′-CCCAGTAGCAGTAAATGGAGTCA-3′	336 bp

[a] Sequences published by Miele, Bandera and Goldstein (1995) except for *vanX* (Manson et al., 2003).

2.4. PCR and sequencing

Except for the type and reference strains, all dairy and clinical isolates were searched for the presence of the genes *vanA* and *vanB* using PCR. Primers used were as presented in Table 1. For the isolates for which *vanA* was detected by PCR eight primer pairs (Table 1) were used to partially map the Tn*1546* transposon. PCR was performed with T-personal Combi thermocycler (Biometra, Goettingen, Germany). Each 50 μl PCR reaction mixture contained 250 ng of DNA, 0.5 μM of each primer, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates (dATP, dCTP, dGTP and dTTP), 1× PCR buffer, 0.005% W1 and 2.5 U of *Taq* DNA polymerase. All reagents were purchased from GibcoBRL (Life Technologies, Paisley, UK), except the primers that were purchased from MWG-Biotech (Ebersberg, Germany). The thermocycler was programmed with the following conditions: 5 min at 95 °C; 30 s at 94 °C, 30 s at 47 °C and 30 s at 72 °C for 30 cycles; 10 min at 72 °C; and 4 °C until analysis. Visualisation of amplicons was done with ethidium bromide (Sigma, Steinheim, Germany) under UV irradiation, after electrophoresis on 2% agarose (GibcoBRL, Life Technologies, Paisley, UK) gels. Image analysis was performed with Kodak Digital Science™ (Stuttgart, Germany). Amplification of the above mentioned genes was confirmed with sequencing of the PCR products, after their purification either with Concert™ Rapid PCR Purification System or with Concert™ Matrix Gel Extraction System, both purchased from GibcoBRL (Life Technologies, Paisley, UK). Sequencing was performed by MWG (Ebersberg, Germany).

2.5. PFGE

PFGE was performed with dairy and clinical isolates carrying either the gene *vanA* or the gene *vanB*. Genomic DNA agarose discs and DNA digestion with *Sma*I were performed according to the method of Chung et al. (2000), except for lysis solution, which contained only RNase A and lysozyme. Agarose discs were electrophoresed on a 1% Seakem GTG agarose gel (FMC Bioproducts, Maine, USA) in 0.5× TBE buffer low EDTA (50 mM Tris, 50 mM boric acid, 0.2 mM EDTA, pH 8.0) in a CHEF apparatus (BioRad, Hertfordshire, UK) at 8 °C for 18 h at 220 V with pulse times of 5 s rising to 35 s during the electrophoresis period. Gels were stained by immersion in ethidium bromide (1 μg/mL) for 30 min and photographed with a UV light source.

3. Results

In this work 109 enterococcal isolates from two different environments, dairy products and clinical settings (both human and pets), and identified as different species, and 26 type and reference strains of the genus *Enterococcus*, were firstly investigated on their resistance to vancomycin using the *E*-test for MIC determination. Teicoplanin MICs were also determined and the study was completed with a survey of the presence of *vanA* and *vanB* genes using PCR. *vanA* positive isolates were subjected to PFGE to ascertain their relatedness and other regions of the Tn*1546* transposon were also searched in these isolates, using eight primer pairs.

3.1. MIC determination

According to results shown in Table 2, no resistant isolates were found among dairy isolates, although 12% (7 isolates) had MIC values of 6 μg/mL and can be considered intermediate. In contrast, among the clinical isolates, 72% behaved as susceptible, 22% were intermediate and 6% were found to be resistant. All type and reference strains behaved as vancomycin susceptible, as they showed MIC values below 4 μg/mL, and 70% of these strains had MIC values below 1 μg/mL (Table 3). Overall, reference strains had lower MIC values than those of the other isolates studied. The clinical isolates showed higher MIC values than dairy isolates, with 28% of the clinical isolates presenting MIC values between 6 and 256 μg/mL, against only 12% of the dairy isolates with MIC values of 6 μg/mL

All MIC values for teicoplanin fell under the susceptible classification, for all the isolates and reference strains studied (results not shown).

