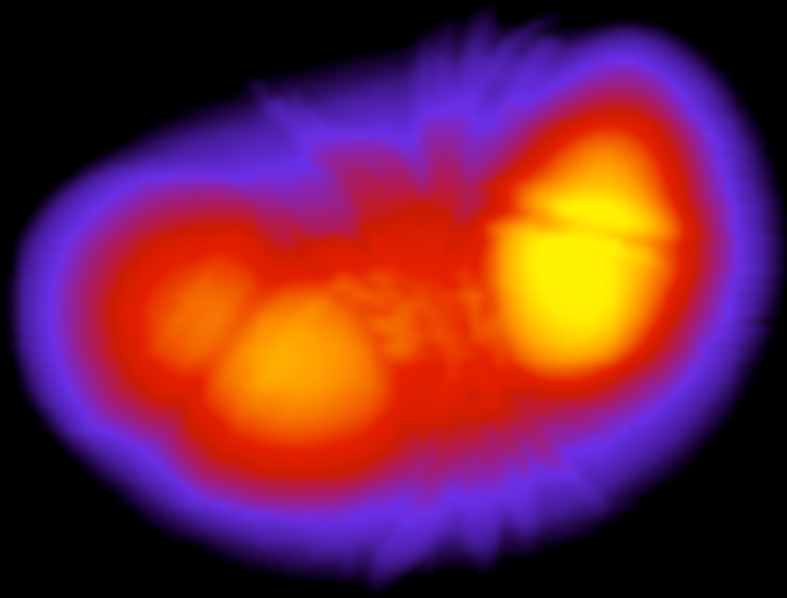
# Non-clinical isolates bring new findings on enterococcal virulence

Frédéric Bustos Gaspar



Dissertation presented to obtain the Ph.D degree in Biology

Instituto de Tecnologia Química e Biológica | Universidade Nova de Lisboa

Oeiras,
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*To my* maman.

*"Thinking is no more than a tiny aspect of the totality of consciousness, the totality of who you are."*

Eckhart Tolle

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Every single person with whom my path has crossed during my PhD has influenced me. I have learned so much and everyone was an essential piece of the puzzle that this journey ended up being.

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ITQB, IBET & IGC @Oeiras.pt / FCUL @Lisbon.pt / INRA @Jouy-en-Josas.fr / Harvard Medical School @Boston.us / University of Freiburg @Freiburg.de / The support that I received from the members of my labs, the division, the research institutes, the universities, and from everyone with whom I have had the opportunity to interact and work was absolutely impressive and out of the ordinary.

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I also want to acknowledge everyone that I feel so close to and that I consider belonging to my families: the acquired and the natural one. Especially my *maman*. To whom I owe my Life… And to whom I owe my life.

# Abstract

Enterococci are Gram-positive lactic acid bacteria, widespread in the environment, present in water, soil, plants and animals, including humans. They typically colonize the skin and mucous membranes, namely the gastrointestinal tract. However, enterococci, and most notably *Enterococcus faecalis* and *Enterococcus faecium*, have become problematic causative agents of several nosocomial infections, including urinary tract infections, bacteraemia, surgical sight infections, and endocarditis. Besides being opportunistic pathogens, the resilient bacteria of the genus *Enterococcus* are key factors contributing to the ripening, flavour, and the organoleptic properties of fermented food products.

The ubiquitous nature of enterococci derives from a number of features, which can be intrinsic to the genus or specific to some species or even strains. These traits allow probing the environment in order to adapt, enabling a survival and fitness advantage. They are encoded in numerous genes that can be easily transferable due to the high genomic promiscuity of enterococci. These genes have been ascribed a role in virulence as they are relevant to different stages of the bacterial infection process, including

adhesion, colonization, invasion, evasion of the immune system and spread through the host's tissues. Enterococcal virulence factors can be either secreted (cytolysin, proteases, hyaluronidase, superoxide), surface associated (enterococcal surface protein, aggregation substance, extracellular polymeric substances, pilin gene clusters, enterococcal microbial surface component recognizing adhesive matrix molecules), or intracellular. At the time this thesis work began, researchers were starting to realize that virulence factors in enterococcal clinical isolates were also present in isolates from other environments, in particular, where enterococci play beneficial roles, namely food. Since dissemination of virulence factors among food isolates was no longer crucial, other issues started to become relevant in the still debated enterococcal virulence.

A thorough search in the literature clearly shows recurrent incongruent results between genotype and phenotype of the virulence factor cytolysin. Moreover, the phenotypic assays are not performed under the same conditions and only a few *cyl* genes are screened for in the majority of the experiments. Therefore, in the first part of this work, we developed a new genotypic and phenotypic screening for cytolysin. Based on our methodology, a much higher correlation was obtained compared to any previously described screening. Complete agreement between genotypic and phenotypic assays was seen for all 55 strains tested, which belong to four enterococcal species, namely *E. faecalis*, *E. faecium*, *Enterococcus durans* and *Enterococcus hirae*. The proposed PCR screening for the complete *cyl* locus gives a measure of the gene reservoir of the strain, while the phenotypic assay is still the only test that allows demonstration of active cytolysin production.

In the second part of this work, the goal was to evaluate the roles of *fsrB* and *gelE,* two genes that encode for well-characterized enterococcal virulence factors, in the potential virulence of *E. faecalis* food strains. Virulence of unrelated *Enterococcus* isolates, including dairy strains carrying *fsr* and *gelE* operons, was compared in the *Galleria mellonella* insect model. *E. faecalis* dairy strains were able to kill larvae and were as virulent as one of the most widely used strains for virulence studies. In contrast, *E. durans* and *E. faecium* strains were avirulent or poorly virulent in *G. mellonella*. To evaluate the role of *fsrB* and *gelE* in the virulence of *E. faecalis* dairy strains, both genes were deleted independently in two strains. Although both mutations significantly attenuated virulence in *G. mellonella*, the *fsrB* mutant strains were more strongly attenuated. Our work demonstrates that the presence of functional *fsrB*, and to a lesser extent *gelE*, significantly contributes to the virulence of *E. faecalis* food isolates, and that the presence of these genes in dairy enterococci should be considered with caution. The simple *G. mellonella* animal model may provide insights for risk assessment of food isolates.

Next, we further characterized the non-starter *E. faecalis* cheese isolate QA29b, which harbours virulence genes and proved to be virulent in a *G. mellonella* virulence model. We looked at traits relevant to the host pathogen interaction, in particular adhesion, colonization and infection. QA29b demonstrated high ability to form biofilms, to adhere to epithelial cells and was readily eliminated by J774.A1 macrophage cells. This work illustrates for the first time that *cps* genes, that are associated with virulence, may be differentially transcribed between isolates, and therefore may not be phenotypically expressed. It also constitutes the first study of

traits important for interaction, colonization and infection in the host performed on a good food isolate representative of *E. faecalis*. Overall, QA29b characterization shows that, despite its virulence potential in an insect model, this food strain is readily eliminated by mammalian macrophages, indicating that fine-tuned approaches combining cellular and mammalian models are needed to address and elucidate the multifactorial aspect of virulence potential associated with food isolates.

In the last part of this work, we were able to associate the extracellular production of autoinducer-2 (AI-2) in *E. faecalis* VE14089, a plasmid-cured derivative of *E. faecalis* V583, to the presence and expression of *luxS* gene. This gene is also present in other clinical and commensal isolates as well as in food isolate QA29b. AI-2-mediated quorum sensing has been extensively studied in relation to the regulation of microbial behaviour and has been recognized as an interspecies communication molecule, which may influence community structure and function. When compared with the wild type, the *luxS* mutant had no apparent phenotype regarding growth, biofilm formation, adhesion to Caco-2 cells, resistance to oxidative stress and survival inside macrophages. However, microarray comparison of gene expression revealed that the *luxS* mutation caused pleiotropic effects in gene expression, affecting genes involved in DNA, fatty acid and intermediary metabolites metabolism, which could not be complemented by extracellular AI-2 addition. This study shows that, in *E. faecalis*, differential gene expression related to the *luxS* mutation cannot be ascribed to quorum sensing and that LuxS has, at least, a role in metabolism.

In conclusion, the main findings presented in this thesis reveal that there is, until now, no virulence trait exclusive to strains isolated from clinical settings, and that the sole presence of these traits does not allow the identification of a strains' habitat. We showed that the presence of virulence associated genes, and therefore an associated virulence potential, does not necessarily translate to a virulent and pathogenic behaviour in *E. faecalis* food isolates. Also, we showed that to determine the outcome of the persistence and resilience of enterococci, not only the presence of a specific gene is important but also the whole genome plays a pivotal role. We revealed for the first time that the LuxS enzyme from the genus *Enterococcus* is responsible for the production of the interspecies communication molecule, AI-2. Taken together, the data reported in this thesis showed the potential for virulence of enterococcal dairy strains in specific virulence models, while being avirulent in other models. This highlights the problem that their pathogenic potential in humans cannot be entirely excluded, and therefore, reiterating the need to keep a close surveillance of their presence in food.

# Resumo

Os enterococos são bactérias lácticas, disseminadas no meio ambiente, presentes na água, solo, plantas e animais, incluindo o ser humano. Para além da colonização da pele e de mucosas, tal como o tracto gastrintestinal, os enterococos, especialmente as espécies *Enterococcus faecalis* e *Enterococcus faecium*, têm-se tornado agentes responsáveis por diversas infecções nosocomiais nomeadamente do tracto urinário, bacterémias, infecções cirúrgicas, e endocardites. Para além de serem agentes patogénicos oportunistas, as resilientes bactérias do género *Enterococcus* são agentes essenciais que contribuem para a maturação, sabor e outras propriedades organolépticas de produtos alimentares fermentados.

A natureza ubíqua dos enterococos pode ser atribuída a diversas características, quer intrínsecas do género quer específicas de certas espécies, que conferem uma vantagem nas suas capacidades de adaptação e sobrevivência. Facilmente transferíveis devido à elevada promiscuidade genómica dos enterococos, estes factores estão codificados em inúmeros genes que lhes permitem sondar o meio

ambiente de modo a adaptar o seu comportamento quer às condições ambientais quer à densidade celular. Estes genes desempenham um papel na virulência pois são relevantes nas diferentes etapas do processo de infecção, nomeadamente na adesão, colonização, invasão, evasão do sistema imunitário e disseminação nos tecidos do hospedeiro. Envolvidos neste processos estão vários factores de virulência, incluindo aqueles estudados nesta tese, nomeadamente citolisina, Fsr, gelatinase, cápsula. No início do trabalho que levou a esta tese, os investigadores começavam a aperceber-se que os factores de virulência presentes em enterococos isolados de ambientes clínicos também podiam ser encontrados em enterococos isolados de outros ambientes, tais como de produtos alimentares onde os enterococos desempenham um papel benéfico. Uma vez que a pesquisa da disseminação de factores de virulência deixara de ser crucial, outros aspectos começaram a tornar-se relevantes na ainda debatida virulência em enterococos, nomeadamente, a incongruência entre genótipo e fenótipo relativamente ao factor de virulência citolisina (Capítulo 2), o potencial de virulência de estirpes alimentares de *E. faecalis* associado à presença dos genes *fsrB* e *gelE* (Capítulo 3), o papel de alguns factores de virulência na relação com o hospedeiro de estirpes de *E. faecalis* alimentares (Capítulo 4) e a contribuição para a virulência da proteína LuxS, responsável pela produção extracelular do autoindutor-2 (AI-2) possivelmente envolvido certamente na comunicação entre *E. faecalis* e outras bactérias dentro e fora do hospedeiro humano (Capítulo 5).

Na primeira parte deste trabalho, e relativamente ao factor de virulência citolisina, uma cuidadosa pesquisa na literatura revelou que não

só os ensaios fenotípicos não eram realizados em condições idênticas como também, na maioria dos trabalhos publicados, poucos dos oito genes do operão *cyl* eram pesquisados, levando frequentemente a resultados incongruentes entre o genótipo e o fenótipo. Entre as 55 estirpes testadas nesta tese, pertencentes a quatro espécies diferentes de enterococos, nomeadamente *E. faecalis*, *E. faecium*, *Enterococcus durans* e *Enterococcus hirae*, e usando uma nova metodologia de pesquisa genotípica e fenotípica, foi possível obter uma concordância perfeita entre os ensaios genotípicos e fenotípicos.

Na segunda parte deste trabalho, o objectivo foi avaliar o papel dos genes *fsrB* e *gelE*, dois genes que codificam para factores de virulência bem caracterizados em enterococos clínicos, no potencial de virulência de estirpes alimentares de *E. faecalis*. A virulência de isolados não relacionados do género *Enterococcus*, incluindo estirpes lácteas portadoras dos operões *fsr* e *gelE*, foi então comparada no insecto modelo *Galleria mellonella*. As estirpes lácteas de *E. faecalis* foram capazes de matar as larvas sendo tão virulentas quanto uma das estirpes mais utilizadas em estudos de virulência. Por oposição, as estirpes das espécies *E. durans* e *E. faecium* mostraram-se avirulentas ou pouco virulentas em *G. mellonella*. De modo a avaliar o papel dos genes *fsrB* e *gelE* na virulência de estirpes alimentares de *E. faecalis,* os dois genes foram independentemente deletados em duas estirpes. Embora ambas as mutações tenham significativamente atenuado a virulência em *G. mellonella*, esse efeito foi mais pronunciado nas estirpes mutantes no gene *fsrB*. Este trabalho demonstrou que a presença de um gene *fsrB* funcional, e de uma forma menos acentuada de um gene *gelE*, contribui significativamente para a virulência de isolados alimentares de *E. faecalis* e

que a presença desses genes em enterococos lácteos deve ser considerada com precaução. *G. mellonella*, sendo um modelo simples, pode apresentar vantagens na avaliação do risco associado a isolados alimentares.

O isolado de queijo *E. faecalis* QA29b, portador de genes de virulência e previamente identificado como virulento no modelo de virulência *G. mellonella*, foi estudado no que respeita a fenótipos relevantes para a interacção com o hospedeiro que podem ter impacto na capacidade de provocar infecções e colonizar o hospedeiro humano. Este trabalho constituiu o primeiro estudo deste tipo de características num *E. faecalis* alimentar, que pertence ao Complexo Clonal 72, sendo, por isso, um bom representante dos *E. faecalis* alimentares. QA29b demonstrou, por um lado, uma elevada capacidade de formar biofilmes e aderir a células epiteliais, e por outro foi prontamente eliminado por células de macrófago J774.A1. Este trabalho evidenciou, pela primeira vez, que os genes *cps*, associados à virulência, podem ser transcritos de forma diferencial entre diferentes isolados, podendo não ser fenotipicamente expressos. Apesar de demonstrar ter um potencial de virulência num modelo de insecto, este isolado alimentar mostrou ser prontamente eliminado por macrófagos de mamíferos. Este trabalho mostra que optimizar métodos que combinem modelos celulares e animais é necessário de modo a abordar e elucidar o aspecto multifactorial do potencial de virulência associado a isolados alimentares.

Na última parte deste trabalho a produção extracelular de AI-2 em *E. faecalis* VE14089, um derivado de *E. faecalis* V583 curado dos seus plasmídeos, foi associada à presença e expressão do gene *luxS*. Este

gene encontra-se também presente em outros isolados clínicos e comensais bem como no isolado alimentar QA29b. A percepção de quórum mediada por AI-2 tem sido extensamente estudada na regulação do comportamento microbiano e foi reconhecida enquanto molécula de comunicação entre espécies, podendo influenciar a estrutura e função da comunidade. Quando comparada com a estirpe selvagem, o mutante *luxS* não demonstrou ter nenhum fenótipo quanto ao crescimento, formação de biofilme, adesão a células Caco-2, resistência ao stress oxidativo ou sobrevivência no interior de macrófagos. No entanto, a análise por microarray da expressão génica revelou que a mutação *luxS* teve efeitos pleiotrópicos, afectando genes envolvidos no metabolismo de DNA, ácidos gordos e intermediários metabólicos, não sendo complementados pela adição extracelular de AI-2. Este estudo mostrou que, em *E. faecalis*, a expressão diferencial génica relacionada com a mutação *luxS* não pode ser atribuída à percepção de quórum e que o LuxS tem, pelo menos, um papel no metabolismo.

Concluindo, o trabalho apresentado nesta tese evidenciou, pela primeira vez, que uma estirpe láctea de *E. faecalis* tem tanto potencial de virulência quanto uma estirpe clinica da mesma espécie. No entanto, ficou também claro com este trabalho que a presença de genes associados com virulência em *E. faecalis* alimentares não se traduz necessariamente num comportamento virulento e patogénico e que a persistência e resiliência de enterococos depende tanto do papel desempenhado pela presença de genes específicos quanto pelo restante genoma. Tendo em conta que os enterococos alimentares, bem como os comensais e clínicos, fazem parte de comunidades polimicrobianas, onde a existência de

comunicação inter-espécies poderá desempenhar um papel de relevo na capacidade para colonizar ou infectar o hospedeiro, estudou-se o papel do LuxS em vários fenótipos relevantes no processo de colonização e infecção por *E. faecalis*. Mostrou-se pela primeira vez que o enzima LuxS, presente no género *Enterococcus*, também em estirpes lácteas, é responsável pela produção da molécula de comunicação entre espécies, AI-2. A totalidade dos dados relatados nesta tese revelam que o potencial patogénico de *Enterococcus* em humanos não pode ser inteiramente excluído, reiterando a necessidade de manter uma estreita vigilância da sua presença em alimentos.

# List of Publications

Gaspar, F.B., Crespo, M.T.B., Lopes, M.F.S., 2009. Proposal for a reliable enterococcal cytolysin production assay avoiding apparent incongruence between phenotype and genotype. J. Med. Microbiol. 58, 1122–1124.

Gaspar, F., Teixeira, N., Rigottier-Gois, L., Marujo, P., Nielsen-LeRoux, C., Crespo, M.T.B., Lopes, M. de F.S., Serror, P., 2009. Virulence of *Enterococcus faecalis* dairy strains in an insect model: the role of *fsrB* and *gelE*. Microbiology (Reading, Engl.) 155, 3564–3571.

# Abbreviations

| | |
|---|---|
| °C | degree Celsius |
| 2×YTGlu | 2×YT supplemented with 0.5 % glucose |
| A | adenine (in a nucleotide sequence) |
| A | alanine (in an amino acid sequence) |
| Ace | adhesin of collagen of *E. faecalis* |
| Acm | adhesin of collagen of *E. faecium* |
| AI | autoinducer |
| AMC | activated methyl cycle |
| AS | aggregation substance |
| ATP | adenosine triphosphate |
| Bap | biofilm-associated protein |
| BCAA | branched-chain amino acid |
| BCKDH | branched-chain alpha-keto acid dehydrogenase |
| BHI | brain heart infusion |
| BLAST | Basic Local Alignment Search Tool |
| bop | biofilm on plastic surfaces |
| bp | base pair |
| BT | bacterial translocation |
| C | cysteine (in an amino acid sequence) |
| C | cytosine (in a nucleotide sequence) |
| CC | clonal complex |
| CcpA | catabolite control protein A |
| CCR | carbon catabolite repression |
| cDNA | complementary deoxyribonucleic acid |
| CFU | colony-forming unit |
| CGH | comparative genome hybridization |
| cod | coding region |

| | |
|---|---|
| CPS | capsular polysaccharide |
| cre | catabolite responsive element |
| Cyl | cytolysin |
| CYS | L-cysteine |
| CYSTA | L-cystathionine |
| D | Aspartic acid |
| Dam | DNA adenine methylase |
| Dcm | DNA cytosine methylase |
| DNA | deoxyribonucleic acid |
| DPD | (S)-4,5-dihydroxypentan-2,3-dione |
| DS | diffusion sensing |
| E | glutamic acid |
| ebp | endocarditis and biofilm-associated pili |
| EcbA | *E. faecium* collagen binding protein A |
| ECF | extracytoplasmic function |
| eDNA | extracellular DNA |
| EDTA | ethylenediaminetetraacetic acid |
| EfaA | *E. faecalis* antigen A |
| Ehk | enterococcal histidine kinase |
| EI | enzyme I |
| EII | enzyme II |
| Epa | enterococcal polysaccharide antigen |
| EPS | exopolysaccharide |
| Err | enterococcal response regulator |
| Ers | enterococcal regulator of survival |
| ES | efficiency sensing |
| Esp | enterococcal surface protein |
| F | phenylalanine |
| FASII | fatty acid synthase II |
| FDR | false discovery rate |
| FRET | fluorescence resonance energy transfer |
| Fsr | *E. faecalis* regulator |
| G | glycine (in an amino acid sequence) |
| G | guanine (in a nucleotide sequence) |
| GBAP | gelatinase biosynthesis-activating pheromone |
| GEI | genomic island |
| GelE | gelatinase |
| GI | gastrointestinal |
| Gsp | general stress protein |
| H | histidine |
| HCY | L-homocysteine |
| HGT | horizontal gene transfer |

| | |
|---|---|
| HJ | Holliday junction |
| HK | histidine kinase |
| HPr | histidine protein |
| HR | homologous recombination |
| HSE | L-homoserine |
| Hyl | Hyaluronidase |
| HypR | hydrogen peroxide regulator |
| I | isoleucine |
| ICE | integrative and conjugative element |
| int | intergenic |
| IS | insertion sequence |
| JCVI | J. Craig Venter Institute |
| K | lysine |
| L | leucine |
| LAB | lactic acid bacteria |
| LB | Luria–Bertani |
| LPS | lipopolysaccharide |
| LTA | lipoteichoic acid |
| M | methionine |
| M17AGlu | M17 agar supplemented with 0.5 % (w/v) glucose |
| M17BGlu | M17 broth supplemented with 0.5 % (w/v) glucose |
| M17Glu | M17 supplemented with 0.5 % (w/v) glucose |
| MET | L-methionine |
| MGE | mobile genetic element |
| mL | millilitre |
| MLST | multi-locus sequence typing |
| MnSOD | manganese superoxide dismutase |
| MOI | multiplicity of infection |
| MRSA | methicillin-resistant *Staphylococcus aureus* |
| MSCRAMM | Microbial Surface Component Recognizing Adhesive Matrix Molecule |
| N | asparagine |
| NAD | nicotinamide adenine dinucleotide |
| NCBI | National Center for Biotechnology Information |
| ng | nanogram |
| nm | nanometre |
| OD | optical density |
| ORF | open reading frame |
| P | proline |
| PAI | pathogenicity island |
| PBS | phosphate-buffered saline |
| PCR | polymerase chain reaction |

| | |
|---|---|
| PEP | phosphoenolpyruvate |
| PerA | pathogenicity island-encoded regulator A |
| PerR | peroxide regulator |
| PFGE | pulsed field gel electrophoresis |
| PGC | pilin gene cluster |
| pH | hydrogen potential |
| PTS | phosphoenolpyruvate transport system |
| Q | glutamine |
| QS | quorum sensing |
| R | arginine |
| RBS | ribosome-binding site |
| RIVET | recombinase-based in vivo expression technology |
| RNA | ribonucleic acid |
| RNase | ribonuclease |
| rpm | rotations per minute |
| RR | response regulator |
| rRNA | ribosomal RNA |
| RT-PCR | reverse transcription PCR |
| s | second |
| S | serine |
| S/I | similarity/identity |
| SAH | S-adenosyl-L-homocysteine |
| SAM | S-adenosyl-L-methionine |
| Scm | second collagen adhesin of *E. faecium* |
| SI | survival index |
| spp. | species |
| SprE | serine protease |
| SRH | S-ribosyl-L-homocysteine |
| ST | sequence type |
| T | threonine (in a amino acid sequence) |
| T | thymine (in a nucleotide sequence) |
| TCA | tricarboxylic acid |
| TCS | two-component system |
| Tpx | thiol peroxidase |
| UTI | urinary tract infection |
| UV | ultraviolet radiation |
| V | valine |
| v/v | volume/volume |
| VBNC | viable but nonculturable |
| VRE | vancomycin-resistant *Enterococcus* |
| W | tryptophan |
| w/v | weight/volume |

| | |
|---|---|
| WT | wild type |
| Y | tyrosine |
| µg | microgram |
| µL | microliter |
| µM | micromolar |

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# Chapter 1

# General Introduction

## WHO

**Description of the genus *Enterococcus* (ex Thiercelin and Jouhaud 1903)**

*Enterococcus* (En.te.ro.coc'cus. Gr. n. *enteron* intestine; Gr. n. *coccus* a grain, berry; M.L. masc. n. *Enterococcus* intestinal coccus) cells are ovoid, occur singly, in pairs, or in short chains, and are frequently elongated in the direction of the chain. Gram positive. Endospores are not formed. May be motile. Facultatively anaerobic. Optimum growth temperature, ca. 35 °C. Strains grow at 10 and 45 °C. Most strains survive heating at 60 °C for 30 min. Grow in 6.5 % NaCl and at pH 9.6. Hydrolyse pyrrolidonyl-β-naphthylamide. Chemoorganotrophs. Metabolism fermentative. The predominant end product of glucose fermentation is L-lactic acid. Oxygen or other hydrogen acceptors may alter the end products of carbohydrate metabolism. Hydrogen peroxide may or may not accumulate in the presence of oxygen. Do not contain haem compounds. Benzidine negative and usually catalase negative, but some strains may produce

pseudocatalase. Some strains synthesize cytochromes or catalase or both when they are provided with haemin. The minimal nutritional requirements are generally complex. React with group D antisera; some strains also react with group Q antisera.
Some strains possess respiratory quinones (menaquinones or demethylmenaquinones). Long-chain fatty acids are predominantly of the straight-chain saturated or monounsaturated types; some strains produce cyclopropane ring acids.
Peptidoglycan type: Lys-D-Asp or Lys-$Ala_{2-3}$.
The G+C content of the DNA ranges from 37 to 45 mol %.
Type species: *Enterococcus faecalis*.
Nucleic acid hybridization studies, in particular DNA-rRNA hybridization studies, demonstrate that members of the genus *Enterococcus* are closely related to each other but not to members of the genus *Streptococcus*.
Enterococci can easily be differentiated from streptococci by their ability to grow in 6.5 % NaCl and at pH 9.6. Moreover, in contrast to most streptococci (exceptions are *Streptococcus lactis*, *Streptococcus cremoris*, and *Streptococcus uberis*), they can grow at 10°C.