3.2. Search for the genes vanA and vanB

vanA gene was found in 37% of the dairy isolates and in 40% of the clinical isolates. *vanB* gene was not found in any dairy isolate, but was found in 18% (seven human isolates and two dog isolates) of the clinical enterococci studied. In four of the *vanB* positive clinical enterococci (two from humans and two from dogs), *vanA* was also detected. Among the dairy isolates, *vanA* was detected in four different species (*E. faecalis*, *E. faecium*, *E. durans* and *E. hirae*), both in milk and cheese isolates. In clinical enterococci, *vanA* was detected in *E. faecalis* and *E. faecium*, and in *E. solitarius*. The four isolates that showed both *vanA* and *vanB* were identified as *E. solitarius*, *E. faecium* and two as *Enterococcus* spp.

Table 2
Distribution of dairy and clinical isolates, carrying *vanA*/*B* genes of resistance, and of type and reference strains, between different MIC values for vancomycin

Strains[a]		MIC (μg/mL)											
		0.38	0.5	0.75	1	1.5	2	3	4	6	8	12	256
Dairy	$vanA^+$	1	0	0	3	0	0	7	7	4	0	0	0
	$vanA^-$	1	0	1	3	9	7	11	2	3	0	0	0
	$vanB^+$	0	0	0	0	0	0	0	0	0	0	0	0
	$vanAB^+$	0	0	0	0	0	0	0	0	0	0	0	0
	Total	**2**	**0**	**1**	**6**	**9**	**7**	**18**	**9**	**7**	**0**	**0**	**0**
Clinical	$vanA^+$	0	0	0	0	0	3	3	6	4	0	0	0
	$vanA^-$	0	0	0	0	2	3	11	5	3	0	0	0
	$vanB^+$	0	0	0	0	0	1	0	1	1	1	0	1
	$vanAB^+$	0	0	0	0	0	1	0	0	2	0	1	0
	Total	**0**	**0**	**0**	**0**	**2**	**8**	**14**	**12**	**10**	**1**	**1**	**1**
Reference Strains	Total	**1**	**3**	**5**	**9**	**1**	**0**	**7**	**0**	**0**	**0**	**0**	**0**

[a] Total number of isolates/strains in each column are in bold; $vanA^{+/-}$, presence/absence of *vanA* gene only; $vanB^+$, presence of *vanB* gene only; $vanAB^+$, presence of both *vanA* and *vanB* genes.

No correlation was evident between the presence of *vanA* gene and higher MIC values for vancomycin (Table 2) or teicoplanin. In fact, both dairy and clinical isolates that were positive for *vanA* had MIC values under 6 μg/mL. Only two clinical isolates had MIC values of 8 and 12 μg/mL and in both of them *vanB* gene was also detected. In fact, the presence of *vanB* gene seems to increase MIC values for vancomycin since six, among nine isolates positive for *vanB*, had MIC values between 6 and 256 μg/mL. In the *E. faecium* isolate with a MIC value of 256 μg/mL, only *vanB* gene was detected.

3.3. PCR Tn1546-like patterns

Strains carrying *vanA*, both clinical and dairy, were surveyed for the presence of other elements of Tn*1546*, the prototype mobile element carrying the *vanA* operon. Eight primer pairs were used in this screen assay. Two of these primer pairs were directed to two intergenic regions, XY and YZ, and the other six pairs of primers were directed to more conserved regions of this transposon, namely genes *vanR*, *vanS*, *vanH*, *vanX* and *vanY*. Among 42 isolates, 23 different PCR patterns were found (Table 4). All the genes and intergenic regions were detected, at least in one isolate, and with the exact size as predicted from the Tn*1546* sequence. The absence of *vanX* was the only common feature among all the 42 isolates. The only two isolates for which all (except *vanX*) the searched elements were detected (pattern B), come from the clinical environment, but from different hospitals and with collection dates 10 years apart. They were both identified as *E. faecalis* and their MIC values for vancomycin were 4 and 6 μg/mL. PCR pattern A, corresponding to absence of all the fragments, was found in four isolates, one from dairy origin (*E. durans*, with a MIC value for vancomycin of 4 μg/mL) and three from clinical environment (two *E. faecalis* and one *Enterococcus* spp., with MIC values for vancomycin of 4 and 3 μg/mL), and none of these strains was highly susceptible. A higher variability in PCR patterns was found among dairy isolates. In fact, seven patterns (from B to H) were only found in clinical enterococci (13 isolates), and 12 different patterns (from L to X) were found in dairy isolates (16 enterococci). Four patterns were common to both environments (A, I, J and K). The only common feature among these four patterns was the absence of the X and S fragment. The S fragment, corresponding to *vanS* gene, was only found in six isolates and is the second most absent after the *vanX* gene. Region H, corresponding to *vanH* gene, was present in 43% of the isolates and the intergenic region XY in 16 isolates (38%). The intergenic region YZ was found in 55% of the isolates and is, overall, the most conserved region of the transposon. Despite the variability of PCR

Table 3
Vancomycin and teicoplanin MIC values for the type and reference strains of the genus *Enterococcus*

Strains [a]	Taxon	MIC (μg/ml)	
		Vancomycin	Teicoplanin
DSMZ 11492^T	*E. asini*	0.5	0.25
DSMZ 20679^T	*E. avium*	0.75	0.75
DSMZ 20680^T	*E. casseliflavus*	0.5	0.094
DSMZ 20682^T	*E. cecorum*	3	0.75
DSMZ 7374^T	*E. columbae*	0.5	0.5
DSMZ 6630^T	*E. dispar*	3	0.5
DSMZ 20633^T	*E. durans*	1	0.094
DSMZ 20478^T	*E. faecalis*	0.75	0.75
CECT 187	*E. faecalis*	3	0.25
CECT 184	*E. faecalis*	3	0.25
DSMZ 20477^T	*E. faecium*	1	0.75
DSMZ 7370^T	*E. flavescens*	0.75	0.094
DSMZ 20628^T	*E. gallinarum*	0.75	0.75
LMG 19487	*E. haemoperoxidus*	1	0.38
DSMZ 20160^T	*E. hirae*	1	0.75
DSMZ 20681^T	*E. malodoratus*	1	0.38
LMG 19486	*E. moraviensis*	1	0.25
DSMZ 4838^T	*E. mundtii*	1	0.38
ATCC 700913	*E. porcinus*	3	0.38
DSMZ 5632^T	*E. pseudoavium*	0.38	0.032
DSMZ 5633^T	*E. raffinosus*	3	0.38
ATCC 700914	*E. ratti*	1	0.25
DSMZ 20726^T	*E. saccharolyticus*	1	1
DSMZ 5634^T	*E. solitarius*	1.5	0.38
DSMZ 6905^T	*E. sulfureus*	0.75	0.38
LMG 12287	*E. villorum*	3	0.38

[a] DSMZ, Deutsch Sammlung von Mikroorganismen and Zellkulturen (Braunschweig, Germany); CECT, Colección Española de Cultivos Tipo (Valencia, Spain); LMG, Laboratorium voor Microbiologie (Gent, Belgium); ATCC, American Type Culture Collection (Rockville, USA).

Table 4
Distribution of *vanA* carrying isolates in the different PCR patterns of the Tn*1546* element found in this work. Patterns are represented as + (presence of the amplicon with the expected size) and − (absence of an amplicon) signs for each of the seven regions searched: genes *vanR*, *vanS*, *vanH*, *vanX* and *vanY* and intergenic regions SH, XY and YZ

Pattern	Region of Tn*1546*								Origin	
	vanR	*vanS*	SH	*vanH*	*vanX*	XY	*vanY*	YZ	Dairy	Clinical
A	−	−	−	−	−	−	−	−	1	3
B	+	+	+	+	−	+	+	+	0	2
C	+	−	+	+	−	−	−	−	0	2
D	+	+	+	+	−	−	−	−	0	1
E	+	+	−	−	−	+	−	+	0	1
F	−	−	+	+	−	−	−	+	0	2 [a]
G	−	−	+	−	−	−	−	+	0	2 [a]
H	−	−	−	−	−	−	−	+	0	3 [b]
I	+	−	−	−	−	−	−	−	2	1 [c]
J	−	−	+	+	−	+	+	+	1	1 [c]
K	+	−	−	+	−	−	−	+	2	2
L	−	−	−	−	−	+	+	+	2	0
M	+	+	−	−	−	+	+	+	1	0
N	−	−	−	+	−	+	−	+	2	0
O	+	−	−	−	−	−	−	+	1	0
P	−	−	+	−	−	+	−	−	1	0
Q	−	−	−	−	−	+	+	−	1	0
R	−	−	−	+	−	+	−	−	1	0
S	−	−	−	−	−	−	+	−	1	0
T	−	−	−	+	−	−	−	−	2	0
U	−	+	−	−	−	+	+	+	1	0
V	+	−	−	−	−	−	+	−	1	0
X	−	−	−	−	−	+	−	−	2	0
SUM [d]	16	6	12	18	0	16	11	23	22	20