(Schleifer et al., 1984)

With the previous description Schleifer and Kilpper-Bälz (Schleifer et al., 1984) revived in 1984 the genus *Enterococcus*. However, Thiercelin was the first to use, in 1899, the term “enterocoque” to indicate the intestinal origin of a Gram-positive diplococcus, and 4 years later, together with Jouhaud, the genus *Enterococcus* was proposed as new (Domig et al., 2003; Khan et al., 2010). Nonetheless, *Enterococcus* as a new genus did not endure, since, in 1906, Andrewes and Horder described Thiercelin’s

“enterocoque” as a group of streptococci so characteristic of the human intestine that the term "*Streptococcus faecalis*" could be applied to it (Andrewes and Horder, 1906). Based on a serological typing system for haemolytic streptococci developed by Lancefield (Lancefield, 1933), Lancefield, this time with colleague Hare, continued, in 1935, that association between the two genus, relating cultures of the intestinal enterococci to group D haemolytic streptococci (Lancefield and Hare, 1935). Sherman was the first to clearly mention, in 1937, that the term "enterococcus" had a somewhat variable and hazy meaning (Sherman, 1937), existing in the literature a synonymous usage of enterococci, faecal streptococci and group D streptococci. At the same time he also stated that, despite being hard at that time to justify a generic segregation of the enterococci from the other streptococci making them an independent genus, enterococci represent one of the clearly defined primary divisions of the streptococci, probably the most clearly marked subdivision of the whole genus, the other three being the pyogenic, viridans and lactic groups (Sherman, 1937).

The genus *Enterococcus* was revived and separated from *Streptococcus sensu lato* based on genomic studies, which relied on DNA-DNA and DNA-rRNA hybridization studies (Schleifer et al., 1984). This separation was further confirmed by 16S ribosomal RNA (rRNA) sequence analysis, which showed that, within the previously described *Streptococcus-Enterococcus* group, organisms fall into three clusters defined by *Enterococcus*, the lactic acid streptococci and streptococci of the pyogenic and oral groups (Ludwig et al., 1985). Since the revival of the genus a total of 40 enterococcal species have been described (Table 1.1), though 5 species might need to be renamed due to being synonyms to

previously described enterococcal species or belonging to other genus ((Euzéby, 1997), related website last visited: 2012.03.20)).

Even if they're not in presence of optimal growth conditions, the enterococcal phenotypic characteristics allow them to grow in diverse and broad range of conditions, conferring them the ability to colonize a wide variety of environments.

WHERE can they be found?

## WHERE / Enterococci can be found in diverse habitats

Bacteria of the genus *Enterococcus* are ubiquitous in nature and their role is often unclear. They can occupy the most varied niches if their viability or basic growth needs are met. Even if some species specificity can be seen in certain habitats (Table 1.1), they are naturally found in animals, plants, and in the environment (Figure 1.1).

### Animals, plants and environment / Habitat diversity

In opposition to parasitism, where one organism benefits at the expense of its host, and mutualism, where both organisms benefit, commensalism is not as easy to define as the previous ones. By definition, commensals benefit from their relationship with their host without causing harm neither benefiting it.

Enterococci have been identified as human commensals since their presence in the gastrointestinal (GI) tract was reported, still in the 19$^{th}$ century. In healthy humans they are mainly associated with the GI tract but, although they are less commonly found at other body sites, enterococci can also be recovered from the skin, the vagina, and the oral cavity (Fisher and Phillips, 2009). They have also been associated with a multitude of other

healthy animals: from warm-blooded animals, such as other mammals and birds (including production animals, such as livestock and fowl), to other vertebrates, like fish (Bulushi et al., 2010; Albesharat et al., 2011), amphibians and reptiles (Cox and Gilmore, 2007), and even invertebrates, like molluscs (Valenzuela et al., 2010) and insects (Devriese and Baele, 2006; Cox and Gilmore, 2007).

Certain host-specific variations in the occurrence of different enterococcal species in different animal hosts are known to exist. Other species variations have also been described regarding the host's age, compartments colonized and the species distribution, as well as the effect due to feeding on species colonization (Devriese and Baele, 2006).

Plants represent a harsh environment with physicochemical conditions that fluctuate widely and rapidly over short periods of time, such as temperature and osmotic conditions within the same day. The aerial plant surfaces are mainly aerobic, overall poor in nutrients, and exposed to UV radiation. Even in these considered non-host conditions, so different from the usual stable temperature, shielded from UV rays, anaerobic and nutrient rich environment of the GI tract of animals where they are commensal, enterococci can find a secondary habitat (Albesharat et al., 2011). Enterococci have been found on the most diverse plant materials, like fruit, root and bulbous vegetables, as well as salads and cereals (Schwaiger et al., 2011).

Enterococci are also widely present in natural environments such as soil, sediments, sand and water (Whitman et al., 2003; Devriese and Baele, 2006; Brownell et al., 2007; Albesharat et al., 2011; Wright et al., 2011). Several enterococcal species have been isolated from these niches, such as *E. faecium*, *E. faecalis*, *E. casseliflavus*, *E. hirae*, *E. durans* and *E. mundtii* (Łuczkiewicz et al., 2010; Albesharat et al., 2011).

**Table 1.1.** Compilation of enterococcal species names, groups, isolation materials, and date. As described by Williams et al. (Williams et al., 1991), De Graef et al. (De Graef et al., 2003) and Koort et al. (Koort et al., 2004) enterococci can be classified in 6 different groups that are indicated by roman numbering (u: for unknown), with the type species of each group indicated in bold type. The species in need of renaming are at the bottom of the table ((Euzéby, 1997), related website last visited: 2012.03.20).

| species goup | year of species publication | species | water | soil | plants | vegetable | crustacean | fish | insect | mollusk | birds | poultry | rodent | carnivor | dog | cat | pig | equid | livestock / cattle | food | milk | milk products | meat | meat products | human | stool | disease |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| i | 1984 | ***Enterococcus avium*** | • | | • | | | | | | | • | | • | • | | • | | • | • | | | | | • | | • |
| i | 1984 | *Enterococcus malodoratus* | | | | | | | | | | | | | | | | | | | | • | | | | | |
| i | 1989 | *Enterococcus pseudoavium* | | | | | | | | | | | | | | | | | • | | | | | | | | |
| i | 1989 | *Enterococcus raffinosus* | | | • | • | | | | | | | | | | • | | | • | | | | | | • | | • |
| i | 2004 | *Enterococcus hermanniensis* | | | | | | | | | | | | | • | | | | | • | | | • | | | | |
| i | 2005 | *Enterococcus devriesei* | | • | | | | • | | | | | | | | | | | • | | | | | | | | |
| ii | 1989 | ***Enterococcus cecorum*** | | | | | | | | | • | • | | • | | • | • | • | • | | | | | | | | |
| ii | 1993 | *Enterococcus columbae* | | | | | | | | | • | | | • | • | • | | | | | | | | | | | |
| iii | 1991 | ***Enterococcus dispar*** | | | • | • | | | | | | | | | | | | | | | | | | | • | | |
| iii | 1998 | *Enterococcus asini* | | | | | | | | | | | | | | | | • | | | | | | | | | |
| iv | 1984 | ***Enterococcus faecalis*** | • | • | • | • | • | • | • | | | • | | • | | | • | • | • | • | • | • | • | • | • | • | • |
| iv | 2001 | *Enterococcus haemoperoxidus* | • | | | | | | | | | | | | | | | | | | | | | | | | |
| iv | 2001 | *Enterococcus moraviensis* | • | | | | | | | | | | | | | | | | | | | | | | | | |
| iv | 2001 | *Enterococcus ratti* | | | | | | | | | | | • | | | | | | | | | | | | | | |
| v | 1984 | *Enterococcus durans* | • | • | • | • | | | • | | | • | | | | | | • | • | • | • | • | • | | • | | • |
| v | 1984 | ***Enterococcus faecium*** | • | • | • | • | • | • | • | • | | • | | • | | | • | • | • | • | • | • | • | • | • | • | • |
| v | 1985 | *Enterococcus hirae* | • | • | • | • | | | | | • | | | • | • | | | • | • | • | • | • | • | | • | | |
| v | 1986 | *Enterococcus mundtii* | • | • | • | • | • | • | | | • | | | | | | • | | • | | | | • | | | | • |
| v | 2001 | *Enterococcus villorum* | | | | | | | | | • | • | | | | | • | | | | | | | | | | |
| v | 2003 | *Enterococcus canis* | | | | | | | | | | | | | • | | | | | | | | | | | | |
| vi | 1984 | *Enterococcus casseliflavus* | • | • | • | • | | | | • | | • | | | | | | | • | • | | | • | | • | | • |
| vi | 1984 | ***Enterococcus gallinarum*** | • | • | • | • | • | • | • | | | • | | | | | | | | • | | | • | | • | | • |
| u | 1991 | *Enterococcus saccharolyticus* | | | | | | | | | | | | | | | | | • | | | | | | | | |
| u | 1991 | *Enterococcus sulfureus* | | | | • | | | | | | | | | | | | | | | | | | | | | |
| u | 2002 | *Enterococcus pallens* | | | | | | | | | | | | | | | | | | | | | | | | | • |
| u | 2003 | *Enterococcus phoeniculicola* | | | | | | | | | • | | | | | | | | | | | | | | | | |
| u | 2004 | *Enterococcus italicus* | | | | | | | | | | | | | | | | | | | • | • | | | | | |
| u | 2005 | *Enterococcus aquimarinus* | • | | | | | | | | | | | | | | | | | | | | | | | | |
| u | 2005 | *Enterococcus canintestini* | | | | | | | | | | | | | • | | | | | | | | | | | | |
| u | 2006 | *Enterococcus caccae* | | | | | | | | | | | | | | | | | | | | | | | | • | |
| u | 2006 | *Enterococcus silesiacus* | • | | | | | | | | | | | | | | | | | | | | | | | | |
| u | 2006 | *Enterococcus termitis* | | | | | | | • | | | | | | | | | | | | | | | | | | |
| u | 2007 | *Enterococcus camelliae* | | | | • | | | | | | | | | | | | | | | | | | | | | |
| u | 2008 | *Enterococcus thailandicus* | | | | | | | | | | | | | | | | | | | | | | • | | | |

*Enterococcus flavescens = E. casseliflavus, Enterococcus porcinus = E. villorum, Enterococcus seriolicida = Lactococcus garvieae, Enterococcus solitarius = Tetragenococus halophilus, Enterococcus saccharominimus = E. italicus.*

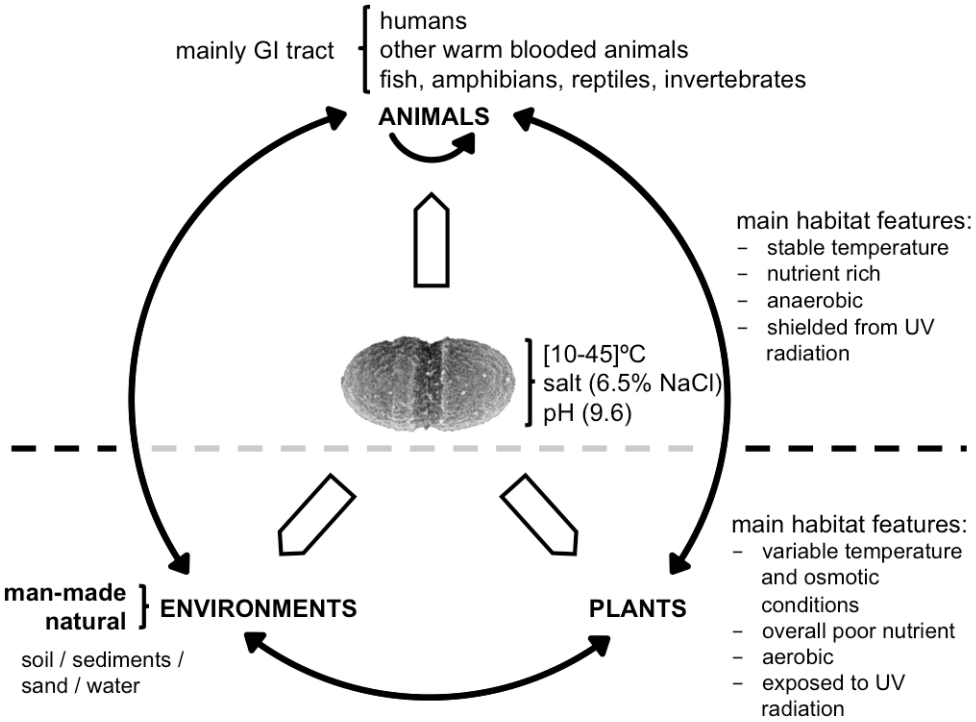


**Figure 1.1.** WHERE enterococci are found: habitat occurrence and features, and flow of presence perpetuation.

In addition to natural environments, in man-made environments enterococci can also resort to their survival mechanisms. Enterococci are capable of surviving on contaminated environmental surfaces for prolonged time periods (Devriese and Baele, 2006; Boyce, 2007). They have been shown to survive for one week to two months on countertops, for greater than seven days on fabric chairs, for seven days to four months on dry polyvinyl chloride surfaces, and for a few days to more than three months on cloth and plastic surfaces (Boyce, 2007).

## Perpetuation of enterococci in nature and man-made environments

Even if they are mainly known as warm-blooded animal commensals, we have seen that enterococci have a great diversity in their ecology and can be found retaining viability in numerous other environments. From the GI tract of animal where they can be found in impressive concentrations, $< 10^7$ CFU/g stool (Fisher and Phillips, 2009), they are excreted and the faeces end up in the environment. These organic wastes of human or production animal origin can be used as manure to fertilize soil for farming. This is a potential source of bacterial cross-contamination of vegetables, along with sewage sludge or irrigation with wastewater (Kühn et al., 2003). Anyhow, they end up being found on plants, in soil, and in the water. Sludge and sewer water may eventually reach lakes, rivers, or the sea, ending up on sandy terrains. The interconnection between all these environments perpetuates the presence of enterococci in nature, from water to soil, from plants to animals (Figure 1.1).

In order to respond to hostile environment and preserve their viability, enterococci can activate several survival strategies including starvation and the viable but nonculturable (VBNC) state. The VBNC state is defined as a survival mechanism activated by bacteria in response to multiple environmental stresses and allowing microorganisms to conserve their viability despite the loss of their own culturability (Lleò et al., 2005). VBNC aquatic enterococci, present in drinking or swimming water, ingested by humans, maintaining the adhesive properties, are capable of binding intestinal cells, resuscitating, and then colonizing the GI tract (Signoretto and Canepari, 2008).

The members of the genus *Enterococcus* can also be found in many food products of vegetable, meat and dairy origin (Ogier and Serror, 2008).

The presence of enterococci in the GI tract of animals may lead to contamination of meat at the time of slaughtering. They have been consistently isolated from beef, poultry, and pig carcasses, as well as from fresh raw meat from those animals. Besides raw meats, enterococci have been associated with processed meats, like several types of fermented sausages, where heating of processed meats during production may confer enterococci a selective advantage.

Enterococci are also recognized to be an essential component of the natural microbiota of many dairy products, predominating in some of them over lactobacilli and lactococci. Dairy products, like traditional European cheeses made from raw ewes' or goats' milk (Khan et al., 2010), contain many different species of enterococci, where *E. faecalis* and *E. faecium* are the species most commonly isolated with *E. durans* being also frequently isolated (Fortina et al., 2008; Ogier and Serror, 2008).

Their recovery and persistence in a variety of cheeses, also produced from pasteurized milk, is justified by their ability to survive under adverse conditions, such as temperatures and salinity (Fortina et al., 2008). Previous studies have shown that in addition to cheese technology, environmental contamination of the milk is also a relevant factor in microbial development in cheeses, particularly traditional raw-milk cheeses (Ogier and Serror, 2008). Contaminating microorganisms may enter the milk either directly from the faecal matter of animals or even humans or indirectly from contaminated water sources, from the surface of the animals, from milking equipment, and from bulk milk holding tanks (Foulquié Moreno et al., 2006; Ogier and Serror, 2008).

Enterococci have been identified as having a role in ripening, flavour development, and bacteriocin production in cheeses. During milk curdling and cheese ripening, complex interactions occur in the microbial community of artisanal products (Carraro et al., 2011). Probably by proteolysis, lipolysis, and citrate breakdown (Ogier and Serror, 2008; Khan et al., 2010), the presence of enterococci throughout ripening positively affects taste, colour, and the sensory profile of the full-ripened cheese (Devriese and Baele, 2006; Ogier and Serror, 2008). In addition to their technological properties and potential contribution to the organoleptic properties of fermented food products, many strains of enterococci may also act as protective agents against various pathogens, such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Clostridium botulinum*, *Clostridium perfringens*, and *Vibrio cholera* (Devriese and Baele, 2006; Ogier and Serror, 2008; Khan et al., 2010).

As well as being considered as normal parts of the food microbiota and an important component of artisanal cultures, enterococci have also been involved in food intoxication and spoilage (Foulquié Moreno et al., 2006; Pérez-Pulido et al., 2006), have the ability to produce biogenic amines in cheese, fermented sausages (Ogier and Serror, 2008) and cured meat products (Foulquié Moreno et al., 2006), and have also been associated as indicators for poor hygiene in the cheese production process (Klein, 2003; Foulquié Moreno et al., 2006). However, in the mammary gland of the healthy animal, milk maintains a microbial load. Fresh milk drawn from a healthy animal normally contains a low microbial load (less than $10^3$ CFU/mL) (Fotou et al., 2011). Even if, until recently, the species diversity and relative abundance of bacteria naturally present in milk were largely unknown, it is now clear that the presence of a microbial load in milk is not necessarily associated with a poor hygiene. Furthermore, the

application of proper sanitary conditions in the milking practice and fermentation processes assures the avoidance of contamination while preserving the natural flora of the milk and hence providing its special characteristics (Fotou et al., 2011).

Even if they have previously been only associated with faecal contamination, enterococci have a great diversity in ecology where they are now considered naturalized. The species distribution seems to be niche dependent, even if we cannot exclude biased isolation and identification results coming from bacteria in the VBNC state, low initial CFU, suboptimal growth conditions or even difficulty in identification of more recent species, which could result in the enrichment of some species. Nevertheless, enterococci are organism whose ubiquitous presence in nature perpetuates its own presence feeding back the cycle.

## WHY & HOW / Characteristics and mechanisms that allow enterococci to colonize and survive diverse habitats

Enterococci are able to inhabit the most diverse habitats. This ubiquitous nature of enterococci is possibly due to a number of features, which can either be intrinsic to the genus or specific to some species or even strains, and that enable a survival and fitness advantage. These characteristics are encoded in numerous genes, which have been associated with virulence and the ability to cause infection, can easily be transferable due to high genomic promiscuity of the genus, and allow to probe the environment in order to adapt the behaviour both to the environmental conditions and to cell density (Figure 1.2).

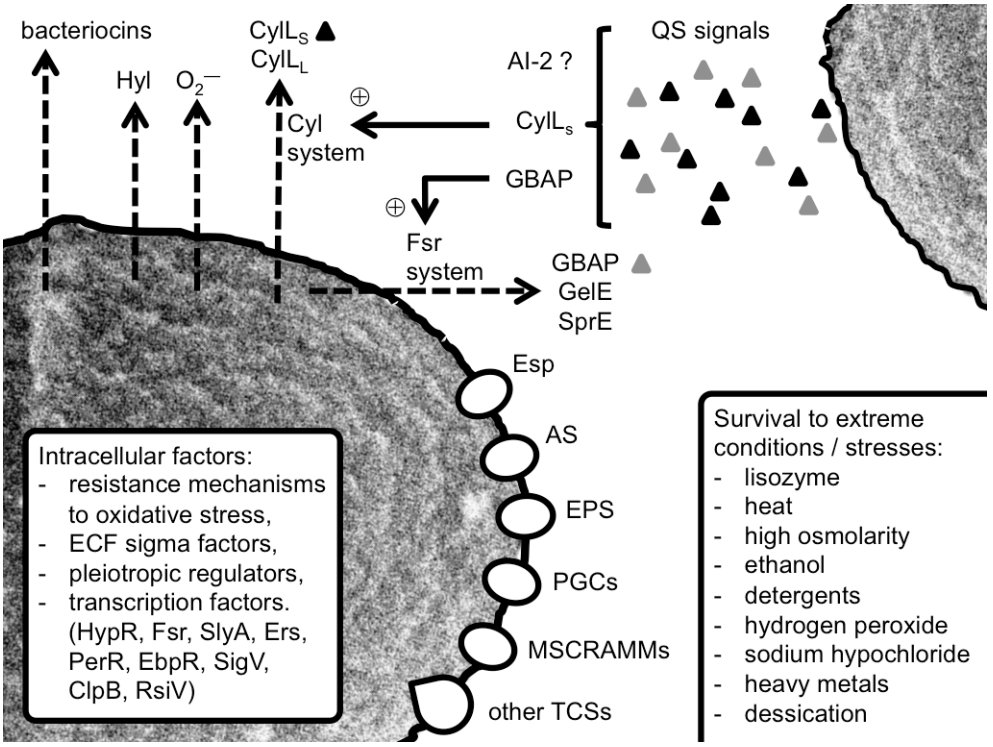


**Figure 1.2.** HOW enterococci are able to interact with their habitat: intrinsic and acquired characteristics and mechanisms. Hyl: hyaluronidase; $O_2^-$: superoxide anion; Cyl: cytolysin; CylL$_s$: small cytolysin subunit; CylL$_L$: large cytolysin subunit; QS: quorum sensing; AI-2: autoinducer-2; GBAP: gelatinase biosynthesis-activating pheromone; Fsr: *E. faecalis* regulator; GelE: gelatinase; SprE: serine protease; Esp: Enterococcal Surface Protein; AS: Aggregation Substance; EPS: exopolysaccharide; PGCs: Pilin Gene Clusters; MSCRAMMs: Enterococcal Microbial Surface Component Recognizing Adhesive Matrix Molecules; TCSs: Two-component systems; ECF: extracytoplasmic function.

The *Enterococcus* genus has been unevenly studied: some strains have not been identified to the species level, there is understandably far less information regarding the newer species, and only two species, *E. faecalis* and *E. faecium*, have been studied in depth. The main focus has

been biased towards fitness characteristics and mechanisms of *E. faecalis* and *E. faecium* since, besides their commensal coexistence with humans, they have been associated with pathogenic behaviours and regarded as a health concern for humans, which will be further discussed in the part WHEN & WHAT of this introduction.

## Intrinsic characteristics of the genus

Enterococci are able to resist hostile condition since strains can grow both in aerobic and anaerobic conditions, between 10 and 45 °C, survive heating at 60 °C for 3 min, and grow in 6.5 % NaCl, at pH 9.6 and tolerate the presence of 40 % (w/v) bile salts (Fisher and Phillips, 2009). They are intrinsically resistant to or tolerant to many antibiotics and are readily able to acquire more resistances (Giridhara Upadhyaya et al., 2010). There are nonetheless several exceptions to these general genus characteristics. Some species, specially the newly identified ones, lack one or more of these features (Benachour et al., 2005), which can make them difficult to identify. However, even lacking some of these extreme survival features, enterococci remain hardy bacteria able to survive in extreme conditions.

In-depth studies revealed more details about *E. faecalis* ability to survive under adverse conditions. Like *S. aureus*, *E. faecalis* is one of the few bacteria that are completely lysozyme resistant (Le Jeune et al., 2010). Exponentially growing cells can resist stresses such as heat, high osmolarity, and the presence of ethanol, detergents, hydrogen peroxide, sodium hypochlorite, and heavy metals, with a cation homeostasis which is thought to contribute to its resistance to pH, salt, metals and desiccation (Benachour et al., 2005; Fisher and Phillips, 2009). Moreover, adaptation by pre-exposure of the culture to sublethal stresses leads to a substantial

increase in resistance to the corresponding, usually lethal, stress (Benachour et al., 2005). When *E. faecalis* is grown at non-stress temperatures, subsequently cultured cells do not have the resilience to warm and cold environments that would occur if the first generation were grown at stressful temperatures (Fisher and Phillips, 2009). Starvation promoted by exhaustion of the carbon and energy source glucose or incubation in an oligotrophic microcosm strongly enhances the resistance of *E. faecalis* to environmental stresses and can be correlated with the increased synthesis of many proteins (Benachour et al., 2005).

## Bacteriocins

Diverse enterococcal species, such as *E. faecalis*, *E. faecium*, and *E. mundtii*, have already been associated with the ability to produce an impressive array of bacteriocins (Franz et al., 2007; Fisher and Phillips, 2009). Bacteriocins are substances elaborated by specific strains of bacteria that are lethal against other strains of the same or related species. They are ribosomally synthesized, small, cationic, amphiphilic (rather hydrophobic), extracellularly released antimicrobial peptides (Foulquié Moreno et al., 2006; Fisher and Phillips, 2009). Their production is favoured during lower growth rates, such as in stressful growth conditions (Fisher and Phillips, 2009), and have the cytoplasmic membrane as their primary target (Foulquié Moreno et al., 2006).

Termed enterocin, cytolysin, enterolysin or mundticin, these enterococcal bacteriocins are preferentially active against Gram-positive bacteria. The lytic effect together with the wide range of targets makes these enterococcal bacteriocins valuable for the fermented food industry, inhibiting Gram-positive food-spoilage and foodborne pathogenic bacteria (Franz et al., 2007; Javed et al., 2010). The production of these bactericidal

peptides provides a competitive advantage to enterococci when competing for an ecological niche and, in combination with the previously mentioned intrinsic characteristics of the genus, may explain why these bacteria are so robust in nature and why they occur in such a wide variety of ecological niches.

## Putative virulence traits

As indicated by case fatality rates and/or the ability to invade the tissues of the host, the degree by which a microorganism is able to cause disease, in man, animals, or plants, beyond that intrinsic to the species background (Michaux et al., 2011), is determined by its virulence factors. Even if they are not required for the viability *per se* of bacteria, these virulence factors have already been described and recognized as being involved in the different steps by which an organism is able to cause disease: adhesion, colonization, invasion, immune response inhibition (immune evasion and/or immunosuppression), obtaining nutrition from the host, and toxin production.

Coded by genes in chromosomal DNA, bacteriophage DNA or plasmids, several virulence factors have already been identified in the genus *Enterococcus*, mainly regarding *E. faecalis* species, but also in *E. faecium*. The association of these factors with virulence has mainly been made through the use of virulence models. However, none of the enterococcal virulence models adequately mimic the particular physiopathological conditions that would clearly distinguish between pathogenic and non-pathogenic strains (Ogier and Serror, 2008), and the virulence attributed to a virulence factor, in its particular virulence model, cannot be necessarily generalized to other virulence models, such as different hosts.

Virulence factors are not exclusive to enterococcal strains isolated from diseased hosts. They are also present in commensal isolates and strains found in other ecological niches, without presenting any pathogenic effect clearly associated to them (Franz et al., 2001; 2003; Lepage et al., 2006; Solheim et al., 2009; Giridhara Upadhyaya et al., 2010). It is still not clear whether the presence of these factors in *E. faecalis* isolates from clinical and commensal isolates contributes to the virulence in humans (Giridhara Upadhyaya et al., 2010), or even if some of them can be beneficial in the commensal relationship with the human host. Enterococcal virulence is subtle and complex, involving both pathogen and host factors, and all of these factors rarely occur in a single strain (Michaux et al., 2011).