[a] One of the isolates is from a dog infection.
[b] Two of the isolates come from dog infections.
[c] It is an enterococcus isolated from a dog infection.
[d] Sum of isolates from each column.

patterns found in these 42 enterococcal isolates, results from Table 4 suggest a higher presence of fragments XY, Y and YZ in dairy isolates and of fragments R, S, SH and H in clinical isolates. In fact, among the total of fragments detected in dairy isolates, 60% were XY, Y and YZ, and in clinical isolates 62% included the R, S, SH and H fragments. No correlation was found between the different PCR patterns and MIC values for vancomycin. The six veterinary isolates carrying *vanA* gene had PCR patterns similar to both human and dairy isolates (Table 4). No association was found between species and PCR pattern. *E. faecalis*, which was the most common species in both samples, were found in 11 of the 23 PCR patterns and shared patterns with either *E. faecium* or *E. durans*.

Among patterns where dairy isolates were assigned, M and U are the most similar, sharing four fragments S, XY, Y and YZ. Isolates assigned to these two patterns are both from cheeses from RDO A. With three fragments in common, namely fragment XY, Y and YZ, are patterns J, L, M and U. Isolates assigned to these patterns come from RDO A cheese (three isolates) and RDO B milk. Although distant geographically, enterococci from these two regions are phenotypically related. Among the nine patterns with only one or two fragments (patterns I and O to X), patterns I, O and V, that share *vanR*, were found only in cheese and milk isolates from RDO B. Fragment S was only detected in RDO A. Fragment SH was not detected in RDO C. However *vanH* was mainly detected in this RDO. These observations point to a possible geographic correlation with the genetic events that led to the observed differences in Tn*1546*.

3.4. Genetic relatedness of isolates

Dairy and clinical isolates for which either *vanA* or *vanB* were detected by PCR were subjected to PFGE and genetic relatedness was determined using criteria defined by Tenover et al. (1995). No relatedness was found between dairy, human clinical and animal clinical enterococcal isolates. Among the 22 dairy isolates with *vanA* gene, only eight were found to be related, in pairs (Table 5). These four pairs were from the three RDOs where *vanA* was detected (RDO A, B and C). Each pair of isolates came from the same cheese manufacturing place, from the same product (milk or cheese) and belongs to the same species, but MIC values were different and so were the Tn*1546* patterns.

Among the 20 clinical isolates carrying *vanA*, only two PFGE groups were found (Table 5). The three isolates in group 5 were isolated in the same Hospital, were identified as *E. faecalis* and presented the same MIC value for vancomycin. Two of them had the same PCR pattern C, and the other had pattern B. Group 6 isolates both came from dog infections, in two consequent years. They both had the same MIC value for vancomycin and their PCR patterns, H and G, although different, are related.

Among *vanB* carriers no genetic relatedness was found.

Table 5
Relevant characteristics of isolates that were grouped according to their genetic relatedness, as determined by their PFGE banding patterns

Group	Origin	Identification	MIC (μg/mL)	PCR pattern [a]
1:				
QA10	Cheese RDO A	*Enterococcus* spp.	6	M
QA41b	Cheese RDO A	*Enterococcus* spp.	4	A
2:				
LN37	Milk RDO B	*Enterococcus* spp.	0,38	T
LN60	Milk RDO B	*E. hirae*	1	I
3:				
QN1	Cheese RDO B	*E. durans*	3	O
QN8	Cheese RDO B	*E. durans*	6	I
4:				
QCB1	Cheese RDO C	*E. faecalis*	4	X
QCB4	Cheese RDO C	*E. faecalis*	3	R
5:				
HSM 3221	Hospital A	*Enterococcus spp.*	6	C
HSM 3720	Hospital A	*E. faecalis*	6	C
HSM 4182	Hospital A	*E. faecalis*	6	B
6:				
Vet 85r	Dog, otite ext, 2000	*E. faecalis*	2	H
344	Dog, urinary infection, 1999	*E. faecalis*	2	G

[a] See Table 4.

4. Discussion

As mentioned before, vancomycin is still at the moment an antibiotic that can be used to treat infections caused by multiresistant enterococci. It is important to have a correct idea of the dissemination of the genes responsible for vancomycin resistance in other environments than the clinical settings, namely in food enterococci since these are microorganisms that contact both with human commensal microorganisms and with those in soil, water, different solid interfaces and animals.