Even if virulence factors have been associated with a pathogenic potential, they are mainly fitness factors for bacteria. The previously described enterococcal virulence factors in the literature can be either secreted, surface associated, or intracellular.

**Secreted factors**

Several secreted factors have been associated with enterococcal virulence in virulence models. Some of these secreted factors have been associated with toxin production, giving a fitness advantage when competing with other organisms for an ecological niche as well as giving an arsenal for attack or defence when in presence of other organisms, but also with obtaining nutrition from the environment, the colonization process, and immune evasion.

Cytolysin (Cyl)

The Cyl (hemolysin/ bacteriocin) system involves production of an extracellular product, the Cyl, that consists of two small lantibiotic-like peptides "$L_L$ and $L_S$", which act extracellularly and in concert to manifest

toxicity or lytic activity and contribute to virulence in infection models (Clewell, 1990).

Cyl is capable of lysing erythrocytes, such as human, horse, sheep and rabbit (Giridhara Upadhyaya et al., 2010). Animal models have suggested that haemolytic *E. faecalis* strains are more virulent than non-haemolytic strains, and an association between a haemolytic phenotype and an increased severity and adverse outcome for patients with bacteraemia caused by *E. faecalis* has been reported (Reynaud Af Geijersstam et al., 2007; Giridhara Upadhyaya et al., 2010).

Proteases: Gelatinase (GelE) and Serine Protease (SprE)

GelE, previously referred in the literature as coccolysin, is a well-known secreted hydrophobic zinc-metalloprotease (Waters et al., 2003; Thurlow et al., 2010; Bøhle et al., 2011). GelE has the capacity to cleave and degrade a broad range of host substrates, including insulin, casein, haemoglobin, gelatine, collagen, fibrinogen, fibrin, endothelin-1, bradykinin, LL-37, and complement components C3 and C3a (Waters et al., 2003; Thurlow et al., 2010). The *gelE* gene, that encodes GelE, is located on the chromosome and is regulated in a cell-density-dependent manner (Fisher and Phillips, 2009).

The quorum sensing (QS) system encoded by the FsrABDC proteins is necessary for the coexpression of GelE and *E. faecalis* virulence-related SprE, located directly downstream, from a promoter located upstream of the *gelE* gene (Fisher and Phillips, 2009). The FsrABDC system and GelE have been shown to be relevant for enterococcal virulence in different animal models (Del Papa and Perego, 2011). SprE has been shown to contribute to disease in animal models but its mechanistic contribution is not known at this time (Thurlow et al., 2010).

Commonly considered a virulence factor of *E. faecalis*, one of the known contributions of GelE producing strains is its involvement in efficient biofilm formation (Benachour et al., 2005; Thurlow et al., 2010; Bøhle et al., 2011; Del Papa and Perego, 2011), functioning to encourage lysis and subsequent extracellular DNA (eDNA) release of a sub-population of cells which could aid in colonization establishment (Pinkston et al., 2011). For the GelE producing population, the co-production of SprE functions as an immunity protein, protecting it from self-destruction (Thomas et al., 2009). Biofilm formation, due to the protease activity of GelE, has been linked to the pathogenesis of enterococcal endocarditis (Giridhara Upadhyaya et al., 2010; Thurlow et al., 2010; Del Papa and Perego, 2011), as well as bacteraemia, urinary infections and oral infections (Zoletti et al., 2011).

In addition to assisting in the establishment of biofilm, it has been suggested that GelE may also play a role in dissemination. Since GelE is known to degrade adhesins, and to also cleave collagen, it has been speculated that GelE could potentially encourage disassociation of *E. faecalis* from collagen through either loss of the corresponding adhesin or by cleaving the collagen to which it is bound. In established vegetations, and directed by QS, GelE, as well as SprE, could aid in the release of cells from the collagen matrix to encourage dissemination of bacteria from the primary site of infection (Pinkston et al., 2011).

Another possible role for GelE mediated cleavage of surface proteins from the surface of *E. faecalis* is that it could provide a method to evade the immune system. Upon establishment of an infection, when initial adherence is no longer required, it may be beneficial for the bacteria to remove surface adhesins, which are known to trigger an immune response in the host. As mentioned previously, GelE is also known to cleave C3 and iC3b, as well as being involved in the degradation of C5a (chemotaxis),

disrupting the PMN-mediated killing of *E. faecalis*, and therefore having a role in evading clearance by the immune system (Pinkston et al., 2011).

A recent study indicates that GelE is loosely associated to the cell surface (Bøhle et al., 2011), which indicates that its possible action is localized, affecting solely the immediate vicinity of the producing enterococcal cell.

Hyaluronidase (Hyl)

Hyl is encoded by the chromosomal *hyl* gene. Along with other hydrolytic enzymes, Hyl is involved in the virulence of *Enterococcus* species (Semedo et al., 2003). This degradative enzyme acts on hyaluronic acid and is associated with tissue damage. Hyl depolymerizes the mucopolysaccharide moiety of connective tissue, thus facilitating spread of enterococci, as well as their toxins, through host tissue (Fisher and Phillips, 2009).

Superoxide

Nearly all *E. faecalis*, but only a few *E. faecium* isolates, produce substantial amounts of extracellular superoxide (Kayser, 2003; Devriese and Baele, 2006). Fumarate reductase is likely to be involved in superoxide production and may thus be an important source of oxidative stress for the host (Bøhle et al., 2011). When *E. faecalis* isolates from patients with endocarditis and bacteraemia were compared with isolates from healthy volunteers, superoxide production was, on average, higher among blood isolates than in commensal strains (Kayser, 2003). It has been demonstrated that superoxide from *E. faecalis* promotes chromosomal instability in mammalian cells, which can lead to colorectal cancer (Bøhle et al., 2011).

**Surface-associated factors**

Apart from the secreted factors, which can also be loosely attached to the surface, some virulence factors are tightly bound to the enterococcal surface. These surface-associated factors have been involved in adhesion, therefore helping further colonization that has been associated to one of the first steps towards a possible infection, hence the association to virulence. They have also been involved in immune evasion, that can be detrimental to the host in case of infection but that is essential when there is the establishment of a commensal relationship.

Enterococcal Surface Protein (Esp)

Of the cell-surface associated factors, the first one discovered to enhance biofilm formation was Esp, which was examined due to its homology to the *S. aureus* protein Bap (biofilm-associated protein) (Garsin and Willems, 2010). *esp* gene is located on a pathogenicity island (PAI) in both *E. faecalis* and *E. faecium*, and the corresponding proteins are expressed in both species at the surface of the bacterium (Sava et al., 2010).

In *E. faecalis*, Esp has been identified as a putative virulence factor involved in colonization of the urinary tract. Conflicting results have been reported about the role of *E. faecalis* Esp in biofilm formation, ranging from significant loss of biofilm formation in *E. faecalis* Esp-deficient mutants to no apparent effect (Sava et al., 2010), showing that its presence is not absolutely required (Garsin and Willems, 2010).

The genetic repertoire and processes that facilitate multicellular cooperative behaviour and biofilm formation in *E. faecium* are not yet well understood (Garsin and Willems, 2010). Recently, more information has been presented about the function of *E. faecium* Esp. Interestingly, in contrast to *E. faecalis esp* gene, which is widely spread among strains, *esp*

*gene* from *E. faecium* is predominantly present in hospital-acquired isolates, suggesting a role in virulence (Sava et al., 2010). The importance of *E. faecium* Esp in urinary tract infections (UTIs) and endocarditis probably results from the fact that these models represent typical biofilm-associated infection models indicating a specific role of Esp in the pathogenesis of *E. faecium* infections (Sava et al., 2010).

Aggregation Substance (AS)

AS is the description used for a group of surface proteins encoded on pheromone-inducible conjugative plasmids. Asa1, Asp1, and Acs10 are the best studied AS proteins and show over 90 % amino acid sequence identity (Sava et al., 2010).

AS has been regarded as a virulence factor in *E. faecalis*. They have been involved in increased adherence, internalization and survival in eukaryotic cells, which can indicate that they might be involved in the translocation of *E. faecalis* through the intestinal epithelia, leading to systemic infection. However AS also promotes conjugation by directing bacterial aggregation, resulting in close cell contact between donor and recipient (Sava et al., 2010).

Extracellular polymeric substances

The presence of extracellular polymeric substances are believed to be associated with bacterial protection against adverse environmental surroundings, such as desiccation or bacteriophages, the sequestration of essential cations, and also to be involved in adhesion (Jolly et al., 2002; Welman and Maddox, 2003; Mozzi et al., 2009). These substances produced by bacterial species are a mixture of various components, including polysaccharides, lipopolysaccharides, proteins, peptides, and

nucleic acids, in which the polysaccharides constitute the major fraction (Suo et al., 2007).

Cell-surface polysaccharides comprise O-antigens lipopolysaccharides (LPSs), lipoteichoic acids (LTAs), capsular polysaccharides (CPSs) and exopolysaccharides (EPSs) (Jolly et al., 2002). EPS are secreted into their surroundings during growth and are generally loosely attached to the surface of the microbial cell or excreted into the environment. This distinguishes them from the structurally similar capsular polysaccharides (CPSs), which do remain permanently linked to the surface of the cell (Jolly et al., 2002; Welman and Maddox, 2003).

The *in situ* production of these cell-surface polysaccharides plays an important role in the manufacture of a diversity of fermented dairy products, such as yogurt, drinking yogurt, cheese, cultured cream, and milk-based desserts (Jolly et al., 2002; Mozzi et al., 2009). Their presence significantly contributes to texture, mouthfeel, taste perception and stability of the final products (Jolly et al., 2002). An additional hypothesized physiological benefit is that EPSs will remain for longer in the GI tract, thus enhancing colonization by probiotic bacteria (Welman and Maddox, 2003). Additionally, beneficial effects for the human health have been assigned to these EPS, such as cholesterol-lowering, antitumoral or immunomodulating activities (Mozzi et al., 2009).

The capsule, which usually contains various pathogenic antigens as virulence factors, plays a critical role in bacterial adhesion and can facilitate the evasion of bacteria from host defences (Suo et al., 2007), by preventing complement activation and/or killing by phagocytes. *E. faecalis* was previously reported to produce one of four capsule serotypes (A, B, C, or D) (Thurlow, Thomas, and Hancock, 2009). The capsule locus in *E. faecalis*, designated *cps*, contains nine genes (*cpsC* – *cpsK*). CpsF encodes a

putative glucosyltransferase which is probably responsible for the serodiversity between serotype C and D strains, modifying the ratio between glucose and galactose in the two types of capsules (Sava et al., 2010). *E. faecalis* strains of either serotype C or D are more resistant to complement-mediated opsonophagocytosis than unencapsulated strains. The capsule has the ability to mask bound C3 from detection on the surface of *E. faecalis*. Similarly, *E. faecalis* capsule masks LTA from detection, which correlates with decreased cytokine production. The altered cytokine response to encapsulated pathogens appears to contribute to pathogenicity and virulence. The capsule has an important role as a virulence factor of *E. faecalis* and provide several mechanisms by which the presence of the capsule influences evasion of the host innate immune response (Thurlow, Thomas, Fleming, and Hancock, 2009). However, to date, the chemical structures of these capsular polysaccharides have not been published (Sava et al., 2010).

Several studies have demonstrated the presence of an enterococcal polysaccharide antigen (Epa) in the *E. faecalis* cell wall. The *epa* locus consists of 16 open reading frames and is widespread among *E. faecalis* strains with the inside of the bacterial cell wall being its possible localization (Sava et al., 2010). This carbohydrate seems to play an important role in enterococcal pathogenicity, since disruption of different genes of the *epa* locus results in decreased biofilm formation (Garsin and Willems, 2010) and enterocyte translocation, lower resistance to killing by PMNs, higher bacterial susceptibility to infection by phages and reduced virulence in mouse peritonitis and UTI models (Sava et al., 2010)

Regarding *E. faecium*, published information about capsular polysaccharides is completely lacking, and it has been suggested there is no capsule expression (Sava et al., 2010).

Pilin Gene Clusters (PGCs)

The multi-factorial processes that have been implicated in adhesion to multiple types of human cells and in biofilm formation, two processes critical in the pathogenesis of many bacterial diseases, also involve the pili of Gram-positive bacteria. *E. faecalis* and *E. faecium* harbour PGCs, comprising genes encoding surface proteins with LPxTG-like motifs and sortases, which are required for pilus assembly (Sava et al., 2010).

*E. faecalis* harbours two PGCs, designated as the *ebp* locus (endocarditis and biofilm- associated pili) and the *bee* locus (biofilm enhancer in enterococci). The *ebp* locus is ubiquitous in *E. faecalis*, and the expressed Ebp pili are important factors involved both in initial adherence and biofilm formation (Garsin and Willems, 2010; Sava et al., 2010). They contribute to the pathogenesis of experimental endocarditis and UTI, which are both biofilm-associated infections. In addition, it was found that Ebp pili are antigenic in humans during endocarditis. The *bee* locus is located on a conjugative plasmid and was only detected in 5% of the *E. faecalis* isolates, whereas the *ebp* operon was found in almost all of the isolates (Sava et al., 2010).

Like *E. faecalis*, *E. faecium* isolates recovered from clinical sites, fruits and vegetables, and water and soil are able to produce pili. *E. faecium* harbours four PGCs, designated PGC-1 to -4 (Garsin and Willems, 2010), that display high similarity with genes of the *ebp* operon of *E. faecalis*, suggesting a possible role of these pili in interactions with the mammalian host (Sava et al., 2010). Although the genetic organization of pilin gene clusters, expression and distribution of pili genes among *E. faecium* from various sources was investigated, the role of pili in *E. faecium* pathogenicity was not defined (Garsin and Willems, 2010).

Enterococcal Microbial Surface Component Recognizing Adhesive Matrix Molecules (MSCRAMMs)

Colonization of human tissues is assumed to occur via interactions between specific proteins in the extracellular matrix and the so-called MSCRAMMs (Sava et al., 2010). By sequence homology with other well-characterized MSCRAMMs, just like the virulence factor Cna in *S. aureus* (Lebreton et al., 2009), several enterococcal MSCRAMMs have been identified in *E. faecalis* and *E. faecium*. The publicly available genome sequences of *E. faecalis* V583 and *E. faecium* TX0016 revealed the presence of 17 and 15 MSCRAMMs, respectively. So far, seven enterococcal MSCRAMMs have been characterized in detail: Ace (adhesion of collagen of *E. faecalis*), Fss1, Fss2, and Fss3 (*E. faecalis* surface protein) of *E. faecalis* and Acm (adhesin of collagen of *E. faecium*), Scm (second collagen adhesin of *E. faecium*) and EcbA (*E. faecium* collagen binding protein A) of *E. faecium* (Sava et al., 2010).

Most of these MSCRAMMs, from both *E. faecalis* and *E. faecium*, have been shown to bind to components of the extracellular matrix. In animal experiments, they have been associated to the pathogenesis of experimental endocarditis (Sava et al., 2010), UTI (Lebreton et al., 2009) or biofilm formation (Hendrickx et al., 2009; Garsin and Willems, 2010). Adding to that, some of these factors were identified as being antigenic in humans during infection, which reinforces the idea of significant expression of these proteins *in vivo* (Lebreton et al., 2009; Sava et al., 2010). Even if some MSCRAMMs are only associated with hospital-acquired enterococcal isolates other are widely found among clinical and non-clinical isolates (Sava et al., 2010).

**Intracellular factors**

As was previously mentioned, enterococci have a number of extracellular and surface factors that give them a fitness advantage when they are presented to harsh conditions in the environment or in the interaction with a host, as well as helping them in the several steps that can lead to infection. The presence of mechanisms that allow countering extreme conditions and regulating stress response are essential for fitness and survival, and are associated to virulence and pathogenicity of the *Enterococcus* genus.

The oxidative burst is one of the major mechanisms by which the host's phagocytes kill pathogenic bacteria. Therefore, an efficient oxidative stress response aiming to increase the cell's antioxidant defence may be crucial, at least in the early stages, for successful infection (La Carbona et al., 2007).

The opportunistic pathogen *E. faecalis* is well equipped to respond to oxidative stress. It harbours an arsenal of genes such as catalase, glutathione reductase, the two subunits of alkyl hydroperoxide reductase, manganese superoxide dismutase (MnSOD) and three loci encoding peroxidatic activities: a NADH peroxidase, an alkyl hydroperoxide reductase and a thiol peroxidase (Tpx) (Verneuil et al., 2005; La Carbona et al., 2007), which are involved in the defence not only towards superoxide but also towards external exposure to $H_2O_2$ and internal generated $H_2O_2$ by aerobic growth (La Carbona et al., 2007). Some of these genes are under direct positive control of the transcriptional regulator HypR, indicating that HypR may be an important virulence factor in *E. faecalis* (Verneuil et al., 2005).

In other organisms, virulence genes are controlled by environmental stresses and involve secondary RNA polymerase sigma factors and

specific transcriptional regulators (Michaux et al., 2011). *E. faecalis* genome, like those of *Streptococcus pyogenes*, *Streptococcus pneumoniae*, and *Lactococcus lactis*, lacks a general stress response regulator homologous to sigmaB of *Bacillus subtilis* (Le Jeune et al., 2010). However, several transcription factors, regulators and extracytoplasmic function (ECF) sigma factor have been shown to be involved in virulence and stress response in *E. faecalis* (Michaux et al., 2011).

The largely studied Fsr system proteins have already been mentioned as being necessary for transcriptionally autoregulating the expression of the extracellular virulence-related proteases, GelE and SprE (Del Papa and Perego, 2011; Pinkston et al., 2011). By strongly influencing the regulation of these proteases, the *fsr* locus is therefore also relevant for enterococcal virulence, as revealed by different virulence models (Del Papa and Perego, 2011). This FsrABDC QS system is a homologue of the AgrABCD virulence system of *S. aureus* and is not uniquely involved in the promotion of biofilm formation, the dispersal of cells in established biofilms and as a contributor to the pathogenesis of enterococcal endocarditis due to its autoregulation of GelE (Del Papa and Perego, 2011; Pinkston et al., 2011). The influence of the Fsr system can also be slightly inhibitory to biofilm formation. Cell death and genomic DNA release — eDNA, dependent on the autolysin, AltA, is necessary for formation of the biofilm matrix. GelE activates AltA, whereas SprE acts as an immunity protein and prevents GelE from accessing AltA. By regulating SprE, the Fsr system adds complexity to the regulation of biofilm formation (Garsin and Willems, 2010). The Fsr system is also known to influence the expression of numerous targets that include other factors, besides GelE, described as important for biofilms (BopD), genes predicted to encode surface proteins,

and proteins implicated in several metabolic pathways (Bourgogne et al., 2006).

Other factors have been identified as being involved in virulence and the processes that may lead to infection. Using different infection models, the transcription factor SlyA was shown to play a role in the virulence of *E. faecalis* as well as in its persistence inside the host (Michaux et al., 2011). The transcriptional regulator Ers (for enterococcal regulator of survival) (PrfA like - the major regulator of virulence genes in *Listeria monocytogenes*) has a pleiotropic effect, especially in the cellular metabolism, like survival to oxidative stress and in mouse macrophages, and virulence of *E. faecalis* (Verneuil et al., 2005). PerR-like (peroxide regulator) regulator is a factor important for *E. faecalis* virulence in a mouse model (Verneuil et al., 2005). PerA (for pathogenicity island-encoded regulator), identified in the PAI, is important as a regulator of biofilm formation and survival within macrophages and is likely a regulator controlling determinants important to pathogenesis, as well as other uncharacterized PAI genes (Coburn et al., 2008). The transcriptional regulator HypR was clearly shown to be involved in the *E. faecalis* stress response (Benachour et al., 2005). The expression of ClpB, regulated by CtsR, appears to play a major role in induced thermotolerance and in pathogenesis in *E. faecalis* (de Oliveira et al., 2011). The transcriptional regulator *bop* (biofilm on plastic surfaces) contributes to the production of biofilm, the regulation of the expression of biofilm and prolonged bacteraemia in mice in *E. faecalis* (Hufnagel, Koch, Creti, Baldassarri, and Huebner, 2004). The transcriptional regulator *ebpR* is important for transcriptional activation of the *E. faecalis* endocarditis and biofilm associated pilus operon, *ebpABC* (Bourgogne et al., 2006). In addition to the pleiotropic effects displayed by the ECF sigma factor SigV, it also

contributes to survival following heat, acid and ethanol treatments and is involved in lysozyme resistance of *E. faecalis* (Le Jeune et al., 2010), just like the factors encoded by *rsiV* (Benachour et al., 2005).

## Emergence of new genetic material

Enterococcal genomes, like other bacterial genomes, evolve through the emergence of new genetic material. This new genetic material can arise in bacteria either *de novo*, by internal genetic mutation, and the genic information is then transmitted from parent-to-offspring, or naturally transmitted from external sources and organisms, related or not, through horizontal gene transfer (HGT). HGT may occur via a variety of naturally occurring processes such as genetic conjugation (a unidirectional transfer of genetic material during fusion of two cells), genetic transduction (transfer of bacterial DNA by phages from an infected bacterium to another bacterium) and transformation (uptake of naked DNA by cells). HGT has a radically different impact from that of spontaneous mutations, which may be responsible for diversification and speciation of microorganisms on an evolutionary time-scale. HGT allows bacteria to rapidly acquire, immediately upon integration, complex and completely new physiological traits. This results in abrupt large-scale alterations in the structure and organization of genomes and has been and remains a key driving force in bacterial evolution, capable of generating new variants of bacterial strains by “genetic quantum leaps” (Dutta and Pan, 2002; Wozniak and Waldor, 2010).

During HGT, mobile genetic elements (MGEs), segments of DNA that can move within genomes, are transferred. These include plasmids, bacteriophages, genomic islands (GEIs), integrons, transposons and insertion sequence (IS) elements (Kelly et al., 2009). Many genetic features

have already been described as being transferred between the same enterococcal species, different species as well as with other genus: plasmids (Coburn et al., 2010; Manson et al., 2010; Palmer et al., 2010), integrative and conjugative elements (ICEs) (Wozniak and Waldor, 2010), the enterococcal PAI (Manson et al., 2010; van Schaik and Willems, 2010; Laverde Gomez et al., 2011). Through HGT, antibiotic resistance transfer allows other bacteria to acquire antibiotic resistance, metabolic traits transfer confers some useful metabolic capabilities to the recipient bacteria of the acquired gene, and PAIs transfer, with the virulence gene clusters therein, is a common strategy of commensal bacteria to acquire pathogenic traits (Dutta and Pan, 2002; Juhas et al., 2009; Laverde Gomez et al., 2011).

## Sensing and probing the environment

Enterococci have a number of traits that allow them to respond and prevail in the most diverse and varying environmental conditions, both inside and outside a host. Their ability to sense changing environmental stimuli and orchestrating a response accordingly is of the utmost importance in its adaptation to these varying conditions (Hancock and Perego, 2004).

### Two-component systems (TCSs)

Bacteria have different mechanisms available for the regulation of stress response. Besides sigma factors and small RNAs, most bacteria monitor and adapt to changing conditions through signal transduction involving two-component signal transduction systems. TCSs generally consist of a sensory histidine kinase (HK) and a cognate response regulator (RR). The HK senses the signal and relays the adaptive response through the transfer of a phosphoryl group to the RR, which can then act as

a transcriptional regulator to modulate gene expression (Hancock and Perego, 2004). Most bacterial genomes encode several TCSs and enterococcal genomes are not unlike. Taken together, studies on *E. faecalis* TCSs can emphasize that two-component signal transduction systems govern important biological parameters of this organism ranging from environmental persistence, virulence and biofilm formation, to antibiotic resistance and stress response (Teng, Wang, Singh, Murray, and Weinstock, 2002; Le Breton et al., 2003; Hancock and Perego, 2004). Although all these roles associated to TCSs contribute together to the opportunistic character of *E. faecalis*, little is known about their target genes.

**Quorum sensing (QS)**

TCSs allow enterococci to sense environmental stimuli and to subsequently coordinate a suitable response. Part of these environmental stimuli can be autoinducers (AIs), molecules secreted by a bacterial population that accumulate extracellularly and induce genes in that same bacterial population. QS is that specific phenomenon: when a sufficient cell density has been reached, microorganisms communicate, coordinate and switch a set of behaviours among all individuals of the population, by the accumulation of AIs over a given threshold concentration (Hense et al., 2007).

As the three key determinants of AI concentration (cell density, mass-transfer properties and the spatial distribution of cells) can vary independently, cells sensing the AI concentration are unable to distinguish cell density from mass-transfer properties or spatial distribution. They can only assess the combination of these factors. This is why QS and diffusion sensing (DS), where cells use AI sensing to measure the mass-transfer properties of their environment, can be integrated. The recent notion of

efficiency sensing (ES) helps unify the concepts of what cells sense, why cells sense and the evolutionary hypotheses of the fitness benefits derived from AI sensing (Hense et al., 2007).

Whether bacteria sense their density to allow them to engage in social behaviour that would benefit the group, in QS, or that sensing evolved as an autonomous activity of single cells to detect mass transfer limitations because of a direct fitness benefit for the individual, in DS, does not change the fact that enterococci are able to sense their specific AIs and adequately regulate gene expression.

It was demonstrated that the previously mentioned Fsr system was activated by AI concentration through the TCS Ehk15-Err15 (enterococcal HK and enterococcal RR) (FsrCA) (Nakayama et al., 2001). The *fsr* locus is comprised of 4 genes, *fsrA*, *fsrB*, *fsrD*, and *fsrC*, whose products form a system that responds to the extracellular accumulation of the gelatinase biosynthesis-activating pheromone (GBAP) peptide encoded by the *fsrD* gene that acts as an AI. FsrB acts as a cysteine protease-like processing enzyme involved in the processing of the FsrD peptide. Accumulation of this peptide in the extracellular space is sensed by the FsrC membrane HK, leading to the activation of the RR and transcription factor FsrA. All the FsrABDC proteins are necessary for autoregulation at a promoter located upstream of *fsrB* and for the expression of two *E. faecalis* virulence-related proteases, GelE and SprE, from a promoter located upstream of the *gelE* gene (Del Papa and Perego, 2011). This QS mechanism, that involves the Fsr system and a TCS, also plays a role in efficient biofilm formation, dissemination, and immune system evasion.

Unrelated to the well-known superfamily of TCS, another two component regulatory system, that involves the membrane component CylR1 and the DNA binding component CylR2, is necessary for the QS

induction of the cytolysin operon, where cytolysin serves as an AI. As previously mentioned, the control of the cytolysin is regulated by a threshold concentration of the posttranslationally modified subunit $CylL_S$. $CylL_L$ has been shown to bind strongly to target cell membranes, allowing free $CylL_S$ to accumulate above a critical induction threshold (Coburn et al., 2004). This QS system provides a means by which the cytolysin is produced in elevated amounts only when a particular target cell is present (Clewell, 2007), contributing to the virulence of enterococcal species harbouring this trait.

Beyond controlling gene expression on a global scale and allowing communication within species, QS also allows bacteria to communicate between species. That communication is achieved via 4,5-dihydroxy-2,3-pentanedione (DPD), one of the conversion products of S-ribosylhomocysteine (SRH) by the enzyme LuxS, which spontaneously cyclizes into several furanones in chemical equilibrium, collectively referred to as autoinducer-2 (AI-2) (Hense et al., 2007). Specifically, the *luxS* gene that encodes the LuxS enzyme is present in roughly half of all sequenced bacterial genomes, AI-2 production has been verified in a large number of these species, and AI-2 controls gene expression in a variety of bacteria (Waters et al., 2003), being implicated in the regulation of many bacterial behaviours including biofilm formation, competence, the production of secondary metabolites like antibiotics, and virulence (Pereira et al., 2009). All enterococcal genomes publicly available (http://www.ncbi.nlm.nih.gov/ and http://www.broadinstitute.org/; related websites last visited: 2012.01.05) carry genes annotated as *luxS* genes, which can allow them to produce AI-2. While in some cases, AI-2 is clearly acting through a canonical QS mechanism, in others no relation to a committed communication between species could be ascribed (Pereira et al., 2009).

## WHEN & WHAT / Is there a straightforward enterococcal associated human health risk?

As seen before, enterococci are widespread among the environment, colonizing water, soil, plants and animals, including humans. Apart from their typical colonization of skin and mucous membranes, such as the GI tract, enterococci have also been linked to human infections. What are the enterococcal associated human health benefits and risks and when did they emerge?

The already mentioned definition of a commensal bacteria is relative because, within the same host species, a given strain can vary its ability to cause disease (Marshall and Ochieng, 2009; Albesharat et al., 2011). The blurring of their definition complicates the study of commensal flora. Selective pressures, including those from antibiotic use and immunosuppressive therapies, further obscure boundaries, leading commensals into the realm of pathogens. This crossover phenomenon arises because the physical state of hosts also has much to do with defining which microorganisms are commensals and which are pathogens (Marshall and Ochieng, 2009). The distinction between ‘‘pathogenic commensals’’, that can cause disease when a patient is vulnerable or the bacterium gains access to a sterile site, and ‘‘non-pathogenic commensals’’, which are incapable of causing disease (Albesharat et al., 2011), allow clarifying the commensal notion. Based on the source of isolation, commensals can also be described as those isolates collected from asymptomatic individuals and from ‘‘typical’’ colonization sites, from environmental, or from non-human animal sources (Albesharat et al., 2011). So, a commensal can be defined as an organism that typically does not cause disease, although some individual strains within a commensal

species may be able to cause disease, and a commensal isolate is defined as an isolate that has not been acquired from a diseased animal or person, even though that isolate could cause disease in an atypical site, and which would not cause disease in humans following typical colonization (Albesharat et al., 2011).

## In human disease

Even though enterococci are considered human commensal, some species, like *E. faecalis,* have been associated to human disease for more than a century, but it was not until the past couple of decades that members of the genus *Enterococcus* emerged as common and problematic nosocomial pathogens. Not present upon admittance to the hospital or other clinical setting, nosocomial infections are acquired by the patient in the clinical setting and enterococci, as a whole, are generally not thought of as community acquired pathogens, but instead are nosocomial pathogens.

Mainly *E. faecalis*, but also *E. faecium*, have been the species associated with hospital acquired infections (Fisher and Phillips, 2009), with *E. faecium*, in recent years, progressively increasing in percentage of infection, owing to being resistant to a large number of antibiotics (Willems and van Schaik, 2009). 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 few reports of infections caused by *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; Mastroianni, 2009), *E. gallinarum* (Reid et al., 2001; Takayama et al., 2003), *E. avium* (Swaminathan and 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). The lower incidence of other enterococcal species may happen because of their possible lower pathogenic potential, but misidentification of unusual enterococcal species in medical centres due to the commercial identification methods cannot be disregarded, requiring accurate identification with molecular methods (Tan et al., 2010).

Regardless of species, enterococci have been associated with several pathological conditions in the hospital environment. The most prevalent infections caused by members of this genus are UTIs, bacteraemia, intra-abdominal and pelvic infections, wound and tissue infections, and endocarditis (Jin et al., 2011).

The emergence of these microbes as nosocomial pathogens coincides with the advent of modern antibiotic therapies. These antibacterial agents can interfere with the cell wall, inhibit metabolic pathways, and interfere with protein and nucleic acid synthesis (Tenover, 2006). To prevent the effect of antibiotics, bacteria have developed four main mechanisms that allow resistance to occur, knowing that more than one may be present in a single bacterium: they can alter the antibiotic target site, decrease its uptake by decreased penetration and/or increased efflux, enzymatically inactivate or modify it, or create “bypass” pathways (Hawkey, 1998).

Enterococci possess a broad spectrum of antibiotic resistances, both intrinsic and acquired. The intrinsic resistance to certain classes of β-lactam antibiotics (e.g. cephalosporins), low-level resistance to aminoglycosides, lincosamides, streptogramins, in the case of *E. faecalis*, and monobactams provided enterococci with a selective advantage in the hospital environment (Willems et al., 2011). The intrinsic and acquired antibiotic resistance mechanisms of the genus can, upon antibiotic therapy with agents inactive against enterococci, lead to an increased enterococcal

burden in the gut by elimination of competitors (Jin et al., 2011), or maybe even by decreasing the host's killing ability (Albesharat et al., 2011). One of the most disturbing resistances seen in enterococci is the acquired resistance to vancomycin, which is often regarded as the antibiotic of last resort for treating various multi-resistant Gram-positive cocci infections, such as enterococcal infections.

However, antibiotic resistance as such cannot explain the virulence of enterococci (Foulquié Moreno et al., 2006), it can only explain their resilience upon treatment of infections. Along with antibiotic resistance, the presence of virulence-associated factors can contribute to the pathogenic potential of these microbes. All the traits that allow the different steps of the process of infection to occur contribute to the success of pathogenesis. One of the most studied phenomena associated with *E. faecalis* infections is the formation of biofilms. When in biofilms, cells are capable of resisting shear forces from blood flow and urine stream (Garsin and Willems, 2010), are more resistant to antibiotics than their planktonic counter parts and are also considered to be in a conducive location to the dissemination of genes, such as antibiotic resistance (Albesharat et al., 2011).

To fully understand the contributions of virulence factors in disease establishment and progression by enterococci, one should also take into account that host factors undoubtedly also play an important role. However, the interplay between enterococci and the human host is only poorly understood (van Schaik and Willems, 2010). Hospitalized patients may have a greater incidence of enterococcal infection not only because of the associated virulence, but because the hospital itself is a hub (Fisher and Phillips, 2009). It was previously thought that infections due to enterococci were endogenously acquired from the patient's own flora. Analysis of enterococcal infections in recent years has shown, however, that most

infecting strains appear to be exogenously acquired. These strains can come from other patients, from the hospital personnel harbouring these strains in their own GI tract, or from environmental sources in the hospital (Jin et al., 2011), but also from patients' mobile phones, patients' companions, and visitors (Tekerekoğlu et al., 2011).

However, even in persons with impaired immunoresistance, enterococci alone are often unable to cause disease. Enterococci frequently are isolated from infectious processes as part of a polymicrobial flora. Elimination of one or several partners of this polymicrobial flora other than enterococci often is enough to cure the infection (Jin et al., 2011). Apparently, in the healthy host, deficiency of one part of the immune system is counterbalanced by other components of the immune system (Sava et al., 2010).

## In human health

Despite being associated with disease as nosocomial pathogens, enterococci can have, not only a commensal relationship, but also a mutualistic one for the human host, therefore being beneficial.

The human GI tract is colonized by microorganisms from the oral cavity to the rectum, comprising nearly 800 species. Bacteria in the gut achieve the highest cell densities recorded for any ecosystem, which makes it one of the most densely populated microbial ecosystems on earth. This population evolves over time, is highly stable within one individual over time, and a unique population is found in each individual (Albesharat et al., 2011).

Microbial colonization in humans varies along the length of the gastrointestinal tract, with a low of $10^1$-$10^3$ bacteria/mL in the stomach and the duodenum, progressing to $10^4$-$10^7$ bacteria/mL in the jejunum and

ileum, and culminating in $10^{11}$-$10^{12}$ bacteria/mL in the colon (Sekirov and Finlay, 2009). Obligate anaerobes make up most of the colonic microbiota, with facultative anaerobes about ~1000-fold fewer (Sekirov and Finlay, 2009). Enterococci are members of the human GI tract consortium, comprising up to 1% of the adult microbiota (Cox and Gilmore, 2007). *E. faecalis* and *E. faecium* are the most common species in the human GI tract. The numbers of *E. faecalis* in human faeces range from $10^5$ to $10^7$ per gram, and those of *E. faecium* from $10^4$ to $10^5$ per gram (Fisher and Phillips, 2009).

The gut microbiota constitutes a complex ecosystem involved in physiologic functions critical for human life (Sanz, 2011). They provide additional metabolic capacities to their host and regulate expression of genes involved in lipid and carbohydrate metabolism, which thereby influences the nutrient supply, energy balance, body weight (Sanz, 2011), as well as achieving an immunological function. The gut microbiota is a critical stimulus for the adequate maturation of the immune system, which contributes to reducing infections and aberrant immune responses (Sanz, 2011). Intestinal microbiota contribute to many aspects of host defence against invading pathogens both through direct microbial antagonism and promotion of maturation of the intestinal immune system.

Aside from the advantageous presence of bacteria, such as enterococci, for human health, this genus can also be a health-promoting agent when specifically consumed in probiotic preparations. A probiotic can be described as a preparation or a product containing viable and defined microorganisms, which, when administered in adequate amounts, alter the microbiota by implantation or colonization, in a compartment of the host, and that exert beneficial health effects in this host (Foulquié Moreno et al., 2006; Vouloumanou et al., 2009). Some of these strains have been chosen

based on selection criteria that are believed to be important for their efficacy such as origin of strain, *in vitro* adherence to intestinal cells and survival during passage through the GI tract (Soccol et al., 2010). A group of requirements have been identified for a microorganism to be defined as an effective probiotic. These include the ability to (a) adhere to cells; (b) exclude or reduce pathogenic adherence; (c) persist and multiply; (d) produce acids, hydrogen peroxide, and bacteriocins antagonistic to pathogen growth; (e) be safe, non-invasive, noncarcinogenic, and non-pathogenic; and (f) coaggregate to form a normal balanced flora (Foulquié Moreno et al., 2006).

Strains that are used as probiotics for man have been isolated from the human GI tract and usually belong to species of the genera *Lactobacillus* and *Bifidobacterium*. However, yeasts and strains belonging to species of other lactic acid bacteria (LAB), among them enterococci, have been used in the past as probiotics too, such as *E. faecium*, *E. faecalis* (Foulquié Moreno et al., 2006; Soccol et al., 2010). Several well studied enterococcal strains, such as *E. faecium* SF 68, *E. faecium* CRL 183, *E. faecium* PR88, *E. faecium* strain ECOFLOR and *E. faecalis* strain Symbioflor 1, have been used for their hypocholesterolemic and anti-carcinogenic effects on individuals, the production of enterocins active towards *L. monocytogenes*, to alleviate the symptoms of irritable bowel syndrome and recurrent chronic sinusitis or bronchitis, and in the prevention of antibiotic-associated diarrhoea and in the treatment of diarrhoea in children (Foulquié Moreno et al., 2006; Domann et al., 2007; Ogier and Serror, 2008). The action attributed to these ingested enterococci is mutualistic just like other enterococcal strains already present in the human GI tract might also be.

At the time this thesis started, researchers were starting to realize that genes found to be associated with increased virulence potential in enterococcal clinical isolates were also present in isolates from other environments, in particular where enterococci play beneficial roles, namely food. Therefore, the quest for dissemination of virulence factors among food isolates was no longer important, but rather other issues started to become relevant. What makes a strain become virulent? Is it enough to carry virulence factors? Can food strains be virulent? Is the mere presence / detection of genes enough to predict a phenotype associated with those genes? Are the traits (phenotype and genotype) detected in a strain enough to predict the outcome of an isolate facing host defences? Is the QS molecule AI-2, a universal communication molecule, eliciting any virulence potential in *E. faecalis*, thus contributing to the complex development of polymicrobial communities where *E. faecalis* naturally inhabit? These questions were addressed in the thesis and the generated knowledge will contribute to a better safety assessment of enterococcal isolates.

# Chapter 2

## Proposal for a reliable enterococcal cytolysin production assay avoiding apparent incongruence between phenotype and genotype

This chapter contains data published in:

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

Enterococci are Gram-positive lactic acid bacteria, which have been known since the end of the 19$^{th}$ century to be associated with humans both as natural colonizers and as infectious agents. At the beginning of the 20$^{th}$ century, these bacteria were also recognized as being able to clot milk. The relevance and the importance of these bacteria are concerned mostly with two particular aspects, which have certainly generated discussion among researchers and have encouraged them to clarify the virulence mechanisms involved in the pathogenesis of infections caused by enterococci. Firstly, enterococci are human commensals that are used in the production of some fermented foods, but are also important nosocomial pathogens. Secondly, their virulence is still debated and puzzling, not only because both infectious and non-infectious strains carry the same putative virulence traits, but also because the bacterial factors mediating virulence may be diverse between different strains and species (Bourgogne et al., 2008).

The cytolysin (haemolysin/bacteriocin) system is one of the best-studied virulence traits in *Enterococcus*. It is encoded by an operon beginning with the *cylL*$_L$ gene and ending at the *cylI* gene (Figure 2.1A).

This cytolysin contributes to virulence in infection models (Clewell, 2007). It is therefore important, for enterococcal isolates from both clinical specimens and food sources, to screen for the production and genetic carriage of this virulence factor. Although several phenotypic techniques for the detection of haemolytic activity have been described (Park et al., 2007), the gold standard method for the production of haemolysin has been for a long time the presence or absence of clear zones (β-haemolysis) surrounding colonies on blood agar plates (Balashova et al., 2006). This method, however, is influenced by factors such as medium composition, the species of red blood cells used, the presence of oxygen and the duration of incubation. In fact, no single standardized method has been described.

The fact that eight described cytolysin genes are considered essential for the production of a functional cytolysin makes the screening for both divergent operons of the *cyl* locus important (Figure 2.1B). The screening for a single gene or part, or parts, of the entire cytolysin determinant will only indicate a strain's potential for production of the haemolysin/bacteriocin activity.

A thorough search in the literature revealed that not only are the phenotypic assays not performed under the same conditions, but also that, in the majority of the experiments, only a few *cyl* genes are screened for, often leading to incongruent results between the genotype and phenotype of strains (Shepard and Gilmore, 2002; Creti et al., 2004; Seno et al., 2005; McBride et al., 2007; and further references therein). Such apparent incongruence needs to be resolved in order to clearly determine whether experimental design accounts for these or whether phenotypic expression of haemolysin/bacteriocin is lacking where the genotypic potential for cytolysin production exists. We believe that unambiguous procedures for

both the genetic and the phenotypic screening of enterococcal isolates are essential and should be the starting point for clarifying the apparent incongruence suggested by the literature.

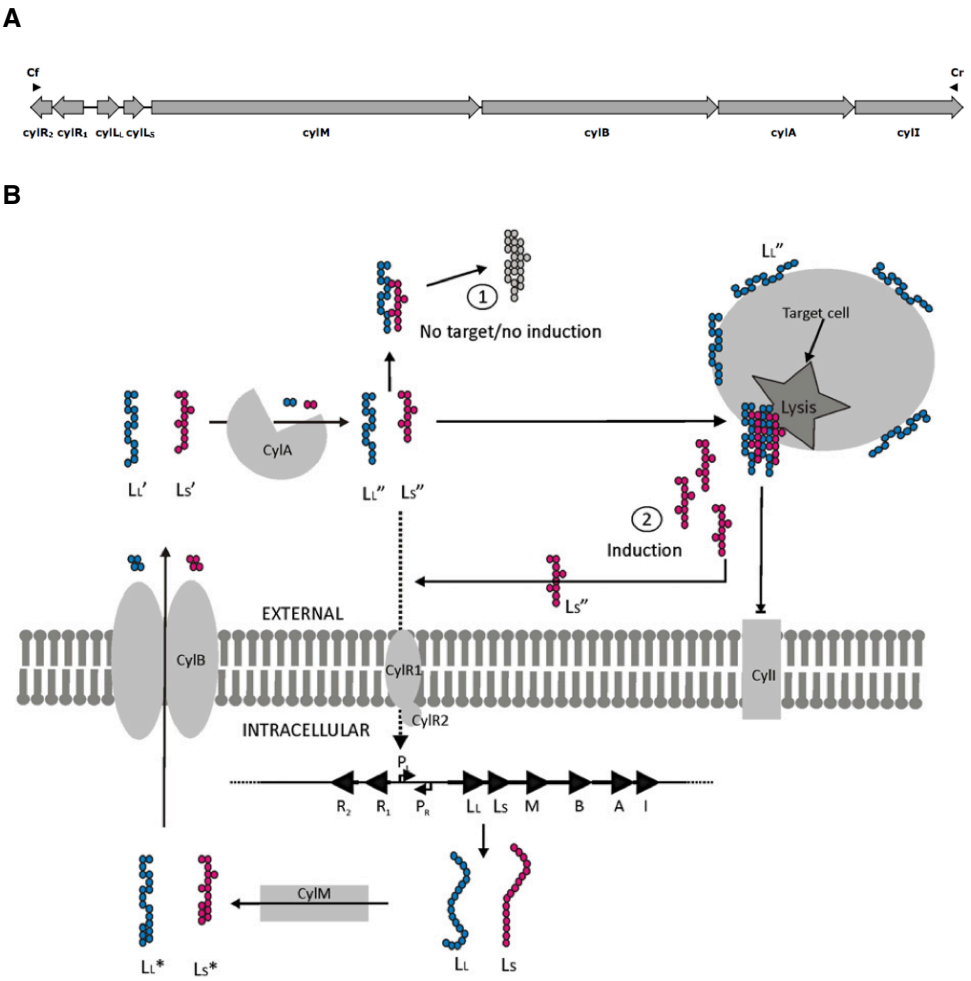


**Figure 2.1.** (**A**) Organization of the *cyl* locus and location of the primers Cf and Cr used for the PCR. (**B**) The *E. faecalis* cytolysin senses and destroys target cells (Roux et al., 2009). The *cylL*$_L$ and *cylL*$_S$ genes encode precursors of two small lantibiotic-like peptides, which are subsequently modified by the *cylM* gene product. The *cylB* gene product is an ATP-binding cassette transporter that facilitates the transport of the peptides out of the cell and is involved in additional processing.

The *cylA* gene product extracellularly processes the peptides and activates them, allowing them to combine to produce the cytolysin. The mechanism signalling the upregulation of the operon involves two regulator proteins corresponding to the *cylR1* and *cylR2* gene products from a divergently transcribed operon. An immunity function is present and is provided by a membrane protein encoded by the *cylI* gene (Clewell, 2007). Cytolysin expression is regulated by $CylL_S$'' through a quorum sensing-like mechanism. In the absence of target cells, the mature subunit $CylL_L$'' interacts with $CylL_S$'' to form an inactive oligomeric complex (1) that is not able to induce expression of the cytolysin operon. In the presence of target cells, $CylL_L$'' binds them preferentially, creating a transient pool of free $CylL_S$'' (2) to induce high- level cytolysin expression prior to the slow reaction of $CylL_S$'' joining the pore complex (Roux et al., 2009).

Knowing that cytolysin is produced by a complex process that requires the products of eight genes, a primer pair was designed (Figure 2.1A) based on the sequence of the cytolysin locus of *Enterococcus faecalis* strain MMH594 from the *cylR2* gene to the *cylI* gene (GenBank accession no. AY032999). In this manner, the two divergent operons of the cytolysin locus were amplified, enabling a genotypic screening that would represent the complete *cyl* locus (Cf, GGT TGC CAT TGA AAA ATA TCT TCT AGT GGA GTA TCC AGG, from bp 194 to 156 of the *cylR2* gene; Cr, GTG ATT GAT TGG CTT ATT TCA TCA TCA TCA CTT TTG AGC, from bp 902 to 863 of the *cylI* gene). The PCR was performed using an Expand Long Template PCR system (Roche) and the conditions recommended by the manufacturer. The thermal cycling parameters for the 8364 bp PCR product consisted of one cycle at 94 ºC for 2 min and 10 cycles at 94 ºC for 10 s, 50 ºC for 30 s, and 68 ºC for 10 min. This was followed by 20 cycles at 94 ºC for 10 s, 50 ºC for 30 s, and 68 ºC for 10 min (with the elongation time increasing by 10 s per cycle) and a final cycle at 68 ºC for 7 min. The

PCR screening for the complete *cyl* locus was performed on 55 strains from nosocomial infections and from food (milk and cheese): 36 *E. faecalis*, 7 *Enterococcus faecium*, 4 *Enterococcus hirae* and 8 *Enterococcus durans*.

The phenotypic screening of the enterococcal strains for cytolysin production was performed on Columbia blood agar supplemented with 5 % (v/v) horse blood (Becton Dickinson) and incubated at 37 ºC for 24 or 48 h, under anaerobic conditions (GasPak EZ Gas Generating Container Systems; Becton Dickinson).

The results are presented in Table 2.1. No differences in haemolytic activity were seen between the durations of incubation (24 and 48 h) at 37 ºC. All strains were also tested on BHI agar supplemented with 5 % (v/v) human blood, incubated at 37 ºC for 24 or 48 h under identical anaerobic conditions. No differences were seen between the results obtained for blood plates with human or horse erythrocytes. Nonetheless, the haemolytic clearing zones were more evident on horse blood (data not shown), making horse erythrocytes more convenient for the haemolysin assay and allowing an assay with a higher sensitivity. All 55 strains tested showed complete agreement between genotypic and phenotypic assays. This correlation was observed for the four enterococcal species tested, namely *E. faecalis*, *E. faecium*, *E. durans* and *E. hirae*.

Based on our genotypic and phenotypic screening methodology, a much higher correlation was obtained compared to any previously described screening (Shepard and Gilmore, 2002; Creti et al., 2004; Seno et al., 2005; McBride et al., 2007; and further references therein). The PCR screening for the complete *cyl* locus described herein gives a measure of the gene reservoir of the strain, while the phenotypic assay is still the only test that allows demonstration of active cytolysin production. As environmental factors could strongly influence gene expression (Finlay and

Falkow, 1997), it is necessary to take into account that the in vitro conditions used to test for phenotypic characters are different from those found *in vivo* in the human host. For these reasons, enterococci should be evaluated not only for the *in vitro* expression of virulence traits, but also using molecular assays for evidence of silent genes that could potentially be activated, thereby changing these bacteria into potential pathogens or augmenting their pathogenicity (Sabia et al., 2008). In the case of negative phenotype results, gene products of another strain or species could complement the lack of expression of an inactive gene. In conclusion, the incongruence previously evidenced between phenotypic and genotypic assays for the haemolysin/bacteriocin of enterococcal species is probably due to the experimental design rather than being attributable to any particular genetic phenomenon.

**Table 2.1.** Results from the genetic and phenotypic screening of the cytolysin system.

| Species | Total no. of strains | Origin (clinical / food) | PCR* | Haemolysis† |
|---|---|---|---|---|
| ***E. faecalis*** | 24 | 20/4 | + | β |
| | 12 | 0/12 | - | γ |
| ***E. faecium*** | 1 | 0/1 | + | β |
| | 6 | 2/4 | - | γ |
| ***E. durans*** | 1 | 0/1 | + | β |
| | 7 | 0/7 | - | γ |
| ***E. hirae*** | 4 | 0/4 | - | γ |

* +/–, The complete *cyl* operon was/was not detected by PCR.

† γ represents the absence of haemolysis; β represents the presence of haemolysis.

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# Chapter 3

## Virulence of *Enterococcus faecalis* dairy strains in an insect model: the role of *fsrB* and *gelE*

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Different researchers contributed to this study. Specifically, the author was involved in the study design, in the gelatinase activity assay and in the mutant construction; N. Teixeira and P. Marujo, from Fátima Lopes Laboratory, did the sequence analysis of the *fsr–gelE* region of *Enterococcus faecium* QSE32; Christophe Buisson helped setting up the *Galleria mellonella* virulence assay at UBLO, L. Rigottier-Gois and C. Nielsen-LeRoux, from Pascale Serror Laboratory, performed the *G. mellonella* mortality assay.

## Abstract

Despite the existence of various virulence factors in the *Enterococcus* genus, enterococcal virulence is still a debated issue. A main consideration is the detection of the same virulence genes in strains isolated from nosocomial or community-acquired infections, and from food products. The goal of this study was to evaluate the roles of two well-characterized enterococcal virulence factors, Fsr and gelatinase, in the potential virulence of *Enterococcus faecalis* food strains. Virulence of unrelated *Enterococcus* isolates, including dairy strains carrying *fsr* and *gelE* operons, was compared in the *Galleria mellonella* insect model. *E. faecalis* dairy strains were able to kill larvae and were as virulent as strain OG1RF, one of the most widely used for virulence studies. In contrast, *Enterococcus durans* and *Enterococcus faecium* strains were avirulent or poorly virulent for *G. mellonella*. To evaluate the role of *fsrB* and *gelE* in virulence of *E. faecalis* dairy strains, both genes were deleted independently in two strains. The Δ*fsrB* and Δ*gelE* deletion mutants both produced a gelatinase-negative phenotype. Although both mutations

significantly attenuated virulence in *G. mellonella*, the Δ*fsrB* strains were more strongly attenuated. These results agree with previous findings suggesting the involvement of *fsrB* in the control of other cell functions relevant to virulence. Our work demonstrates that the presence of functional *fsrB*, and to a lesser extent *gelE*, in dairy enterococci should be considered with caution.

## Introduction

*Enterococcus* is a peculiar and controversial genus of Gram-positive lactic acid bacteria. It includes commensal species that inhabit the gastrointestinal tracts of humans and animals, which can be used as starter cultures in food fermentations, as animal health supplements and/or as probiotics. However, they are also capable of causing opportunistic infections, including bacteraemia, endocarditis, meningitis, and wound, urinary tract and nosocomial bloodstream infections. Owing to their robustness, these bacteria are able to contaminate and maintain viability in diverse environments such as soil, sand, water, plants and food (Mundt, 1986). In the particular case of fermented food products, enterococci belong to the non-starter flora, although they have been proposed as starters for the production of certain cheeses and other fermented milk products (Aarestrup et al., 2002). They are of particular relevance for traditionally made cheeses in south European countries, where they play an important role in the ripening process, through proteolysis, lipolysis and citrate metabolism, therefore contributing to the organoleptic characteristics of the product.