The present work has demonstrated that *vanA* is equally disseminated in dairy enterococci, as it is in clinical (human and pets) environments. However, its presence does not correspond to a resistant behaviour. In order to understand the reason for this discrepancy between the susceptible behaviour and the presence of *vanA* gene, some elements of the Tn*1546* transposon were searched for in *vanA* carrying isolates. Among 42 isolates, 23 different patterns were found, showing either a high variability and heterogeneity in this element, as also reported by other works (van den Braak, 2001; Woodford et al., 1998), or that we are in the presence of remnant forms of Tn*1546*. In fact, if this transposable element had been present in these isolates, it had to suffer some independent deletions or insertions that would prevent the primers used from annealing with DNA. This is a very likely possibility in the light of some reports on the instability of enterococci genomes. For example, the strain V586, vancomycin resistant and appearing to have identical pulsed field gel electrophoretic banding patterns to the vancomycin resistant V583 strain, has been shown to derive from this strain as a result of high-frequency spontaneous excisions by an as yet unknown mechanism (Tendolkar et al., 2003). This genetic instability of the Tn*1546* transposon is also evident even in genetically undistinguishable isolates. The lack of complete and functional Tn*1546* may therefore account for the lack of resistance found both in dairy and clinical enterococci studied. In the particular case of pattern B, the fact that no full resistance

was observed can be ascribed to the absence of *vanX*, that code for a dipeptidase essential for the vancomycin resistance phenotype. Although presenting only *vanA* gene, strains with pattern A were not fully susceptible. This fact suggests that the absence of *vanX* cannot, per se, explain the absence of resistant phenotype in the strains carrying pattern B. If the presence of *vanA* alone can account for the absence of full susceptibility in strains with pattern A remains to be established.

No genetic association was found between dairy and clinical (human or animal) isolates, despite the equal presence of *vanA* in both environments. This excludes the hypothesis of clonal dissemination of the VanA resistance. This is in accordance with previous reports stating that in Europe there is a high degree of heterogeneity among VRE isolates (van den Braak, 2001). However, the fact that dairy and clinical isolates share some Tn*1546* PCR patterns is indicative of a possible genetic trading and share of a resistance genetic pool between these two environments. Despite these differences in PCR patterns, reasonable explanations based on deletion/insertions events can be hypothesised. Pair no. 1 presents patterns A, characterized by the absence of all the fragments searched for, and M, that present all the genes, except *vanH* and the intergenic region SH. One molecular event, like a deletion and/or an insertion in the region upstream from *vanH* and downstream from *vanS* would have been sufficient to prevent one of the primers of either primer pair to anneal to DNA. Pair no. 2 isolates present patterns T, with only *vanH*, and pattern I, with only *vanR*. Although apparently not related, both these patterns are probably the result of multiple insertion/deletion events. Pair no. 3 isolates present patterns O and I, which are, as mentioned above, similar. Pair no. 4 isolates present patterns X and R, which are also related.

The scenario is different with *vanB* genes. It seems that dairy enterococci have not been brought into contact with any VanB resistant isolate. This is in agreement with other reports showing that *vanA* resistance is more disseminated in the environment than *vanB* resistance. On the other hand, *vanB* carrier isolates, both human and animal, were found not to be genetically related, demonstrating that dissemination of *vanB* resistance is not clonal.

VRE are often identified as *E. faecalis*, *E. faecium*, *E. gallinarum* and *E. casseliflavus*. The present work reports detection of *vanA* gene in VSE also from other species, like *E. durans* and *E. hirae* and *E. solitarius*, not often associated with infectious processes in clinical settings, and therefore neglected. This again shows that species is not a barrier for trading antibiotic resistance genes among the genus *Enterococcus*.