*Enterococcus faecalis* is the predominant species in human/ animal-associated environments, and therefore the most studied species of this

genus (Tannock and Cook, 2002). *E. faecalis*, together with *Enterococcus faecium*, are also the most frequently found species in dairy food products, with varying prevalence as a function of the country and cheese type (Aarestrup et al., 2002). *E. faecalis* is also responsible for up to 80 % of enterococcal-associated nosocomial infections, followed by *E. faecium*. One explanation for the over-representation of *E. faecalis* among clinical isolates may relate to its natural abundance, or to the presence of virulence factors (Hancock and Gilmore, 2000). Although harmless in healthy individuals, enterococcal clinical isolates become pathogenic in patients in intensive care units and in hospitalized patients with impaired immune systems. This has been associated with a variety of virulence factors carried by *E. faecalis* (Ogier and Serror, 2008).

One of the most studied *E. faecalis* virulence factors is gelatinase, a metalloprotease able to degrade several substrates, such as gelatine, casein, haemoglobin and other bioactive peptides, including *E. faecalis* sex pheromones (Kayaoglu and Ørstavik, 2004). Gelatinase is encoded by *gelE*, which is in an operon with *sprE*, which encodes a serine protease (Qin et al., 2000). Its phenotypic expression requires the regulatory system encoded by the *fsrABC* operon (Qin et al., 2000; Lopes et al., 2006), recently renamed *fsrABDC* (Nakayama et al., 2006). *fsr* is homologous to the *agr* quorum sensing system of *Staphylococcus aureus*, and has also been implicated in virulence of *E. faecalis* independently of *gelE* (Bourgogne et al., 2006). The role of *gelE* and *fsr* loci in *E. faecalis* virulence has been demonstrated in different mammalian infection models (Mohamed and Murray, 2006), in the *Caenorhabditis elegans* nematode model (Sifri et al., 2002) and in the *Arabidopsis thaliana* plant model (Jha et al., 2005). More recently, gelatinase has been implicated in evasion of the immune system of the insect *Galleria mellonella*, suggesting that gelatinase

may participate in virulence in this system (Park et al., 2007). Screening of both *gelE* and *fsr* in *E. faecalis* isolates from different origins revealed their dissemination in fermented milk products (Semedo et al., 2003; Lepage et al., 2006; Lopes et al., 2006). However, the roles for Fsr and gelatinase in the potential virulence of *E. faecalis* food strains have never been established. In the present study, two gelatinase-positive *E. faecalis* dairy strains carrying *gelE* and the complete *fsr* operon, and their respective isogenic *gelE* and *fsrB* deletion mutants, were tested for virulence in the *G. mellonella* infection model.

## Materials and Methods

### Bacterial strains, plasmids, and culture conditions

Bacterial strains used in this study are listed in Table 3.1. Enterococci were grown in M17 broth supplemented with 0.5 % (w/v) glucose (M17BGlu) or M17 agar supplemented with 0.5 % glucose (M17AGlu) at 37 ºC without aeration. *Escherichia coli* strains were grown aerobically in Luria–Bertani broth or on LB agar at 37 ºC. Antibiotics were used at the following concentrations: erythromycin, 30 mg/mL for *E. faecalis* and 150 mg/mL for *E. coli*; ampicillin, 80 mg/mL.

### General DNA techniques

General molecular biology techniques were performed by standard methods (Sambrook et al., 1989). Restriction enzymes, polymerases and T4 DNA ligase were used according to manufacturers' instructions. PCR amplification was performed using a Biometra or Eppendorf thermocycler. When necessary, PCR products and DNA restriction fragments were

purified with QIAquick purification kits (Qiagen) or Montage Life Science kits (Millipore). Plasmids were purified using the QIAprep Spin Miniprep kit (Qiagen). Electrotransformation of *E. coli* and *E. faecalis* was carried out as described by Dower et al. (Dower et al., 1988) and Dunny et al. (Dunny et al., 1991), using a Gene Pulser apparatus (Bio-Rad). Plasmid inserts were sequenced at Baseclear.

**Table 3.1.** Strains used in this study

| Strains | Relevant characteristics | Reference or source |
|---|---|---|
| ***E. coli*** | | |
| **DH5α** | $F^-$ Φ80d*lacZ*ΔM15 Δ(l*acZYA-argF*)U169 *deoR recA1 endA1 hsdR17*($r_K^-$ $m_K^+$) *phoA supE44* $\lambda^-$ *thi-1 gyrA96 relA1* | (Grant et al., 1990) |
| **TG1 *repA*** | *supE hsdD5 thi* (Δ*lac-proAB*) $F^-$ (*traD36 proAB-lacZ*ΔM15) *repA* | (Law et al., 1995) |
| ***E. faecalis*** | | |
| **OG1RF** | $Fus^r$ $Rif^r$, plasmid free wild-type strain, $Gel^+$ | (Dunny et al., 1978) |
| **QA29b** | Wild-type *E. faecalis*; isolated from cheese, $Gel^+$ | This study |
| **LSE4a** | Wild-type *E. faecalis*; isolated from milk, $Gel^+$ | This study |
| **LN68** | Wild-type *E. faecalis*; isolated from milk, $Gel^-$ | (Lopes et al., 2006) |
| **TX5264** | *gelE* in-frame deletion in OG1RF, $Gel^-$ | (Sifri et al., 2002) |
| **TX5266** | *fsrB* in-frame deletion in OG1RF, $Gel^-$ | (Qin et al., 2001) |
| **QA29bΔ*fsrB*** | *fsrB* in-frame deletion in QA29b, $Gel^-$ | This study |
| **QA29bΔ*gelE*** | *gelE* in-frame deletion in QA29b, $Gel^-$ | This study |
| **LSE4aΔ*fsrB*** | *fsrB* in-frame deletion in LS4E, $Gel^-$ | This study |
| **LSE4aΔ*gelE*** | *gelE* in-frame deletion in LS4E, $Gel^-$ | This study |
| ***E. faecium*** | | |
| **QSE32** | Wild-type; isolated from cheese, $Gel^+$ | (Alves et al., 2004) |
| ***E. durans*** | | |
| **QN1** | Wild-type; isolated from milk, $Gel^-$ | (Ribeiro et al., 2007) |

## Construction of in-frame *gelE* and *fsrB* deletion mutants of strains QA29b and LSE4a

Markerless *gelE* and *fsrB* deletion mutants of *E. faecalis* were constructed essentially as described by Brinster et al. (Brinster, Furlan, and

Serror, 2007). Briefly, 5' and 3' flanking regions of *fsrB* and *gelE* were amplified from chromosomal DNA of each strain by PCR with primers OEF-232, OEF-233, OEF-234 and OEF-235, and OEF- 236, OEF-237, OEF-238 and OEF-239, respectively (Table 3.2). The two cognate PCR fragments were fused by PCR using the external primers OEF-232 and OEF-235, and OEF-236 and OEF-239 for *fsrB* and *gelE*, respectively, and the resulting product was cloned into pGEM-T (Promega). The inserted PCR fragment was removed from its cloning vector by restriction enzymes and subsequently cloned into pG+host9 plasmid (Maguin et al., 1996), which was then electroporated into *E. faecalis*. The *fsrB* and *gelE* single- and double- crossover mutants were selected as described by Brinster et al. (Brinster, Furlan, and Serror, 2007). Successful targeted mutations of *fsrB* and *gelE* in strains LSE4a and QA29b were first identified by PCR screening and were confirmed by Southern blot analysis.

## Gelatinase activity assay

The phenotypic assay of gelatinase activity was performed as described by Lopes et al. (Lopes et al., 2006). Briefly, *Enterococcus* strains were grown on agar plates containing 3 % (w/v) gelatine (Oxoid) and flooded with a saturated solution of ammonium sulphate (Merck). A transparent halo around cells indicated gelatinase activity.

**Table 3.2.** Primers used in this study

| Primer name | Sequence (5'-3')* | Position of the primer 5' end | Reference |
|---|---|---|---|
| **OEF-232** | GAAAGGGATGAGTGAACAAATG | bp 839 upstream of the *fsrB* start codon | (Sifri et al., 2002) |
| **OEF-233** | CTTTGTCCATTGTGTTTTTCCTG | bp 78 in *fsrB* | (Sifri et al., 2002) |
| **OEF-234** | CAGGAAAAACACAATGGACAAAGT GGATGGGACAAACTGAAAAACC | bp 54 before the *fsrB* stop codon | This study |
| **OEF-235** | TAGCCAACAAACGAATCACAACC | bp 1020 after the | (Sifri et al., |

| | | | |
|---|---|---|---|
| | | *fsrB* stop codon | 2002) |
| **OEF-236** | CTAAAAGTGATTGTTGATGTGC | bp 639 upstream of the *gelE* start codon | (Qin et al., 2001) |
| **OEF-237** | ATCAACAGTAACGCCTTCC | bp 258 in *gelE* | (Qin et al., 2001) |
| **OEF-238** | GGAAGGCGTTACTGTTGATATTCAGGTAAACCAACCAAGTG | bp 45 upstream of the *gelE* stop codon | This study |
| **OEF-239** | GATTATTTGCCTTCTTTTCAGC | bp 1047 after *gelE* stop codon | (Qin et al., 2001) |
| **sprE_2** | TCAAACAAACGAAACTGGC | 1 761 697† | This study |
| **sprE39** | TGCCTTCTTTTCAGC | 1 761 921 | This study |
| **sprE_3** | CATTCTTAAAACTTTCAGCCAC | 1 762 591 | This study |
| **gelE39** | CAAGCTAAAACCGGC | 1 762 832 | This study |
| **msprE** | TAACTTTGATCGCCGG | 1 762 858 | This study |
| **gelE** | TCATTCATTGACCAG | 1 762 961 | This study |
| **sprE59** | TGAATCTGTTCTGGTC | 1 762 986 | This study |
| **msprE_3** | AAAGTTTGAATCTTCAGATACCC | 1 763 120 | This study |
| **msprE_2** | AAAGTTTGAATCTTCAGATACCC | 1 763 120 | This study |
| **gelE_3** | CAACACTCTGAGTATCCGCACC | 1 763 362 | This study |
| **mgelE_2** | AACGGATAACACAGGGG | 1 763 829 | This study |
| **gelE_2** | GTGTAAAGCAATTCCCG | 1 763 831 | This study |
| **mgelE_3** | TCTTCGCCAACTGGTGACC | 1 764 008 | This study |
| **fsrC39** | TTGCTTTATCCTCCC | 1 764 545 | This study |
| **mgelE** | AATATTTACGCAGGG | 1 764 605 | This study |
| **gelE59** | TTGAGTTATGAGGGG | 1 764 700 | This study |
| **fsrC_3** | TTTCTTTTTACATATAACAATCCCC | 1 764 840 | This study |
| **mfsrC_2** | TGAAGAAACGATTGCACCAACC | 1 765 263 | This study |
| **fsrC_2** | TTTATAATCATGACGAAACATCGC | 1 765 328 | This study |
| **mfsrC_3** | TTTTTGTGTTTTTGATTTCGCC | 1 765 573 | This study |
| **fsrB39** | AACATTAATGCCGC | 1 765 917 | This study |
| **mfsrC** | TTTGTTCGTTTGCGGC | 1 765 942 | This study |
| **fsrB** | GTCCAAATATATTGGGC | 1 766 115 | This study |
| **fsrC59** | GACAATGGATGGGAC | 1 766 118 | This study |
| **fsrA3I** | TTCGCTTAACGTCCC | 1 766 635 | This study |
| **mfsrB** | TTGAAGAGGAGGGCG | 1 766 814 | This study |
| **fsrB59** | TTACTTAGGGAGGG | 1 766 893 | This study |
| **fsrA_2** | CTAGGAAAAAGATATTTAGTTGGGC | 1 767 442 | This study |
| **mfsrA** | ATGAGTGAACAAATGGC | 1 767 638 | This study |
| **fsrA59** | GTTTTTGTTTGCAGG | 1 767 788 | This study |
| **mfsrA_2** | CAAGAACAGTTTGGCGGTTG | 1 767 908 | This study |

*Sequences added for fusion PCR are underlined.
†Position of the primer 5' end in the V583 genome.

## *G. mellonella* mortality assay

*G. mellonella* eggs were hatched at 25 ºC, and the larvae were reared on bee's wax and pollen (Naturalim) until the last instars which were

used for the infection experiments. *Enterococcus* strains were grown in M17BGlu and collected by centrifugation 1 h after they had reached stationary phase. Bacterial cells were washed with 0.9 % saline solution and stored as a dry, frozen pellet at -80 ºC. Before inoculation, the frozen bacterial pellet was suspended in 1 mL saline solution and serial dilutions were plated on M17AGlu plates in order to determine the bacterial count of the pellet.

Groups of 20–30 *G. mellonella* larvae, starved for 24 h and weighing about 200 mg, were injected at the base of the last proleg with 10 mL each bacterial inoculum (~$2 \times 10^8$ cells/mL) using a microinjector (KDS 100; KD Scientific) with a 1 mL syringe and 0.45 × 12 mm needles (Terumo). A control group of larvae received saline solution only. The size of the inoculum was confirmed by determining the number of CFU on M17AGlu. Five infected larvae were kept per Petri dish, without food, at 37 ºC and survival was monitored every 24 h for 2–5 days, depending on the experiment. Experiments were repeated at least three times. The mortality rate was compared using a two-tailed unpaired t-test at the 95 % confidence interval. Survival curves were constructed by the Kaplan–Meier method and compared by log-rank analysis (GraphPad Prism, version 4.0; GraphPad Software). *P*-values of $< 0.05$ were considered statistically significant.

## Sequence analysis of the *fsr–gelE* region of *E. faecium* QSE32

PCR amplification of overlapping fragments of the *fsr–gelE* region was carried out using Expand High Fidelity DNA polymerase (Roche) and the primers indicated in Table 3.2. Sequencing was performed at Baseclear. The sequence was analysed using Vector NTI 10.3.0 (Invitrogen) and the final DNA sequence, and the deduced protein

sequence were analysed using Vector NTI 10.3.0 (Invitrogen) and BLAST from the NCBI website (http://blast.ncbi.nlm.nih.gov/).

## Results

### Killing of *G. mellonella* by isolates of *E. faecalis* and other enterococcal strains

Increasing interest in using *G. mellonella* as a surrogate model to study virulence of various microorganisms led us to compare virulence of enterococcal strains. We determined whether strains of enterococci other than *E. faecalis* GM could kill *G. mellonella* (Park et al., 2007). Mortality rates at 48 h post-infection are presented in Figure 3.1. All *E. faecalis* strains tested (LN68, V583, LSE4a, OG1RF and QA29b) were able to kill between 60 and 98 % of *G. mellonella* larvae with inocula of about $2 \times 10^6$ CFU. The most virulent *E. faecalis* strains for *G. mellonella* were the clinical strain V583 and milk isolates LN68 and LSE4a. Maximum killing by LN68 was reached within 24 h (data not shown), indicating that this isolate was more rapidly lethal for *G. mellonella*. The clinical strain OG1RF and the cheese isolate QA29b were significantly less virulent with ~72 and ~60 % killing after 48 h, respectively. Interestingly, *E. durans* strain QN1 and *E. faecium* strain QSE32 did not kill *G. mellonella* larvae significantly. These data show that *E. faecalis* strains from both clinical and food origin have the ability to kill *G. mellonella* larvae, whereas *E. durans* and *E. faecium* species seem to be avirulent or of low virulence for *G. mellonella* under the conditions used.

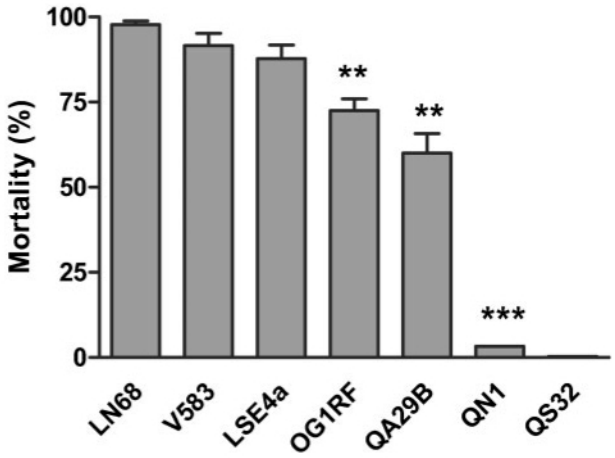


**Figure 3.1.** Killing of *G. mellonella* larvae by various enterococci isolates. Percentage mortality of *G. mellonella* larvae at 48 h post-infection with *E. faecalis* strains LN68 ($2.07 \times 10^6 \pm 0.5 \times 10^6$ CFU), V583 ($1.67 \times 10^6 \pm 0.06 \times 10^6$ CFU), LSE4a ($2.45 \times 10^6 \pm 0.13 \times 10^6$ CFU), OG1RF ($2.0 \times 10^6 \pm 0.2 \times 10^6$ CFU) and QA29b ($2.07 \times 10^6 \pm 0.11 \times 10^6$ CFU), *E. durans* strain QN1 ($1.94 \times 10^6 \pm 0.19 \times 10^6$ CFU) and *E. faecium* strain QSE32 ($1.8 \times 10^6 \pm 0.1 \times 10^6$ CFU). Larvae were infected with $\sim 2 \times 10^6$ bacteria as indicated in parentheses. Data were obtained from three independent experiments and are expressed as the mean values ± SD. Asterisks indicate a significant difference (** $P < 0.005$, *** $P < 0.0005$) relative to the LN68 strain.

## Gelatinase and Fsr affect *E. faecalis* virulence in *G. mellonella*

We asked whether the *G. mellonella* model could discriminate between the roles of *gelE* and *fsrB* in virulence, using OG1RF and isogenic strains TX5264 and TX5266 (kindly provided by B. Murray) deleted for *gelE* and *fsrB*, respectively. Larvae were infected with $\sim 2 \times 10^6$ CFU OG1RF, TX5264 and TX5266. As shown in Figure 3.2, the three strains have similar killing rates of *G. mellonella* at 48 h. However, killing was significantly

delayed for larvae injected with the Δ*gelE* strain (TX5264); 50 % of the larvae were dead after 24 h, compared to 80 % of those injected with OG1RF or TX5266 ($P < 0.05$). This result indicates that *gelE*, but not *fsrB*, contributes to OG1RF virulence in the *G. mellonella* infection model.

To study the role of *gelE* and *fsrB* in the potential virulence of *E. faecalis* food isolates, we collected strains QA29b and LSE4a in two distant areas of cheese production in Portugal (Semedo et al., 2003; Lopes et al., 2006). According to PFGE and MLST analysis, these two isolates are not genetically related (data not shown). Besides the *fsrABDC* and *gelE-sprE* operons, both strains carried the virulence genes *agg*, *esp* and *efaAfs*, none of which was haemolytic (results not shown). We successfully generated independent in-frame *gelE* and *fsrB* deletion mutants by allelic exchange in *E. faecalis* QA29b and LSE4a, as described by B. Murray and co-workers (Qin et al., 2001; Sifri et al., 2002). As expected, the resulting Δ*fsrB* and Δ*gelE* deletion mutants failed to produce detectable gelatinase in a standard plate assay (data not shown). Next, their virulence was examined using the *G. mellonella* infection model. As for OG1RF, inactivation of the *gelE* gene in the two strains significantly decreased killing of *G. mellonella* larvae; 65 and 32 % of larvae infected with the *gelE* mutants were dead after 48 h, compared to 88 and 60 % of those infected with LSE4a and QA29b, respectively ($P < 0.05$). Moreover, inactivation of *fsrB* in LSE4a and QA29b clearly reduced virulence compared to the isogenic wild-type (WT) strains (Figure 3.3). After 48 h post-infection, the QA29b Δ*fsrB* mutant killed 15 % of the *G. mellonella* larvae, versus 60 % killing by the isogenic WT strain. Thus, in contrast to results in OG1RF, *fsrB* appears to be implicated in infection by certain *E. faecalis* isolates.

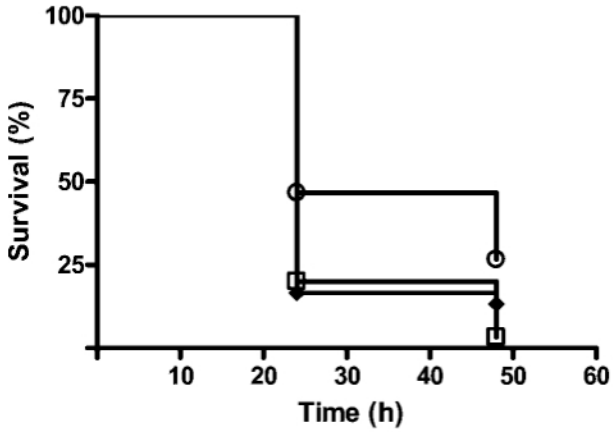


**Figure 3.2.** Role of *E. faecalis* gelatinase in killing of *G. mellonella* larvae. Survival of *G. mellonella* after injection of *E. faecalis* strains OG1RF ($2.1 \times 10^6$ CFU per larva), Δ*gelE* (TX5264, $1.89 \times 10^6$ CFU per larva) and Δ*fsrB* (TX5266, $1.71 \times 10^6$ CFU per larva). Larva infection doses are indicated in parentheses. ◆, OG1RF; ○, Δ*gelE*; □, Δ*fsrB*. One representative experiment of three independent experiments is shown.

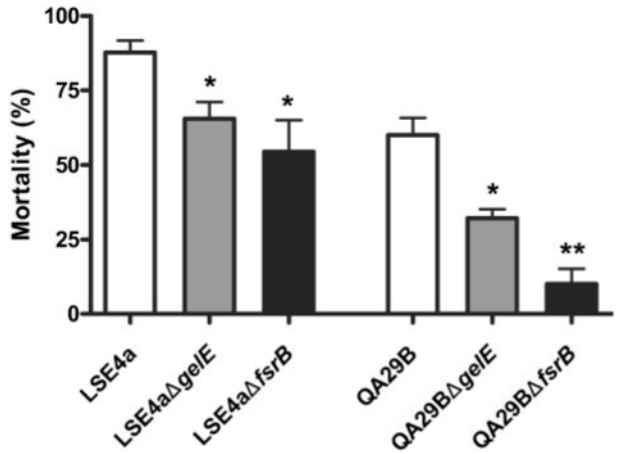


**Figure 3.3.** Effects of *gelE* and *fsrB* inactivation on killing of *G. mellonella*. Percentage mortality of *G. mellonella* larvae at 48 h post-infection with *E. faecalis* strains LSE4a ($2.45 \times 10^6 \pm 0.14 \times 10^6$ CFU), LSE4aΔ*gelE*

($1.91 \times 10^6 \pm 0.02 \times 10^6$ CFU) and LSE4aΔf*srB* ($1.94 \times 10^6 \pm 0.49 \times 10^6$ CFU), QA29b ($2.07 \times 10^6 \pm 0.11 \times 10^6$ CFU), QA29bΔ*gelE* ($2.32 \times 10^6 \pm 0.42 \times 10^6$ CFU) and QA29bΔ*fsrB* ($2.32 \times 10^6 \pm 0.29 \times 10^6$ CFU). Larvae were infected with ~$2 \times 10^6$ bacteria as indicated in parentheses. Data were obtained from three independent experiments and are expressed as mean values ± SD. Asterisks indicate a significant difference (* $P < 0.05$, ** $P < 0.005$) relative to the wild-type strain.

### Sequence analysis of the *E. faecium* QSE32 *fsr–gelE* region

To check whether the low virulence of the *E. faecium* QS32 strain in *G. mellonella* could result from the absence of a functional Fsr system, we sequenced the entire *fsr–gelE* region of this strain. The gene organization was identical to *E. faecalis* V583, and the sequence shared 98 % identity at both the nucleotide and amino acid sequence level with *gelE* of *E. faecalis* V583 (data not shown). Therefore, we concluded that the Fsr system of *E. faecium* QS32 is most probably functional and that *gelE* conferred gelatinase activity. The low virulence of *E. faecium* QS32 seems to be independent of the functionality of the *fsr–gelE* region.

## Discussion

Simple invertebrates, the nematode *C. elegans* and insects *Drosophila melanogaster* and *G. mellonella* have recently attracted interest as models for screening of virulence factors of pathogenic microbes, including *E. faecalis*, or for elucidating their effects in the host (Maadani et al., 2007; Park et al., 2007; Schneider et al., 2007). Although adaptive immunity is unique to vertebrates, the innate immune response seems to

be well conserved between vertebrates and invertebrates. In contrast to the nematode, which lacks the cellular immune response involved in phagocytosis of bacteria (Fares and Greenwald, 2001), insects have both cellular and humoral responses, making them attractive models to study bacteria–host interactions (Vallet-Gely et al., 2008). Although the *G. mellonella* infection model has been efficiently used to identify virulence factors in Gram-negative bacteria and in fungi (Xu et al., 1991; Cotter et al., 2000; Brennan et al., 2002; Choi et al., 2002; Mylonakis et al., 2005; Schell et al., 2008), this model has been mainly used to characterize virulence factors in *Bacillus cereus* and *Bacillus thuringiensis*. These bacteria are virulent to *G. mellonella* by oral infection (Salamitou et al., 2000; Fedhila et al., 2006) and by injection into the haemocoel (Bouillaut et al., 2005). A recent study demonstrated killing of *G. mellonella* larvae by at least one *E. faecalis* strain isolated from larval cadavers (Park et al., 2007). In the present study, we used the *G. mellonella* model to compare virulence of several *Enterococcus* strains from food and clinical origins. Using this simple infection model, we demonstrated the efficacy of the *G. mellonella* model, and establish for the first time that gelatinase and Fsr virulence factors of *E. faecalis* contribute to the virulence of food isolates.

*E. durans* and *E. faecium* strains were relatively avirulent or of low virulence, killing only 0–3 % of larvae, whereas *E. faecalis* strains had killing rates of 60–98 %. Although we used different strains of *E. faecalis* and *E. faecium* in this study, our results with *G. mellonella* correlate well with the *C. elegans* model, for which *E. faecium* strains were less virulent than *E. faecalis* strains (Garsin et al., 2001).

Attenuated virulence of *gelE*-deficient *E. faecalis* strains in the *G. mellonella* infection model is consistent with results in mammal and nematode models (Qin et al., 2000; Sifri et al., 2002; Engelbert et al., 2004).

This result correlates with the recent finding that gelatinase degrades cecropin, an antimicrobial peptide important for the immune system in the haemolymph of larvae of *G. mellonella* (Park et al., 2007).

Since inactivation of *gelE* does not abolish *E. faecalis* virulence, other factors might be involved in *G. mellonella* virulence. Although SprE protease is still expressed in *gelE*-inactivated strains, it does not seem to have insecticidal activity (Park et al., 2007). Based on the high genome diversity between *E. faecalis* isolates (Lepage et al., 2006; Bourgogne et al., 2008), it is likely that the strains used in this study carry other factors involved in virulence in *G. mellonella* or differentially express them. Interestingly, the OG1RF genome encodes a putative extracellular protease (OG1RF_0194), which remains to be characterized (Bourgogne et al., 2008).

The major regulatory system of gelatinase, the *fsr* operon, is among the most probable candidates to be involved in virulence in *G. mellonella*. While no significant difference in virulence was observed between larvae inoculated with the OG1RF and the *fsrB* isogenic mutant, we found that *fsrB* contributes to virulence in *G. mellonella* in the two food isolates studied. The result with OG1RFΔ*fsrB* is in agreement with a previous study in which *fsrB* inactivation in OG1RF did not modify virulence in a rat endocarditis model (Singh et al., 2005). The authors proposed that residual production of gelatinase was sufficient to allow induction of endocarditis. In this case, however, residual gelatinase production would have had to be strongly induced *in vivo*, especially as virulence was evaluated at just 24 h post-infection. As proposed above, *fsrB* may directly or indirectly regulate other factors important for virulence in OG1RF. Since inactivation of *fsrB* in LSE4a and QA29b food isolates appears to be more relevant for virulence, we propose that OG1RF has additional factors that contribute to its

pathogenic potential, as suggested by its intrinsic virulence in a mouse peritonitis model (Bourgogne et al., 2008), or that these food isolates lack factors important for virulence in such a model. Taken together, present results and previous work suggest that the genetic background of the strain may be as important as the infection or host model used to assess virulence. Singh et al. (Singh et al., 2005) used an endocarditis model to demonstrate that a *gelE sprE* double mutant significantly decreased the evolution of endocarditis compared to infection by WT OG1RF; the *fsrB* mutant was not significantly attenuated compared to wild-type OG1RF. Using different models, i.e. rabbit endophthalmitis, mouse peritonitis, *C. elegans* and *G. mellonella* (this study), the *fsrB* mutant strain of OG1RF was more attenuated than the double protease mutant (Sifri et al., 2002; Engelbert et al., 2004). An important conclusion of this work is that the role of virulence genes studied in one model cannot always be extrapolated to other models.

Transfer of virulence or antibiotic resistance genes has been observed *in vitro* (Eaton and Gasson, 2001) and *in vivo* (Mater et al., 2005; Lester et al., 2006). Although our finding does not preclude that enterococcal food isolates will be virulent in other infection or animal models or pathogenic for humans, it indicates the *in vivo* activity of the gelatinase and Fsr present in food isolates, and calls for surveillance in monitoring virulence traits in food strains which represent a potential reservoir for virulence genes.

In summary, we used the *G. mellonella* infection model as a surrogate virulence model to compare virulence of three *Enterococcus* species, and *E. faecalis* isolates and isogenic deletion mutants. Our data show that *fsrB* and to a lesser extent *gelE* significantly contribute to the

virulence of *E. faecalis* food isolates. This simple animal model may provide insights for risk assessment of food isolates.

## Acknowledgments

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The GenBank/EMBL/DDBJ accession number for the *fsr–gelE* region sequence of *E. faecium* QS32 is FJ858146.

# Chapter 4

## Incongruence between the *cps* type 2 genotype and host-related phenotypes of an *Enterococcus faecalis* food isolate

Submitted for publication to the International Journal of Food Microbiology and in revision:

Incongruence between the *cps* type 2 genotype and host-related phenotypes of an *Enterococcus faecalis* food isolate

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The author was involved in the design and execution of most of the experimental work presented in this chapter, except for the adherence and macroarray experiments. Adherence assays were performed by Natalia Montero, in collaboration with Bruno Gonzalez-Zorn Laboratory, UCM, Spain; macroarrays were done by Elodie Akary and Pascale Serror, in collaboration with Pascale Serror Laboratory, INRA, France. The macrophage survival assay was performed together with Renata Matos, from Fátima Lopes Laboratory.

## Abstract

*Enterococcus faecalis* is a nosocomial opportunistic pathogen, but is also found in fermented food products where it plays a fundamental role in the fermentation process. Previously, we have described the non-starter *E. faecalis* cheese isolate QA29b as harbouring virulence genes and proven to be virulent in *Galleria mellonella* virulence model. In this study, we further characterized this food strain concerning traits relevant for the host-pathogen relationship. QA29b was found to belong to sequence type (ST) 72, a common ST among food isolates, and thus we consider it as a good representative of food *E. faecalis* strains. It demonstrated high ability to form biofilms, to adhere to epithelial cells and was readily eliminated by J774.A1 macrophage cells. Despite carrying the *cps* locus associated with the capsular polysaccharide CPS 2 type, *cps* genes were not expressed, likely due to an IS*6770* insertion sequence existing in the *cpsC-cpsK* promoter region. This work constitutes the first study of traits important for interaction, colonization and infection in the host performed on a good representative of *E. faecalis* food isolates. Reported results stress the need

for a reliable serotyping assay of *E. faecalis,* as *cps* genotyping may not be reliable. Overall, QA29b characterization shows that despite its virulence potential in an insect model, this food strain is readily eliminated by mammalian macrophages. Thus, fine tuned approaches combining cellular and mammalian models are needed to address and elucidate the multifactorial aspect of virulence potential associated with food isolates.

## Introduction

Enterococci are at the crossroad of food safety and while they are used as markers associated with faecal contamination they also have been ascribed a beneficial role in foods. In cheese, the presence of enterococcal flora affects taste, aroma, colour, structure, as well as the overall sensory profile (Giraffa, 2003), therefore contributing to the organoleptic characteristics of the full-ripened product (Gaspar et al., 2009). Bacteria from this genus belong to the non-starter flora (Aarestrup et al., 2002) that originates from raw milk and the environment, they proliferate among other bacteria which occur in the same environment, resist the manufacturing processes involved in cheese making and can therefore be found in the final product (Fortina et al., 2008). Using food as a vehicle, and having the ability to survive the harsh conditions in the stomach, enterococci gain access to the gastrointestinal tract (Magalhaes et al., 2007), and therefore interact with the host and the microbiota already present.

While harmless in healthy individuals, some enterococcal isolates may become pathogenic in hospitalized patients in intensive care units and with impaired immune systems. They are capable of causing opportunistic infections, including bacteraemia, endocarditis, meningitis, and wound, urinary tract and nosocomial bloodstream infections. This has been

associated with a variety of virulence factors carried by enterococci (Gaspar et al., 2009), particularly by *Enterococcus faecalis*. About a dozen of these putative virulence traits have been reported from virulence analyses in various animal models. They are involved in attachment both to host cells and to extracellular matrix proteins (AS, Esp, EfaA), in resistance to macrophages (AS, HypR), in cell and tissue damage (Cyl, GelE, SprE) and in immune system evasion (capsular polysaccharides) (Ogier and Serror, 2008). Encapsulated strains of serotype C or D are more resistant to complement-mediated opsonophagocytosis (Hufnagel et al., 2005; Thurlow, Thomas, Fleming, and Hancock, 2009). Serodiversity between serotype C and D isolates is attributed to the presence or absence of the putative glycosyltransferase encoded by *cpsF* gene of the nine-genes capsule-encoding *cps* locus (*cpsC*-*cpsK*), respectively (Hufnagel, Hancock, Koch, Theilacker, Gilmore, and Huebner, 2004; Thurlow, Thomas, and Hancock, 2009). The cognate loci with or without *cpsF* correspond to *cps* type 2 (*cps2*) and type 5 (*cps5*), respectively. Recent studies, indicate that *cps2* isolates are associated with an enrichment of virulence traits suggesting that *cps2* isolates may be more prone to survive and/or colonize hospitalized patients (McBride et al., 2007). In particular, ST6 *E. faecalis* isolates of the high-risk enterococcal clonal complex (CC) 2 predominate among hospital acquired isolates; they seem to be uniformly of CPS2 type (McBride et al., 2007). That is the case of *E. faecalis* V583 isolated from a patient suffering from a persistent bloodstream infection. It was the first vancomycin-resistant clinical isolate reported in the United States (Sahm et al., 1989). This isolate is a good reference as clinical strain causing human infections.

Numerous studies report that enterococcal food strains carry and express virulence determinants (Franz et al., 2003; Foulquié Moreno et al.,

2006; Ogier and Serror, 2008). We have recently described the non-starter *E. faecalis* QA29b strain isolated from raw milk traditional Portuguese ewe's cheese as being able to express some virulence traits in *G. mellonella* insect model (Gaspar et al., 2009) and the *Caenorhabditis elegans* nematode model (unpublished data). Even if the presence of non-starter enterococcal flora is advantageous for the processes of fermentation, preservation and maturation of food products, the association of this genus with nosocomial infections indicates the requirement of a risk assessment for the presence of these strains in food.

In this study we were interested in further characterizing the non-starter *E. faecalis* food isolate QA29b, regarding gene content and traits involved in host recognition, adhesion and survival, using vancomycin-resistant *E. faecalis* CC2 clinical isolate V583, and it's cured derivative VE14089, as a reference.

## Material and Methods

### Bacterial strains and culture conditions

Bacterial strains used in this study are listed in Table 4.1. VE14089 strain is a derivative of V583 strain and was used in assays with animal cells, instead of V583, because it is gentamicin sensitive.

For biofilm assay experiments strains were grown for 16 h in 2×YT (BD, Franklin Lakes, NJ) supplemented with 0.5 % glucose (2×YTGlu) broth, at 37 °C, and the culture was subsequently diluted 1:100 (v/v) in pre-warmed to 37 ºC 2×YT broth. For reverse transcription PCR (RT-PCR) experiments the strains were inoculated in 5 mL 2×YTGlu broth and grown for 16 h without shaking at 37 ºC. The culture was then diluted 100 times in pre-warmed to 37 ºC 2×YTGlu medium and incubated at 37 ºC until

reaching mid exponential phase (optical density (OD) at 600 nm of approximately 0.5). A sample from this culture was washed twice in phosphate-buffered saline (PBS) (pH 7.4) (Gibco, Life Technologies, Carlsbad, CA) and resuspended in one tenth of its initial volume. The washed bacterial culture was diluted 50 times and incubated for 1 h at 37 ºC in two different conditions: 2×YT and 2×YTGlu.

**Table 4.1.** *E. faecalis* strains used in this study.

| Strains | Relevant characteristics | Reference or source |
|---|---|---|
| **QA29b** | Isolated from a Portuguese cheese; *fsrABDC*$^{+}$, *gelEsprE*$^{+}$, *agg*$^{+}$, *esp*$^{+}$ and *efaAfs*$^{+}$; Cyl$^{-}$, Gel$^{+}$ | (Gaspar et al., 2009) |
| **V583** | Sequenced strain containing PAI and plasmids pTEF1, pTEF2, and pTEF3 | (Sahm et al., 1989) |
| **VE14089** | Plasmid-free derivative of V583; Ery$^{S}$, Gen$^{S}$ | (Rigottier-Gois et al., 2011) |

## Multi-locus sequence typing (MLST)

MLST was performed by amplifying and sequencing the internal regions of seven housekeeping genes *gdh*, *gyd*, *pstS*, *gki*, *aroE*, *xpt* and *yqiL*, as described (http://efaecalis.mlst.net). The allele number for each gene was assigned based on the *E. faecalis* MLST database. The combination of the allelic sequences for the seven genes yielded the allelic profile and the corresponding ST for QA29b isolate.

## Macroarray

The macroarray was constructed, performed and analysed as described by Lepage et al. (Lepage et al., 2006). A set of 308 additional probes were added to the previously 202 described probes, making a total of 506 and 4 chromosomal gene probes from V583 and MMH594, respectively, that were used for the screening of QA29b genome. MMH594

is an *E. faecalis* blood isolate carrying the *E. faecalis* pathogenicity island (Huycke and Gilmore, 1995; Shankar et al., 2002).

## CPS type polymorphism determination by PCR

The CPS type polymorphism was determined by PCR, in a total volume of 20 µL with 100 ng of genomic DNA, extracted as previously described (Fouet and Sonenshein, 1990), and using primers cpsE1 (5' - GTGTTGTCATGCCGATTCACAATGC - 3') and cpsG2 (5' - CTGCTGGACCACCAACAAAATTCG - 3'). The PCR was performed using a thermostable DyNAzyme™ II DNA Polymerase (Finnzymes, Vantaa, Finland) and the conditions recommended by the manufacturer. The thermal cycling parameters were adapted in order to amplify the 4069 bp PCR product.

## Adherence assay

The ability of *E. faecalis* strains VE14089 and QA29b to adhere to Caco-2 cells was determined as previously described (Olier et al., 2003), with minor modification. On 24-well tissue-culture plates, an almost confluent monolayer of Caco-2 cells was infected with an *E. faecalis* bacterial suspension, with a corresponding multiplicity of infection (MOI) of ~50. Adhesion of *E. faecalis* cells to Caco-2 cells was allowed to occur for 2 h at 37 °C. Cells were then washed with PBS and adherent bacteria were harvested after lysis of the cell monolayers with Triton X-100 and suitable dilutions of the lysates were plated. The plates were subsequently incubated for 24-48 h at 37 ºC and colony forming unit (CFU) values for viable bacteria were determined.

Adherence assays were done in duplicate, and the overall significance of the differences was determined by a two-tailed unpaired *t*-test.

## Biofilm assay on polystyrene microtiter plates

Biofilm formation on polystyrene was quantified with crystal violet staining method as previously described (Thomas et al., 2009). 200 µL of the diluted cell suspension was used to inoculate sterile 96-well polystyrene microtiter plates (Sarstedt, Nümbrecht, Germany). Biofilms were processed after 24 h incubation at 37 ºC, as described above. Each assay was performed in quintuplicate, repeated three times, and the overall significance of the differences was determined by a two-tailed unpaired *t*-test. All experiments included a blank well (medium without any inoculum).

## Macrophage Survival Assay

The macrophage survival assay was mainly performed as described by Bennett et al. (Bennett et al., 2007) with some modifications. Confluent J774.A1 (mouse monocytes - macrophages) monolayers were infected with an overnight bacterial culture. Approximately $4 \times 10^6$ bacteria were added to J774.A1 monolayers, to yield a MOI of approximately 10, and were incubated at 37 °C in 5 % $CO_2$ atmosphere for 1 h to allow bacterial adherence and entry, after which gentamicin (250 µg/mL) was added to the cultures to kill extracellular bacteria. At various time points after infection, 1 % Triton X-100 in PBS was used to lyse cells. Lysates were then serially diluted and inoculated on BHI plates to enumerate viable intracellular bacteria. The assays were performed 5 times and results are reported as intracellular Survival Index (SI), i.e. the per cent (mean) of the internalized CFUs at each analysed time post-infection that survived after phagocytosis.

## RT-PCR of *cps* locus

For each studied condition, culture samples for RNA isolation were collected and processed accordingly. Immediate RNA sample stabilization and protection was achieved using the RNAprotect Bacteria Reagent (Qiagen, Hilden, Germany), total RNA purification was performed using the RNeasy Mini Kit (Qiagen, Hilden, Germany), DNA digestions were executed after RNA isolation using DNase I recombinant, RNase-free (Roche Applied Science, Penzberg, Germany), and final RNA clean-up and concentration was carried out with RNeasy MinElute Cleanup Kit (Qiagen, Hilden, Germany), all according to the manufacturer's instructions. The integrity and overall quality of the total RNA preparations was evaluated by native agarose gel electrophoresis and their corresponding RNA concentration was measured using a NanoDrop spectrophotometer (NanoDrop Technologies, Inc.).

cDNA was synthesized using Transcriptor High Fidelity cDNA Synthesis Kit (Roche Applied Science, Penzberg, Germany) according to the standard procedure for qualitative RT-PCR from the manufacturer's instructions. Specificity and efficiency for the primer pair (5'-GATGTTGTCCGTTGTAATTGG-3') (5'-CCACATTTCTTGCGTGTCC-3') were confirmed by amplifying an internal gene fragment of 852 bp of the *cpsE* gene (*ef2491*) on genomic DNA. cDNA solutions were added to PCR reactions, which were performed using a thermostable DyNAzyme™ II DNA Polymerase (Finnzymes, Vantaa, Finland), using conditions recommended by the manufacturer. The previously described MLST housekeeping gene *gdh* and *gyd* were used as positive control (http://efaecalis.mlst.net).

For each condition, the qualitative RT-PCR tests were independently performed twice and showed similar results.

### Sequencing

The region between *cdsA* and *cpsC* genes, which encompasses the *cpsC-cpsK* promoter, was amplified from QA29b DNA, using the primer pair (5'- CTGAGCTCTTGGCATCAACC-3') (5'-TATGGCGTGAAAGACTCTGG-3'). Based on V583 genome sequence, the expected size for this amplicon was 865 bp. For QA29b we obtained an amplicon with 1933 bp, which we sequenced (STAB Vida, Portugal) using primer walking. Sequence is deposited in GenBank under the accession number JX119099.

## Results

### MLST typing, gene content and *cps* typing

The combination of the allelic sequences for the seven housekeeping genes in QA29b yielded the allelic profile, which corresponded to the ST72. This ST is closely related with 162, 168 and 196, differing only by one allele type, and belonging to the CC72. MLST typing of QA29b thus shows that it is clonally distinct from V583, which belongs to the ST6 and to the CC2 (differing in all alleles of the seven housekeeping genes).

Gene content of QA29b was evaluated by comparative genome hybridization (CGH) using a macroarray, which covered approximately 16 % of the whole V583 chromosomal genome with gene probes scattered around the chromosome (Figure 4.1). More than 30 % of the V583 chromosomal genes probed were missing from QA29b genome. They included 51 genes that were recently reported as CC2-enriched (Solheim et al., 2011). With the exception of prophage 4 genes, which were present in QA29b, 80 % of the absent genes were localized in mobile genetic

elements predicted in V583 genome (Lepage et al., 2006). Of the 115 core variable genes probed as defined by Solheim et al. (Solheim et al., 2009), 30 were absent in QA29b. They comprised genes *ef2164*, *ef2166*, *ef2168*, *ef2169*, *ef2172*, *ef2174*, *ef2175*, *ef2176* located downstream of the enterococcal polysaccharide antigen gene (*epa*) cluster, the rhamnopolysaccharide biosynthesis operon, which encode predicted functions involved in polysaccharides biosynthesis.

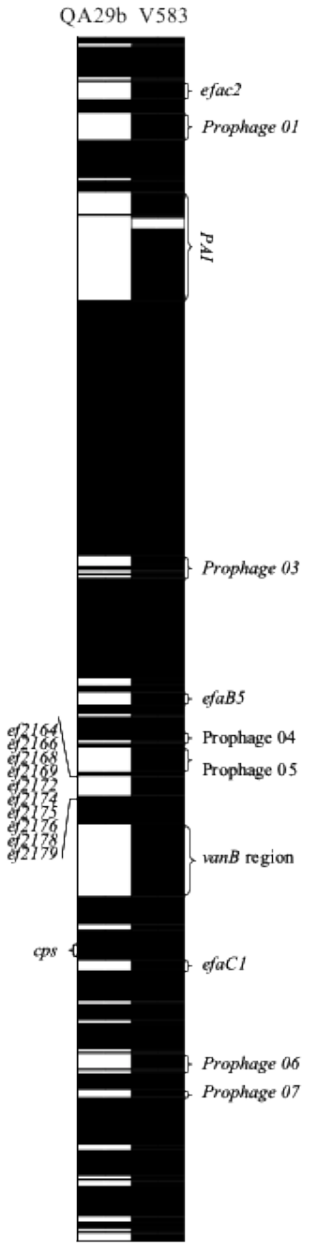


**Figure 4.1.** CGH analysis of QA29b strain. The genes are ordered according to the organization of the V583 genome. Black and white bars indicate present and absent genes, respectively. The mobile genomic elements and regions are indicated at the top. Genes located in the *cps* locus and *epa* region are displayed on the left.

CGH data indicated that QA29b carries the full-length *cps* locus (*ef2487* to *ef2492*). This result was confirmed by PCR using primers cpsE1 and cpsG2 that were designed to amplify the region between *cpsE* (*ef2491*) and *cpsG* (*ef2489*), to detect the presence of *cpsF* (*ef2490*), which distinguishes *cps2* and *cps5* loci. Detection of an amplification product of 4069 bp for QA29b strain and V583 used as positive control confirmed the presence of *cpsE* and *cpsG* genes along with *cpsF* gene. Accordingly to this result QA29b carries a *cps* 2 locus associated with serotype C (Thurlow et al., 2009).

## Biofilm assay on polystyrene microtiter plates

Ability to form biofilm on a polystyrene surface was quantified (Figure 4.2). For the same conditions used to quantify the biofilm production of the *E. faecalis* clinical strain, QA29b exhibited an increased ability to form biofilm. The observed differences between the two strains were not a result of growth differences as both strains displayed similar growth curves (data not shown).

## Adherence assay

Since enterococci are normal commensals of the gastrointestinal tract, the ability to adhere to Caco-2 cells (a human colon carcinoma cell line) was investigated. For this purpose, we compared adhesion of strain QA29b and strain VE14089, a plasmid-cured derivative of V583 clinical strain that allows gentamicin protection assay. Figure 4.3 shows that attachment of QA29b to Caco-2 cells was three times higher than the clinical isolate derivative VE14089.

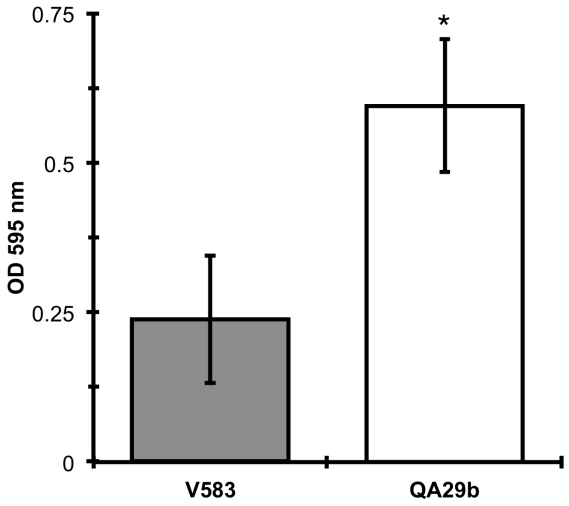


**Figure 4.2.** Biofilm formation on polystyrene. The quantification of biofilm formation was assayed as a function of crystal violet stain (measured at 595 nm) retained by the biofilm biomass grown for 24 h. Data are mean of two independent hexuplicate trials, and error bars indicate standard deviations. *, significant *P* values of less than 0.05 relative to V583, determined using a two-tailed unpaired *t*-test.

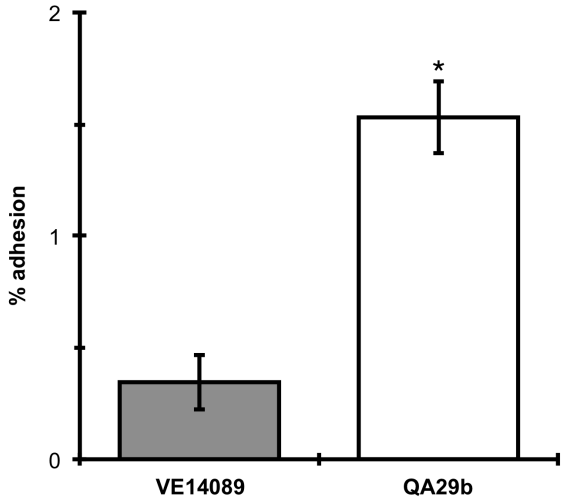


**Figure 4.3.** QA29b and VE14089 adhesion to Caco-2 cells. The results are presented as per cent adhesion ± standard deviation. *, significant *P* values of less than 0.05 relative to VE14089, determined using a two-tailed unpaired *t*-test.

## Macrophage Survival Assay

Phagocytosis and survival of QA29b were evaluated in murine J774-A1 macrophage-like cell line. As shown in Table 4.2, uptake of bacteria of strain VE14089 was almost 4 fold lower than uptake of QA29b at a comparable multiplicity of infection. This result indicates that bacteria of strain QA29b are more efficiently internalized by macrophages. A time course study of bacterial survival inside macrophages (Figure 4.4) showed a significant decrease of QA29b viability compared to VE14089. Altogether these data show that QA29b is more efficiently internalized by murine macrophages and has a higher susceptibility to macrophage bactericidal activity than the clinical strain.

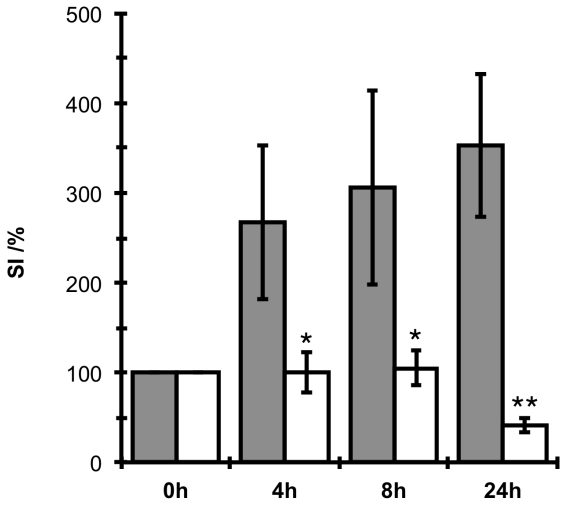


**Figure 4.4.** Time course of intracellular survival of QA29b and VE14089 strains within murine J774A.1 macrophages. Results correspond to the per cent means ± standard deviations of intracellular Survival Index (SI) determined at 0, 4, 8 and 24 h post-killing of external bacteria (grey bars: VE14089; white bars: QA29b). 0 h post-killing corresponds to 2 h post-infection, so time points mentioned in the figure correspond to 2, 6, 10 and 26 h post-infection; asterisks indicate a significant

difference (* $P < 0.05$, ** $P < 0.005$) relative to VE14089 for each time point, determined using a two-tailed unpaired *t*-test.

**Table 4.2.** Macrophage Survival Assay: MOI, uptake percentage at 0 h of infection, and standard deviation values.

| Strain | MOI (± SD) | % Uptake (± SD) |
|---|---|---|
| **QA29b** | 12.0 (± 0.4) | 24.1 (± 3.8) |
| **VE14089** | 14.3 (± 0.8) | 6.3 (± 0.9) |

## Expression of the *cps* locus

As described by (Hancock et al., 2003), the expression of the large transcript that encompasses the *cpsC* to -*K* genes, is controlled by one promoter. The screening for the presence of an internal fragment of the *cpsE* gene would therefore allow the detection of the presence of the transcript, which codes for the *cps* locus. The *cps* transcript was detected for V583 strain, regardless of the growth conditions. In contrast to the clinical isolate, no amplification product was detected in the dairy strain QA29b in any of the tested conditions (data not shown). This indicates that for the two different media used no detectable amount of the *cps* transcript was present, suggesting the absence of a capsule formed from the expression of *cps* locus. Indeed, sequencing of the *cpsC-cpsK* promoter region revealed a 1068bp insertion, identified by BLAST as an IS*6770* of the IS*30* family (Thorisdottir et al., 1994). This insertion was located between the RBS and the predicted -10 and -35 positions (Hancock et al., 2003), and explains the fact that we did not detect expression of the *cps* genes in QA29b strain.