In conclusion, this work demonstrated that, although vancomycin resistant enterococci are not relevant in clinical and food environment in Portugal, resistance genes, like *vanA* and *vanB*, are disseminated in both environments, in the case of *vanA*, and only in the clinical settings, in the case of *vanB*. Although the presence of *vanA* genes is generalized, it does not imply a resistance phenotype, which is probably due to lack of a complete and functional Tn*1546* element. The heterogeneity of *vanA* carrying element in the isolates studied, even in genetically undistinguishable isolates, is once again described. But, as opposite to the generally published works, it describes a genetically variable unfunctional Tn*1546*. This is probably due to the lack of selective pressure of the studied isolates. Both the antibiotic and the resistance genes are needed to produce a clinical resistance problem (Levy, 2002). Low use of vancomycin in Portugal, and in Europe in general, has so far protected people from vancomycin resistance in hospitals. However, from what has been shown with our work, the resistance gene is, or has been, present in both dairy food and in clinical environments. Therefore, if vancomycin use increases in Portuguese hospitals, the resistance gene will become more prevalent and the resistance problem will rise. This work also shows that MIC determinations together with the search for *vanA* or *vanB* genes is not enough to categorize and predict the isolate behaviour to vancomycin.

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SHORT REPORT

Enterococcus hirae causing wound infections in a hospital

Tânia C Ribeiro, Vera Pinto, Frèdèric Gaspar, Maria F S Lopes

[**Abstract**] Reports of nosocomial infections caused by *Enterococcus hirae* (*E. hirae*) are rare. To our knowledge this is the first description of *E. hirae* associated with wound infections in humans. Two *E. hirae* strains were isolated from wound infections in different patients. They were both resistant to methicillin, kanamycin, netilmycin, clindamycin, lincomycin, colistin and polymyxin B, described as associated to the genus Enterococcus. The screening of virulence factors associated with enterococcal infectious ability revealed that both *E. hirae* strains carried the cyl, fsr and gelE-sprE operons, all ascribed a role in virulence in *E. faecalis*. Although not clones the two strains were related, as revealed by PFGE analysis. Overall, our results demonstrate the potential of *E. hirae* strains both to cause hospital outbreaks and as virulence trait carriers.

[**Key words**] *Enterococcus hirae*; wound infections; antibiotic resistance; cyl; fsr; gelE

INTRODUCTION

Enterococci are widespread in nature and are part of the commensal flora of human gastrointestinal and genitourinary tracts. Enterococci are often implicated in infections of the urinary tract and abdomen or of superficial wounds in hospitalized patients, but can also cause severe infections such as bacteraemia, endocarditis and occasionally meningitis or pneumonia. As opportunistic pathogens they are emerging as an important cause of hospital-acquired infections. Nosocomial infections by enterococci are mainly due to two species, *E. faecalis* and *E. faecium*. Frequently other species are neglected because they are rarely associated with severe infections in humans. However, there are some reports of infections due to *E. gallinarum*, *E. casseliflavus*[1,2], *E. raffinosus*[3] and *E. hirae*[4,5]. The *E. hirae* species is widely known to cause infections in animals[6,7], but is rarely associated with human infections. Several authors have reported the isolation of *E. hirae* from clinical specimens from various sources[8,9]. In all studies published the isolation of *E. hirae* corresponds, on average, to 1% of all the enterococci isolates identified. Since this is such a rare species in clinical environment it is comprehensible that it is infrequently associated with infections in humans.

MATERIALS AND METHODS

From November 2004 to January 2006, 109 Enterococcus spp. isolates were recovered from patients in a Portuguese Hospital. The isolates were identified by VITEK 120 system (BioMérieux, Marcy-l'Etoile, France) as *E. faecalis* (81/109), *E. faecium* (26/109) and *E. hirae* (2/109), 48 corresponding to 74.3%, 23.9% and 1.8%, respectively. These clinical isolates were recovered from wound infections (54.1%), blood (25.7%), catheters (10.1%), urine (5.5%) and the remaining 4.6% were recovered from other biological products and devices (unpublished results). The two *E. hirae* isolates, named SAVE H1 and SAVE H2, were both responsible for wound infections in two different patients and were isolated within one week. These two *E. hirae* isolates were tested for their susceptibility towards 30 antibiotics as previously described by Lopes, *et al*.[10] PFGE of SmaI digested total DNA of both

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strains was performed according to the method of Chung, *et al.*[11], except for the lysis solution, which contained only RNase A and lysozyme. The presence of virulence traits (namely cyl operon, fsr and gelE-sprE operons and the genes ace, asa1 and efaA) in these strains was screened by PCR using the primers described in Table 1 and Immolase DNA polymerase (Bioline, Germany), according to manufacturer's instructions.