## Discussion

*E. faecalis* QA29b is a previously studied non-starter lactic acid bacteria, which harbours several known virulence traits, and was proven to be virulent in non-mammalian virulence models. In the present study, QA29b was further characterized genetically and regarding traits involved in the interaction with the host in order to enable the risk assessment associated with its presence in food. QA29b belongs to CC72, which, according to the MLST database, includes other dairy food isolates. Although few CC72 hospital-associated isolates were reported they are not the most prevalent (Willems et al., 2011). Large-scale genomic comparison with V583 chromosomal genes, used as a clinical reference strain, established that QA29b lacks many of the genetic mobile elements of V583, including the pathogenicity island. Moreover, QA29b lacks also most of the CC2-enriched genes probed. Compared to the reference strain, QA29b had increased ability to form biofilm, to adhere to epithelial cells and to be engulfed by macrophages. Inversely, QA29b is unable to survive in J774.A1 macrophages and shows no expression of the *cps* genes.

The most abundant *E. faecalis* exopolysaccharides are the enterococcal polysaccharide antigen (EPA) and the serotype-determining CPS. They both seem to play an important role in enterococcal pathogenicity. Disruption of the *epa* locus decreased biofilm formation, survival in polymorphonuclear leukocytes, translocation across human-derived enterocytes and reduced virulence (Xu et al., 1998; Teng, Jacques-Palaz, Weinstock, and Murray, 2002; Zeng et al., 2004; Mohamed and Huang, 2007; Singh et al., 2009). *E. faecalis* CPS contributes to resistance to complement-mediated opsonophagocytosis (Thurlow, Thomas, Fleming, and Hancock, 2009), but there is no knowledge on its effect upon survival

inside macrophages. Comparative genomics indicates that QA29b lacks V583 genes located downstream the *epa* operon, which could contribute to EPA biosynthesis (Rigottier-Gois et al., 2011). In contrast, like V583, QA29b carries the full-length *cps2* locus. According to Thurlow et al. (Thurlow, Thomas, and Hancock, 2009) who established a correlation between *E. faecalis* serotypes and the *cps* locus polymorphism, QA29b is expected to belong to serotype C. However, in contrast to V583, QA29b isolate did not express detectable amounts of the transcripts corresponding to the *cps* cluster in the conditions tested, raising the question of its serotype and moreover of the CPS-related traits. Indeed, lack of CPS could explain efficient internalization of QA29b by macrophages, adhesion to epithelial cells and increased biofilm formation. Like proposed for opsonophagocytosis (Thurlow, Thomas, Fleming, and Hancock, 2009), absence of the capsule could make other bacterial cell surface ligands more accessible for recognition by either macrophages or epithelial cells. This is in agreement with the findings of Soriani et al. (Soriani et al., 2006) that state that in group B *Streptococcus* infections of Caco-2 cells, the absence of capsule led to better adhesion to cells and, consequently, to an increased capacity to invade them. Previous studies showed that the expression of the *cps* locus is regulated in order to modulate the presence of polysaccharide antigens during colonization or infection (Corcionivoschi et al., 2009; Vebø et al., 2009). Absence of expression of the *cps* locus could allow optimal adherence to host cells.

*E. faecalis* isolates were reported resistant to killing by mouse peritoneal macrophages (Gentry-Weeks et al., 1999; Verneuil et al., 2005). However, QA29b strain was particularly sensitive to killing by J774.A1 macrophages. Although the role of the *cps* locus in *E. faecalis* survival inside macrophages has not been investigated, it would be tempting to

speculate that absence of expression of *cps* locus could contribute to QA29b sensitivity to macrophages. *E. faecalis* survival mechanisms inside macrophages have not been elucidated; however oxidative stress response and EPA synthesis appear to be important (Brinster, Posteraro, Bierne, Alberti, Makhzami, Sanguinetti, and Serror, 2007). They both could contribute to QA29b sensitivity to macrophages.

The results of this work illustrate for the first time that the mere presence of *cps* genes, associated with virulence, is not sufficient to predict the corresponding phenotype. Although this fact has been previously established on other genes (Shepard and Gilmore, 2002; Hew et al., 2007; Carlos et al., 2010), our results on the *cps* locus highlight the need to improve and deepen the knowledge on enterococci serotypes, as CPS 2 type may not necessarily be expressed in a *cps* 2 genotype. It would be interesting to determine if silencing of *cps* 2 genes also applies in clinical isolates. Overall, even if the presence of virulence-associated genes in food isolates has to be evaluated, their expression and cognate phenotypic traits have to be considered for accurate safety assessment. Moreover, the two strains studied in this work have different genetic backgrounds. If, on the one hand, this may explain phenotypic differences, on the other hand, it highlights the importance of gathering more genetic and functional data on genetically related strains from different origins.

## Acknowledgments

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cooperation and coordination of genome sequencing and functional genomics of human-pathogenic microorganisms. The authors acknowledge Fundação para a Ciência e Tecnologia for project grant PTDC/CVT/67270/2006, co-financed through FEDER, and PEst-OE/EQB/LA0004/2011, and for grant SFRH/BD/18757/2004 attributed to Frédéric Gaspar.

# Chapter 5

# Role of LuxS in *Enterococcus faecalis*

In preparation:

Role of LuxS in *Enterococcus faecalis*

Frédéric Bustos Gaspar, Natalia Montero, Bruno Gonzalez-Zorn, Maria Teresa Barreto Crespo, and Maria de Fátima Silva Lopes.

The author was involved in the design and execution of the experimental work presented in this chapter, except for the adherence assay, performed by Natalia Montero from Bruno Gonzalez-Zorn Laboratory, UCM, Spain. The macrophage survival assay was performed together with Renata Matos, from Fátima Lopes Laboratory, and the quantification of AI-2 was done in collaboration with Catarina Pereira, from Karina Xavier Laboratory. Microarray cDNA synthesis, labelling, hybridization, and data acquisition were performed by NimbleGen (Reykjavik, Iceland).

## Abstract

Interspecies communication may influence community structure and function. That is likely true in the gut, which contains a complex collection of bacterial species. Autoinducer-2 (AI-2)-mediated quorum sensing (QS) has been extensively studied in relation to the regulation of microbial behaviour and has been recognized as an interspecies communication molecule. Its synthase, LuxS, is an enzyme in the activated methyl cycle (AMC) that catalyses the conversion of S-ribosylhomocysteine (SRH) to homocysteine. The by-product of the reaction catalysed by LuxS is (S)-4,5-dihydroxy-2,3-pentanedione (DPD), which spontaneously forms the furanones known collectively as AI-2. Despite belonging to the normal microbiota of the gastrointestinal (GI) tract of hosts ranging from mammals to insects, *Enterococcus faecalis* remains an important opportunistic pathogen and represents one of the main causes of nosocomial infections in the USA and Europe. *E. faecalis* VE14089, a plasmid-cured derivative of *E. faecalis* V583, harbours the *luxS* gene. This work was thus designed to check for the ability of VE14089 to produce AI-2, understand the role of both AI-2 and

LuxS in the biology of *E. faecalis*, and evaluate its ability to relate with the host. The role of LuxS in *E. faecalis* VE14089 has been examined by phenotypic experiments and transcriptomic profiling. VE14089 was found to produce AI-2 through the activity of LuxS protein. When compared with the wild type (WT), the *luxS* mutant had no apparent phenotype regarding growth, biofilm formation, adhesion to Caco-2 cells, resistance to oxidative stress and survival in a macrophage survival assay. However, microarray comparison of gene expression revealed that the *luxS* mutation caused pleiotropic effects in gene expression, which could not be complemented by extracellular AI-2 addition. These global differences in gene expression affected several gene functional roles, namely energy, DNA, fatty acid and intermediary metabolites metabolism, transport and regulatory functions, cell envelope, and hypothetical and unknown function proteins. This study shows that differential gene expression related to the *luxS* mutation cannot be ascribed to quorum sensing (QS) and that the role of LuxS appears to be limited to metabolism.

## Introduction

Despite a dozen putative virulence factors having been reported from virulence analyses in various animal models, they are not exclusive to clinical strains and, *per se*, are not accountable for understanding the opportunistic behaviour of *Enterococcus faecalis*.

*E. faecalis* belong to the normal microbiota of the GI tract of hosts ranging from mammals to insects (Brinster, Posteraro, Bierne, Alberti, Makhzami, Sanguinetti, and Serror, 2007). They are also found in a variety of food products, namely milk and cheese produced in the south of Europe (Ogier and Serror, 2008). However, *E. faecalis* remains an important

opportunistic pathogen and represents one of the main causes of nosocomial infections in the USA and Europe. Especially for immunocompromised patients, these infections include endocarditis, meningitis, pneumonia, peritonitis, visceral abscesses, urinary infections or septicaemia (Lebreton et al., 2009).

Pathogenic bacteria produce virulence factors when they sense they are in a location in which the energy required for pathogenesis is warranted. One environmental factor monitored by pathogens is population density, either of its own population or of the population of a host's endogenous flora (Parker and Sperandio, 2009).

Intercellular communication is not the exception but, rather, the norm in the bacterial world. The process of sensing population density, called QS, is fundamental to coordinate certain behaviours of microbes.

As a population of QS bacteria grows, a proportional increase in the extracellular concentration of the signalling molecule occurs. When a threshold concentration is reached, the group detects the signalling molecule and responds to it with a population-wide alteration in gene expression. Processes controlled by QS are usually the ones that are unproductive when undertaken by an individual bacterium but become effective when undertaken by the group (Bassler and Losick, 2006). QS has been shown to regulate a variety of functions, including symbiosis, virulence, competence, motility, sporulation, mating, conjugation, antibiotic production, and biofilm formation (Zhu and Pei, 2008).

The small molecules that are produced, released and detected, and which mediate QS are called autoinducers (Zhu and Pei, 2008). Most autoinducers are species specific; however, one autoinducer, AI-2, and its synthase, LuxS, have been identified in many bacteria, (Pereira et al., 2008) and implicated in the regulation of many bacterial behaviours

including biofilm formation, competence, the production of secondary metabolites like antibiotics, and virulence. While in some cases, AI-2 is clearly acting through a canonical QS mechanism, in others a possibly primary and sometimes sole role in central metabolism has been proposed (Pereira et al., 2009).

LuxS protein is an integral metabolic component of the AMC (Figure 5.8). The AMC is a key metabolic pathway that generates S-adenosylmethionine (SAM) as an intermediate product. SAM bears a methyl group with a relatively high transfer potential, and is used by numerous methyltransferases to carry out cellular processes including nucleic acid and protein methylation, and detoxification of reactive metabolites. The product of the methyltransferase reaction is S-adenosylhomocysteine (SAH), and in the complete AMC, SAM is regenerated from SAH via homocysteine and methionine, ready for another round of methylation/transmethylation. The role of LuxS in the AMC is to catalyse the cleavage of SRH to yield homocysteine and a by-product DPD. DPD is the precursor of the family of related, inter-converting molecules collectively termed 'AI-2' (Doherty et al., 2010). A gene, annotated as *luxS,* is also present in the genome of *E. faecalis* V583, the first vancomycin-resistant clinical isolate reported in the United States (Sahm et al., 1989) to be fully sequenced.

In this study we investigated the production of AI-2 in *E. faecalis*, determined *E. faecalis* ability to sense the extracellular presence of AI-2, and explored the role of LuxS in the bacterial cell and in its interaction with the host.

# Materials and Methods

## Bacterial strains and general culture conditions

Bacterial strains used in this study are listed in Table 5.1. Enterococci were grown in M17 broth (BD, Franklin Lakes, NJ) supplemented with 0.5 % (w/v) glucose (M17Glu) or M17Glu agar at 37 ºC, unless stated otherwise. *Escherichia coli* strains were grown in Luria–Bertani (LB) broth or on LB agar at 37 ºC. Antibiotics were used at the following concentrations: erythromycin, 30 µg/mL for *E. faecalis* and 150 µg/ml for *E. coli*; ampicillin, 80 mg/ml.

**Table 5.1.** Strains used in this study

| Strains | Relevant Characteristics | Reference or Source |
|---|---|---|
| ***E. coli*** | | |
| **DH5α** | F$^-$ *Φ*80d*lacZ*ΔM15 Δ(l*acZYA-argF*)U169 *deoR recA1 endA1 hsdR17*($r_K^-$ $m_K^+$) *phoA supE44* λ$^-$ *thi-1 gyrA96 relA1* | (Grant et al., 1990) |
| **TG1 *repA*** | *supE hsdD5 thi* (Δ*lac-proAB*) F$^-$ (*traD36 proAB-lacZ*ΔM15) *repA* | (Law et al., 1995) |
| ***E. faecalis*** | | |
| **V583** | Sequenced strain containing PAI and plasmids pTEF1, pTEF2, and pTEF3 | (Sahm et al., 1989) |
| **VE14089** | Plasmid-free derivative of V583; Ery$^S$, Gen$^S$ | (Rigottier-Gois et al., 2011) |
| **VE14089Δ*luxS*** | VE14089 with *luxS* in-frame deletion | This study |
| **QA29b** | Isolated from a Portuguese cheese; *fsrABDC*$^+$, *gelE-sprE*$^+$, *agg*$^+$, *esp*$^+$ and *efaAfs*$^+$; Cyl$^-$, Gel$^+$ | (Gaspar et al., 2009) |

## General DNA techniques

General molecular biology techniques were performed by standard methods (Sambrook et al., 1989). Restriction enzymes, polymerases and

T4 DNA ligase were used according to manufacturer's instructions. PCR amplification was performed using a thermocycler (Biometra GmbH, Goettingen, Germany). When necessary, PCR products and DNA restriction fragments were purified with QIAquick purification kits (Qiagen, Hilden, Germany). Plasmids were purified using the QIAprep Spin Miniprep kit (Qiagen, Hilden, Germany). Electrotransformation of *E. coli* and *E. faecalis* was carried out as described by Dower et al. (Dower et al., 1988) and Dunny et al. (Dunny et al., 1991), using a Gene Pulser apparatus (Bio-Rad Laboratories, Hercules, CA). Genomic DNA fragments and plasmid inserts were sequenced at Baseclear (Netherlands).

## Construction of in-frame *luxS* deletion mutant in strain VE14089

Markerless *luxS* deletion mutant of *E. faecalis* strain VE14089 was constructed essentially as described by Gaspar et al. (Gaspar et al., 2009). Briefly, 5' and 3' flanking regions of *luxS* were amplified from chromosomal DNA of each strain by PCR with primers luxS1, luxS2, luxS3, and luxS4 (Table 5.2). The two cognate PCR fragments were fused by PCR using the external primers luxS1 and luxS4, and the resulting product was cloned into pGEM-T (Promega Corporation, Fitchburg, WI). The inserted PCR fragment was removed from its cloning vector by restriction enzymes and subsequently cloned into pG+host9 plasmid (Maguin et al., 1996), which was then electroporated into *E. faecalis*. The *luxS* single- and double-crossover mutants were selected as described by Brinster et al. (Brinster, Furlan, and Serror, 2007). Successful targeted mutations of *luxS* in strains VE14089 was first identified by PCR screening and then confirmed by Southern blot analysis.

**Table 5.2.** Primers used in this study

| primer name | sequence (5'-3') | position of the primer 5' end | Reference |
|---|---|---|---|
| **luxS1** | TCAATCAACCTTTGCTGACG | bp 1011 after the *luxS* stop codon | This study |
| **luxS2** | ATTAGTTTAGATCCATTTGAACG | bp 33 before the *luxS* stop codon | This study |
| **luxS3** | CGTTCAAATGGATCTAAACTAATTTC AAAACTTTCTACGCGTGC* | bp 24 in *luxS* | This study |
| **luxS4** | AGGTGGCAACGACTTTTAGC | bp 936 upstream of the *luxS* start codon | This study |
| **luxS_seq** | TTACCCATCAAAGGACTATCC | bp 64 upstream of the *luxS* start codon | This study |

*Sequences added for fusion PCR are underlined.

## Quantification of AI-2

To monitor extracellular AI-2 activity in cell cultures during growth, aliquots were collected at the indicated times and cell-free culture fluids were prepared by filtration of liquid cultures and then analysed for AI-2 activity. AI-2 quantification was done using a LuxP-FRET-based reporter (FRET — fluorescence resonance energy transfer), as established by Rajmani et al. (Rajamani et al., 2007) and optimized for 96-well plate reading by Marques et al. (Marques et al., 2011). The binding of AI-2 to the CFP-LuxP-YFP chimeric protein causes a dose-dependent decrease in the FRET signal, and concentration can be determined by comparing the FRET ratios (527 nm/485 nm) of each sample with a calibration curve performed with AI-2 samples of known concentration (Figure 5.3A). Concentrations between 1 and 60 µM DPD (Omm Scientific, Inc., Dallas, TX) were used for the calibration curve, corresponding to the linear range of this assay. All assays were performed in duplicate.

## Biofilm assay on polystyrene microtiter plates

Biofilm formation on polystyrene was quantified with crystal violet staining method as previously described (Thomas et al., 2009). Briefly, strains were grown for 16 h in 2×YT (BD, Franklin Lakes, NJ) supplemented with 0.5 % glucose (2×YTGlu) broth, at 37 °C, and the culture was subsequently diluted 1:100 (v/v) in pre-warmed to 37 ºC 2×YT broth. 200 µL of the diluted cell suspension was used to inoculate sterile 96-well polystyrene microtiter plates (Sarstedt, Nümbrecht, Germany). Biofilms were processed after 24 h incubation at 37 ºC, as described above. Each assay was performed in hexuplicate, repeated twice, and the overall significance of the differences was determined by a two-tailed unpaired *t*-test. All experiments included a blank well (medium without any inoculum).

## Adherence assay

The ability of *E. faecalis* strains VE14089 and VE14089Δ*luxS* to adhere to Caco-2 cells was determined as previously described (Olier et al., 2003), with minor modifications. On 24-well tissue-culture plates, an almost confluent monolayer of Caco-2 cells was infected with an *E. faecalis* bacterial suspension, with a corresponding multiplicity of infection (MOI) of ~50. Adhesion of *E. faecalis* cells to Caco-2 cells was allowed to occur for 2 h at 37 °C. Adherent bacteria were harvested after lysis of the cell monolayers with Triton X-100 and suitable dilutions of the lysates were plated. The plates were subsequently incubated for 24-48 h at 37 ºC and CFU values for viable bacteria were determined. Adherence assays were done in triplicate, and the overall significance of the differences was determined by a two-tailed unpaired *t*-test.

## $H_2O_2$ challenge conditions

$H_2O_2$ challenge was performed based on the method described by Giard et al. (Giard et al., 2006), with some adaptations. Briefly, WT and mutant cells were inoculated in M17Glu broth and grown for 16 h without shaking at 37 ºC. The culture was then diluted 1:100 (v/v) in pre-warmed to 37 ºC M17Glu broth and incubated, with 150 rpm agitation in an orbital shaker (Innova, Edison, New Jersey), at 37 ºC, until reaching an OD at 600 nm of approximately 0.5. The cells were harvested by centrifugation and resuspended in M17 broth with 7 mM $H_2O_2$. These cultures were placed into a 37 ºC water bath and, every 2 h for 6 h, samples were taken and plated in M17Glu agar. The number of CFU was determined after incubation at 37 ºC. The growth of the mutant and WT cells in the absence of peroxide stress was previously determined and did not reveal any difference. Each point is the mean of four independent experiments, each with duplicate plating, and the statistical comparison of means was performed using a two-tailed unpaired *t*-test. Survival at any given time point was determined as the ratio of the number of CFU after treatment to the number of CFU at the zero time point.

## Macrophage Survival Assay

The macrophage survival assay was mainly performed as described by Bennett et al. (Bennett et al., 2007) with some modifications. Confluent J774.A1 (mouse monocytes - macrophages) monolayers were infected with an overnight bacterial culture. Approximately $4 \times 10^6$ bacteria were added to J774.A1 monolayers, to yield a MOI of approximately 10, and were incubated at 37 °C in 5 % $CO_2$ atmosphere for 1 h to allow bacterial adherence and entry, after which gentamicin (250 µg/mL) was added to the

cultures to kill extracellular bacteria. At various time points after infection, 1 % Triton X-100 in PBS was used to lyse cells. Lysates were then serially diluted and inoculated on Brain heart infusion (BHI) (Oxoid Ltd, Basingstoke, England) plates to enumerate viable intracellular bacteria. The assays were performed 5 times and results are reported as intracellular Survival Index (SI), i.e. the per cent (mean) of the internalized CFUs at each analysed time post-infection that survived after phagocytosis.

## Microarray Analysis

For all experiments, the strains were inoculated in 5 mL 2×YTGlu broth and grown for 16 h without shaking at 37 ºC. The culture was then diluted in pre-warmed to 37 ºC 2×YT broth, adjusting the bacterial suspension density to the 1,0 McFarland standard. This pre-culture was diluted 1:100 (v/v) in 50 mL pre-warmed to 37 ºC 2×YT broth, and incubated, with 150 rpm agitation in an orbital shaker (Innova, Edison, New Jersey), at 37 ºC, until, for each condition studied, samples for RNA isolation were collected and processed accordingly. When needed 10 µM DPD was added 15 min prior to the RNA collection. The whole process was performed twice, independently.

Immediate RNA sample stabilization and protection was achieved using the RNAprotect Bacteria Reagent (Qiagen, Hilden, Germany). Total RNA purification was performed using the RNeasy Midi Kit (Qiagen, Hilden, Germany), DNA digestions were executed after RNA isolation using DNase I recombinant, RNase-free (Roche Applied Science, Penzberg, Germany) and repeated when necessary, and final RNA clean up and concentration was carried out with RNeasy MinElute Cleanup Kit (Qiagen, Hilden, Germany), all according to the manufacturer's instructions.

Integrity and overall quality of the total RNA preparations, but also DNA contamination, were evaluated by native agarose gel electrophoresis and by PCR, respectively, and their corresponding RNA concentration was measured using a NanoDrop spectrophotometer (NanoDrop Technologies, Inc., Thermo Fisher Scientific Inc., Wilmington, DE).

Microarray comparative genomic hybridization analysis was carried out using the microarray analysis platform of NimbleGen Technologies (Roche NimbleGen, Madison, WI). The chosen expression microarray, 4x72k format (Catalogue Number: A7980-00-01, Design Name: 080625 Efae V583 EXP X4), covers 3114 genes represented with 11 60mer probes per gene and 2 replicates per probe.

cDNA synthesis, labelling, hybridization, and data acquisition were performed by NimbleGen (Reykjavik, Iceland).

Image analysis was performed with the NimbleScan software (v2.6) (Roche NimbleGen Inc., Madison, WI), and feature intensities were exported as .pair files, after background correction and quantile normalization. All data analysis was carried out using the ArrayStar 3.0 software package (DNAStar, Madison, WI). Robust multichip averaging (RMA) algorithm and quantile normalization were used for probe summarization and normalization and applied to the entire data set, which consisted of two biological replicates for each condition. Statistical analyses were carried out with the normalized data using a moderated *t*-test with false discovery rate (FDR) multiple-test correction (Benjamini-Hochberg) to determine differential transcript abundance. Changes in transcript abundance were considered significant if they met the following criteria: *P* value $< 0.05$ and $| \log_2\text{-ratio} | > 5.0$ in at least one of the three performed comparisons.

# Results and Discussion

## Identification of the *luxS* gene in *E. faecalis*

Genomic database analysis has previously revealed (Surette et al., 1999; Paulsen et al., 2003) that *E. faecalis* possessed the gene encoding the putative LuxS protein, S-ribosylhomocysteine lyase (EC: 4.4.1.21), a 152 amino-acids protein encoded by the *luxS* gene (*ef1182*) homologous to *luxS* from *V. harveyi*. The LuxS protein of *V. harveyi* ATCC BAA-1116 was described as having a per cent similarity/identity (S/I) with the putative one present in *E. faecalis* V583 of (60/44) (Surette et al., 1999).

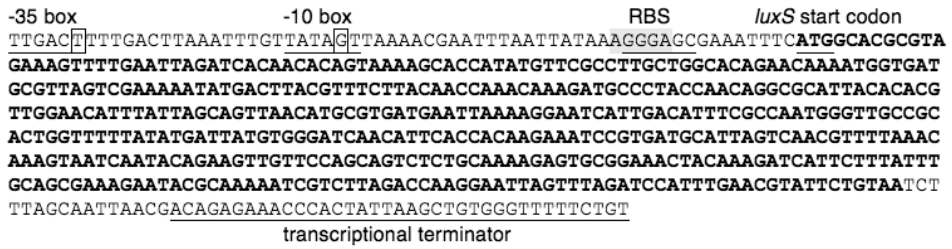
```
1    MPLLDSFKVDHTKMPAPAVRLAKVMKTPKGDDISVFDLRFCIPNKDIMSEKGTHTLEHLF   60  Q9PN97   LUXS_CAMJE
1    MPLLDSFTVDHTRMEAPAVRVAKTMNTPHGDAITVFDLRFCVPNKEVMPERGIHTLEHLF   60  P45578   LUXS_ECOLI
1    MPLLDSFKVDHTKMNAPAVRIAKTMLTPKGDNITVFDLRFCIPNKEILSPKGIHTLEHLF   60  P44007   LUXS_HAEIN
1    MARVESFELDHNTVKAPYVRLAGTEQNG-DALVEKYDLRFLQPNKDALPTGALHTLEHLL   59  Q836D3   LUXS_ENTFA
1    MPSVESFELDHNAVVAPYVRHCGVHKVGTDGVVNKFDIRFCQPNKQAMKPDTIHTLEHLL   60  O34667   LUXS_BACSU
     *. ::** :**. : ** ** . .     .  :  :*:**  ***: :     ******:

61   AGFMRDHLNS-NSVEIIDISPMGCRTGFYMSLIGTPDEKSIAKAWEAAMKDVLSVSDQSK  119  Q9PN97   LUXS_CAMJE
61   AGFMRNHLNG-NGVEIIDISPMGCRTGFYMSLIGTPDEQRVADAWKAAMEDVLKVQDQNQ  119  P45578   LUXS_ECOLI
61   AGFMRDHLNG-DSIEIIDISPMGCRTGFYMSLIGTPNEQKVSEAWLASMQDVLGVQDQAS  119  P44007   LUXS_HAEIN
60   AVNMRDELKG-----IIDISPMGCRTGFYMIMWDQHSPQEIRDALVNVLNKVINTE---V  111  Q836D3   LUXS_ENTFA
61   AFTIRSHAEKYDHFDIIDISPMGCQTGYYLVVSGEPTSAEIVDLLEDTMKEAVEIT---E  117  O34667   LUXS_BACSU
     *  :*.. :      *********:**:*: : .      : .     ::..:

120  IPELNIYQCGTCAMHSLDEAKQIAQKVLNLGISIINNKELKLENA-------  164  Q9PN97   LUXS_CAMJE
120  IPELNVYQCGTYQMHSLQEAQDIARSILERDVRINSNEELALPKEKLQELHI  171  P45578   LUXS_ECOLI
120  IPELNIYQCGSYTEHSLEDAHEIAKNVIARGIGVNKNEDLSLDNSLLK----  167  P44007   LUXS_HAEIN
112  VPAVSAKECGNYKDHSLFAAKEYAKIVLDQGISLDPFERIL-----------  152  Q836D3   LUXS_ENTFA
118  IPAANEKQCGQAKLHDLEGAKRLMRFWLSQ--DKEELLKVFG----------  157  O34667   LUXS_BACSU
     :*  .  :**    *.*  *:   :  :           :
```

**Figure 5.1.** Sequence alignments of putative LuxS protein from *E. faecalis* V583 (ENTFA) and selected organisms for which there is evidence of the existence of LuxS at the protein level. The four representatives are *Bacillus subtilis* (BACSU)(Hilgers and Ludwig, 2001; Ruzheinikov et al., 2001; Zhu et al., 2003), *Campylobacter jejuni* (CAMJE) (Elvers and Park, 2002), *Escherichia coli* (strain K12) (ECOLI) (Surette and Bassler, 1998; Surette et al., 1999) and *Haemophilus influenzae* (strain ATCC 51907 / DSM 11121 / KW20 / Rd) (HAEIN) (Lewis et al., 2001). Under aligned protein sequences there is a line containing characters that mean that the column of the alignment contains: "*", identical amino acid residues

in all sequences; ":", different but highly conserved amino acids; ".", different amino acids that are somewhat similar; blank, dissimilar amino acids or gaps. Metal ligands are highlighted in grey. Multiple alignment performed with ClustalW (Thompson et al., 1994).