Table 1 Primers (f-forward; r-reverse) used in the PCR reactions to detect the genes described

Gene	Primer (5′ – 3′)		Amplicon size (bp)
cylR1	GCTATTTTTCTTTTTCTACCTAA – f	TATGAAGTCCTCTTGTTGAAA – r	128
cylR2	GCTTTATTAGAAGTTAGCAGACAA – f	TGGAGTATTCAGGTAGTAAGCAA – r	105
cylLL	AAATGGAAGCGATTCAAGGT – f	CAGCCACAAGCAGCACTACT – r	100
cylLS	AACTTGTTGGTCCTTCTTTTGA – f	CCTATGGTAAAACATGCTGGAG – r	106
cylM	TGGTGTAAGGGAACTGTAGGTG – f	ATGGCACAGACAATCTTTATTTTTA – r	117
cylB	GGAGAATTAGTGTTTAGAGCG – f	TATCCCTAAAAAAAGACTATCTATTA – r	99
cylA	TGCTCGTGAAATGATGATGAC – f	CGCTTACTTCTGGAGTTG – r	125
cylI	TGATGATGATGAAATAAGCCA – f	AACCCATTGTTTTTCTGCTAT – r	103
fsrA	GCATCCAGATTTGATTAGAGTC – f	TGGTACTTCCGTTCCGTC – r	112
fsrB	AAGTCAGTGATGGTGTGGGAA – f	ATTCTTTTTAGCTTTTTCAGTTTGTC – r	98
fsrC	TAATACCGCAAAGCAAGCAA – f	AGCCAACAAACGAATCACAA – r	103
gelE	TCTTCACTTTTTGTAGCCGC – f	TGCCTCAGAAATTGCCTC – r	96
sprE	ACAGGCTTTGTTGTTGGAA – f	ATCACGACCTGGATAAAACC – r	123
ace	AATCAAAATGTGGAAATGCC – f	CACTTGTTTCTGTTGCCTGTT – r	109
asa1	AGCCAATGTGGTTCCTGTTC – f	AGTTCTGTCGTATCCCCTGTC – r	126
efaA	ACCTGAACAAATGACCACGA – f	TCTTTTGAGACCCGTTCCAT – r	108

RESULTS AND DISCUSSION

Both isolates were susceptible to most of the antibiotics tested. They presented resistance to only 7 antibiotics (methicillin, kanamycin, netilmycin, clindamycin, lincomycin, colistin and polymyxin B) described as associated to the genus Enterococcus[10]. Although not carrying important antibiotic resistances, these strains had the genes coding for three of the most relevant virulence factors described in the genus Enterococcus, namely the cyl operon, coding for the citolysin, the gelE-sprE, coding for gelatinase and a serine protease, and fsr, reported as controlling both gelatinase and serine protease expression and, among others, biofilm development in *E. faecalis*[12]. The cyl operon was not complete, since both cylR1 and cylR2 were not detected. However, these results demonstrate that other species then *E. faecalis*, namely *E. hirae*, are acquiring the same virulence genes which are most probably responsible for the *E. hirae* success as an infectious agent.

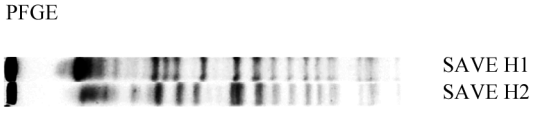

Figure 1 PFGE of SmaI digested total DNA of *E. hirae* strains SAVE H1 and SAVE H2

The PFGE profiles of the two *E. hirae* strains (Figure 1) indicate that they are genetically related, but not clones since they present distinct macrorestriction patterns, differing in less than 7 bands. The two strains were isolated with just one week apart and were both isolated from the same kind of biological product (pus). Therefore, the PFGE results suggest that the first isolated strain (SAVE H1) was able to persist in the hospital, suffer a few genetic events that originated the second strain (SAVE H2) which was again able to cause an infection in another patient. However, the distinct PFGE patterns clarify that they are two distinct strains. Consequently we can rule out the hypothesis that the isolates could belong to the patients own commensal flora in the case of the second strain. It is widely known that enterococcal infections are most frequently caused by patient's endogenous flora, which might be the case of the first infection, although we are not able to prove it. In Portugal, the colonization by *E. hirae* is yet to be described. A recent study describes the presence of *E. hirae* in healthy poultry and pets, but not in humans[9]. To our knowledge this is the first description of *E. hirae* infection in humans in Portugal and the first report of *E. hirae* associated with wound infections. Moreover, this work calls the attention to a yet unknown, or not previously described, ability or potential of *E. hirae* to cause hospital outbreaks.

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