The three highly conserved amino acids H54, H58, and C126 reported to be the metal ligands from the catalytic centre of LuxS protein of *Bacillus subtilis* (Hilgers and Ludwig, 2001) are also conserved in *E. faecalis* putative LuxS, in the corresponding H53, H57, and C120 positions (Figure 5.1). Moreover the amino acids involved in hydrogen bonding and electrostatic interactions with the substrate of *B. subtilis* LuxS (S6, K35, E57, R65, D78, I79, S80, C84, and G127, except for Q125) (Hilgers and Ludwig, 2001; Ruzheinikov et al., 2001; Zhu et al., 2003; Rajan et al., 2005) are also conserved in *E. faecalis* LuxS. In *E. faecalis* V583 genome, *ef1182* (*luxS*) locus is flanked by a 735 bp gene (*ef1181*) encoding a nitroreductase family protein, 84 bp downstream of *ef1182* stop codon, and a 1068 bp gene (*ef1183*) encoding an aspartate-semialdehyde dehydrogenase (*asd*), 207 bp upstream of the start codon. Potential ribosome-binding site (RBS) was examined using JCVI's RBS finder (Delcher et al., 1999) and the rest of the promoter sequences were examined manually (Figure 5.2A). The *luxS* locus appeared to be followed by an inverted repeat downstream of the stop codon, which may be a transcriptional terminator (Figure 5.2B). This is consistent with the prediction of http://biocyc.org (Karp et al., 2005) in which *luxS* gene does not appear to form an operon with any of the genes that surround it.

**A**




**B**

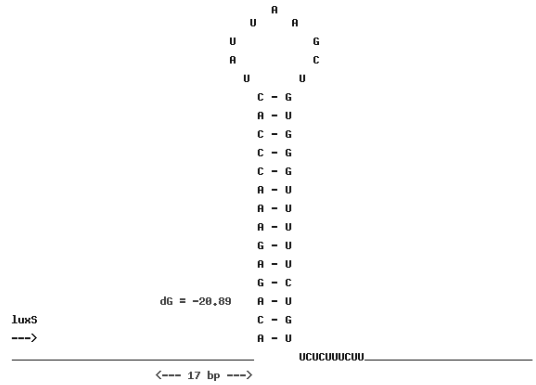



**Figure 5.2.** *E. faecalis* V583 *ef1182* (*luxS*) promoter and terminator sequences. (**A**) Potential -10 and -35 regions, the RBS, and transcriptional terminator sequences are underlined, the start codon is indicated by boldface type and underlining, and the *luxS* gene is indicated by boldface type. The mismatches between the *luxS* locus -10 and -35 regions and the canonical -10 and -35 sequences are boxed. (**B**) Putative transcriptional terminator of *luxS* locus. According to the predicted terminators tool WebGeSTer DB (Mitra et al., 2011), the *luxS* locus appeared to be followed by an inverted repeat ($\Delta G$ = –20.89 kcal/mol), 17 bp downstream of the stop codon, which may be a transcriptional terminator.

Performing a nucleotide BLAST (Altschul et al., 1997; 2005) with all the 55 finished and unfinished *E. faecalis* genomes and genome projects available in the microbial BLAST database from the National Center for Biotechnology Information (NCBI) we found that *ef1182*, the putative *luxS*

gene, has a per cent identity of 99 % or higher, in all the 55 hit results obtained. The genomes available in the databases are mainly from clinical, unknown or commensal origin. We also analysed the previously amply studied food strain *E. faecalis* QA29b (Gaspar et al., 2009; Gaspar et al., 2011, submitted), isolated from a Portuguese cheese, for the presence of the *luxS* gene. QA29b has the *luxS* gene, which sequence is identical to the one of V583 strain. Very similar upstream and downstream regions were found (data not shown). A 100 % identical nucleotide sequence for potential promoter sequence, RBS, and terminator can be found in the up- and downstream sequences from the putative QA29b *luxS* gene. In this isolate the putative *luxS* gene has the same adjacent gene organization as in V583: a downstream nitroreductase and an upstream aspartate-semialdehyde dehydrogenase, both with high amino acid sequence identity to the ones in V583.

Further *in vitro* analysis using purified LuxS proteins from *E. faecalis* overexpressed in *E. coli* strain BL21 revealed its activity to produce AI-2 (Schauder et al., 2001).

## *luxS* mutagenesis depletes AI-2 activity

VE14089, a plasmid-cured derivative of *E. faecalis* V583 (Rigottier-Gois et al., 2011), was used as the genetic background for a *luxS* mutant construction. Besides removing the effect of plasmid contained genes and making easier the markerless in-frame *luxS* deletion mutagenesis, this plasmid-cured derivative was used in assays with animal cells because it is gentamicin sensitive.

In order to conclude on LuxS activity in VE14089, we started by measuring the ability of this bacterium to produce AI-2 molecules. Cell-free culture supernatants of VE14089 were tested for the presence of AI-2 using

LuxP-FRET-based reporter method (Figure 5.3A), using the correlation between AI-2 concentration and FRET ratios (527 nm/485 nm) presented in Figure 5.3B. As shown in Figure 5.4A, cell-free culture supernatants of VE14089 induced a growth phase dependent signal, above the detection method threshold, indicating that AI-2 molecules were produced by VE14089. The AI-2 activity in the culture supernatant of the WT was detectable from the mid-exponential phase onward, reaching maximum levels during late-exponential/early-stationary phase and then decreasing, while remaining higher than the basal levels detected during lag phase.

AI-2 internalization and possible subsequent modification, through an ATP-binding-cassette transporter (Lsr of *Salmonella enterica* serovar Typhimurium) (Hardie and Heurlier, 2008), has been described for other bacteria (Rezzonico and Duffy, 2008), and has been hypothesized to be a mechanism to control AI-2 levels in the vicinity of a cell or to prevent AI-2 signalling by other bacterial species in its environment (Taga et al., 2003; Xavier and Bassler, 2005a; Xavier et al., 2007; Pereira et al., 2009). As an alternative to QS, after being released as a waste product, AI-2 may be reused subsequently as a metabolite, or the molecule may be used as a borate scavenger by bacteria (Rezzonico and Duffy, 2008). When internalization occurs, extracellular AI-2 concentration typically increases during exponential growth and begin to decline during the transition from exponential phase to the early stationary phase, by which time there is no detectable levels of AI-2 (Xavier and Bassler, 2005b; Azakami et al., 2006; De Keersmaecker et al., 2006; Zhu and Pei, 2008; Han and Lu, 2009; Marques et al., 2011). The extracellular accumulation of AI-2 described for VE14089, as well as its level remaining considerably high, has also been described for other bacteria (Learman et al., 2009; Zhao et al., 2010) and may indicate the absence of AI-2 transport into the cells, which is in

agreement with the genomic predictions previously made for *E. faecalis* (Rezzonico and Duffy, 2008).

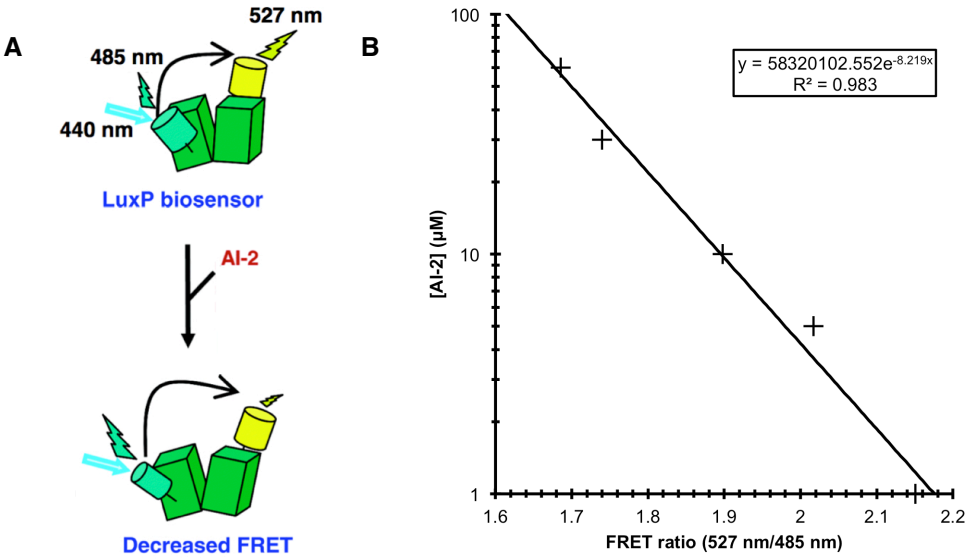



**Figure 5.3.** (**A**) Mechanism for ligand-induced fluorescence resonance energy transfer (FRET) changes in the cyan fluorescent protein (CFP), LuxP, yellow fluorescent protein (YFP) fusion. Schematic representation of the CFP-LuxP-YFP protein as a CFP in cyan, LuxP in green, and YFP in yellow fusion protein. In the absence of AI-2, CFP exhibits high fluorescence. Following ligand binding a conformational-induced change in the distance between CFP and YFP occurs, altering the level of FRET between the terminal fluorophores (Rajamani et al., 2007). (**B**) Calibration curve performed with AI-2 samples of known concentration. Concentrations of 1, 5, 10, 30 and 60 μM AI-2 were used for the calibration curve, corresponding to the linear range of this assay. Binding of AI-2 to the CFP-LuxP-YFP fusion protein causes a dose-dependent decrease in the FRET signal, and concentration can be determined by comparing the FRET ratios (527 nm/485 nm) of each sample with the calibration curve.

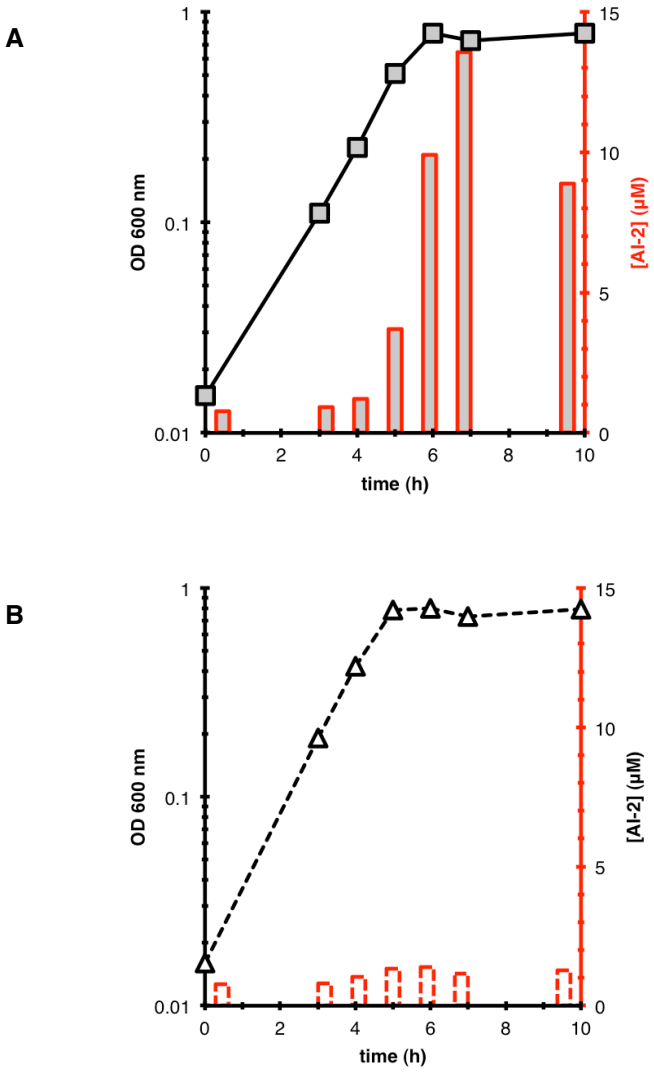



**Figure 5.4.** Growth curves and AI-2 production from VE14089 (**A**) and VE14089Δ*luxS* (**B**).

A markerless in-frame *luxS* deletion mutant was constructed by allelic exchange in VE14089 to successfully produce VE14089Δ*luxS*, so that the physiological role of the *E. faecalis* LuxS as well as the production

of AI-2 and its involvement in cell-to-cell communication mechanisms could be examined.

The resulting VE14089Δ*luxS* mutant generated near-background signal levels (Figure 5.4B). No AI-2 activity was detectable in the supernatant of the *luxS* mutant, regardless of cell density. The *luxS* deletion abolished the production of AI-2, demonstrating that LuxS is the key determinant in the AI-2 production process in VE14089.

Even if there is no internalization of the AI-2 molecule, a different mechanism of AI-2 detection in bacteria, like the one found in *V. harveyi*, can occur (Rezzonico and Duffy, 2008), where just the signal but not the AI-2 molecule is transduced inside the cell. This mechanism is triggered by the interaction of AI-2 with a two-component signal regulator pair (LuxP/LuxQ in *V. harveyi*), followed by a dephosphorylation cascade (Hardie and Heurlier, 2008). However, none of the published *E. faecalis* genomes contains potential homologues for the LuxP/LuxQ AI-2 signal transduction system found in *Vibrio* spp., which also happens for other bacteria (Altschul et al., 1997; Doherty et al., 2006; Rezzonico and Duffy, 2008). Thus, if AI-2 is deployed as an extracellular signal by *E. faecalis*, it must be sensed via a different, yet to be found, mechanism, for instance the reported two-component systems in *E. faecalis* genome.

## Biological role of *luxS* in *E. faecalis*

To characterize the effect of the *luxS* deletion in VE14089 regarding traits that may be involved in the host-pathogen relationship, like host recognition, adhesion and survival, four phenotypic experiments were carried out in this study: biofilm formation on an abiotic surface, adherence

assay to Caco-2 cells, resistance to oxidative stress, and macrophage survival assay.

When using a two-tailed unpaired *t*-test, no significant differences were observed between VE14089 and VE14089Δ*luxS* regarding biofilm formation (Figure 5.5A), adhesion to Caco-2 cells (Figure 5.5B), $H_2O_2$ challenge (Figure 5.5C), uptake by macrophages (Table 5.3) or macrophage intracellular survival (Figure 5.5D). Also, even if the lag phase is longer for VE14089 than VE14089Δ*luxS*, when normalization of growth conditions was conducted, the *luxS* deletion did not affect growth of the mutant, having no obvious difference in growth from mid-exponential onwards and reaching stationary phase at the same time (Figure 5.6). All these results indicate that, at least in the conditions used here, *E. faecalis* LuxS does not seem to be involved in the regulation of growth, biofilm formation, adherence to epithelial cells, resistance to oxidative stress, and survival inside macrophages.

**Table 5.3.** Macrophage Survival Assay: MOI, uptake percentage at 0 h post-killing, and standard deviation values.

| Strain | MOI (± SD) | % Uptake (± SD) |
|---|---|---|
| **VE14089** | 12.2 (± 2.7) | 8.2 (± 2.5) |
| **VE14089Δ*luxS*** | 14.3 (± 2.6) | 7.7 (± 2.2) |

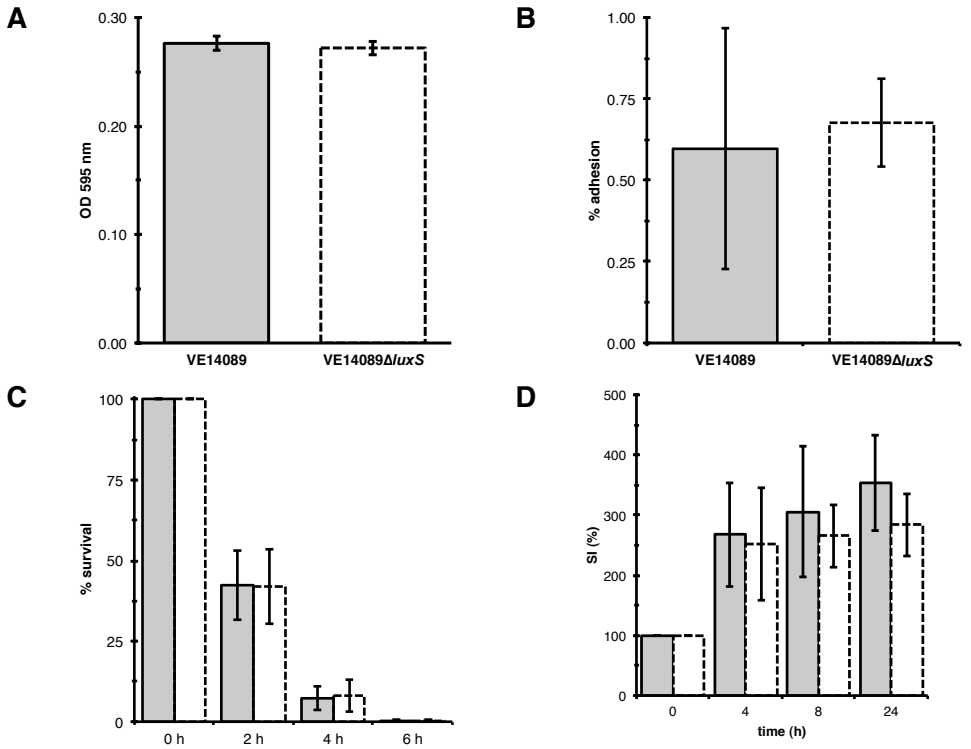



**Figure 5.5.** Phenotypic characterization of *luxS* mutant: VE14089 in grey bars with full line outline and VE14089Δ*luxS* in white bars with a dashed line outline. (**A**) Biofilm formation on polystyrene microtiter plates. The quantification of biofilm formation was assayed as a function of crystal violet stain (measured at 595 nm) retained by the biofilm biomass grown for 24 h. Data are mean of hexuplicate trials, and error bars indicate standard deviations. (**B**) VE14089 and VE14089Δ*luxS* adhesion to Caco-2 cells. The results are presented as per cent adhesion ± standard deviation. (**C**) Percentage (± standard deviation) survival of growing cells of *E. faecalis* VE14089 and VE14089Δ*luxS* at 2, 4 and 6 h of a challenge with 7 mM $H_2O_2$. One hundred per cent corresponds to the number of CFU before $H_2O_2$ treatment. All data are means (± standard deviation) of four independent experiments. (**D**) Time course of intracellular survival of VE14089 and VE14089Δ*luxS* strains within murine J774A.1 macrophages. Results correspond to the per cent means ± standard deviations of intracellular Survival Index (SI) determined at 0, 4, 8 and 24 h post-killing of external bacteria, of five independent

experiments. 0 h post-killing corresponds to 2 h post-infection, so time points mentioned in the figure correspond to 2, 6, 10 and 26 h post-infection.

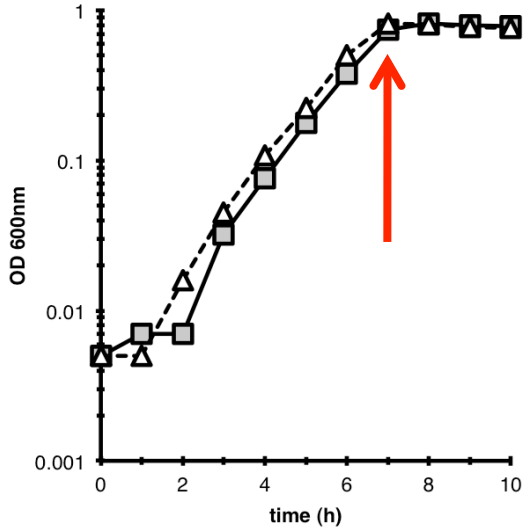



**Figure 5.6.** Growth of *E. faecalis* strains VE14089 (-□-) and VE14089Δ*luxS* (-Δ-) in 2×YT medium, after growth conditions optimization. The red arrow indicates the growth phase point used for the sampling of RNA used in the transcriptomic analysis. The average OD at 600 nm and AI-2 concentration, in µM, with the corresponding standard deviation (±SD), for each RNA sampling point was the following: VE14089 (OD 600 nm = 0.844 (±0.045); [AI-2] = 5.71 (±0.52)), VE14089Δ*luxS* (OD 600 nm = 0.833 (±0.017); [AI-2] = 0.80 (±0.21)), and VE14089Δ*luxS* + 10 µM DPD (OD 600 nm = 0.842 (±0.008); [AI-2] = 15.09 (±1.71)).

Similar phenotypes have been studied in other Gram-positive and in Gram-negative bacteria. However a thorough analysis of the literature reveals no trend for *luxS* role in growth (Challan Belval et al., 2006; Zhang et al., 2008), biofilm formation (Wen and Burne, 2004; Challan Belval et al.,

2006; Shemesh et al., 2008), adherence to Caco-2 cells (Buck et al., 2009; Holmes et al., 2009), and resistance to oxidative stress (Wen and Burne, 2004; Gao et al., 2009; Holmes et al., 2009). No trend for a *luxS* role was also evident regarding invasion of epithelial cells (Siller et al., 2008; Perrett et al., 2009), overall fitness in intracellular growth (Doherty et al., 2006; Zhao et al., 2010) and virulence in a mouse infection model (Stroeher et al., 2003; Perrett et al., 2009). The *luxS* mutation has been described as being able to enhance, reduce or have no detectable effect on these different phenotypes. In some cases the supplementation with AI-2 like molecules was able to complement the altered phenotypes (Doherty et al., 2006; Sztajer et al., 2008; Zhang et al., 2008), but in others AI-2 addition did not restore the detected defects (Tannock et al., 2005; Sela et al., 2006; van Houdt et al., 2006; Zhang et al., 2008; Learman et al., 2009)

As *luxS* and AI-2 production appears to have no role in *E. faecalis* in the phenotypes studied, we decided to look for the intracellular role of *luxS* and AI-2 by performing a transcriptomic analysis of VE14089 and VE14089Δ*luxS*.

Because both products of LuxS have potentially important functions, disruption of the activity of LuxS can have extremely different consequences. They can range from the regulation of specific factors (a defined QS repertoire of changes), due to cell-cell communication disruption, to global influences on transcription (the consequences of major metabolic imbalance), affecting cell fitness (Hardie and Heurlier, 2008).

## Impact of *luxS* at the transcriptional level

From production experiments (Figure 5.4), we know that high AI-2 levels were achieved in the transition between late-exponential and early-stationary phase. Thus, cells were collected at late-exponential phase,

which guarantees that, for the tested conditions, AI-2 is present extracellularly for the parental strain and that a possible effect for its presence may be monitored. The *luxS* mutant supplementation with DPD was performed 15' prior to the cell harvesting, which did not notably interfere with VE14089Δ*luxS* growth, and to a level that would come to the one of the parental strain. We chose the use of synthetic pre AI-2 molecules in the form of DPD, as previously done in other studies (Ahmed et al., 2009; Kint et al., 2009; Armbruster et al., 2010), to avoid the use of purified culture filtrates that might have been misleading, as they contain complex mixture of other signals to which bacteria could respond to (Vendeville et al., 2005).

Genes regulated by AI-2 were identified by performing a pair-wise comparison of gene expression in VE14089Δ*luxS* and VE14089Δ*luxS* supplemented with 10 µM DPD (Table 5.4). Pair-wise comparisons of gene transcription in VE14089 and VE14089Δ*luxS*, supplemented or not with 10 µM DPD, allowed to determine changes in gene expression affected uniquely by the *luxS* mutation or by the *luxS* mutation along with the lack of extracellular *in vitro* presence of AI-2, respectively (Table 5.4).

These comparisons would allow to clarify the role of *luxS* gene, investigate its effect on gene transcription, and attempt to discriminate between the transcriptional response induced by this extracellular signal and the metabolic role of the LuxS protein.

With a cutoff value of 5 for the n-fold change of expression (*P*-value $< 0.05$) in at least one of the three pairwise comparisons, the data revealed fundamental changes in gene expression affecting a total of 113 genes of all 3114 chromosomal genes present in the microarray, corresponding to 3.6 % of the whole genome. It is acknowledged that changes in expression of lesser magnitude can nonetheless be of profound biological significance

and, conversely, statistically significant changes that met the 5-fold threshold may not represent biological change of great consequence.

Transcription patterns remain unaffected by addition of DPD to VE14089Δ*luxS*, when compared to VE14089Δ*luxS*. No genes were significantly up- or downregulated in the *luxS* mutant upon addition of DPD, indicating that there weren't any genes that could be assumed to be responding to the signalling molecule AI-2.

**Table 5.4.** List of differentially regulated genes in at least one of the pairwise comparisons: VE14089 and VE14089Δ*luxS*, and VE14089 and VE14089Δ*luxS* supplemented with 10 µM DPD. No significantly regulated genes were obtained in the pairwise comparisons between VE14089Δ*luxS* and VE14089Δ*luxS* supplemented with 10 µM DPD, therefore the data was omitted from this table. Genes belonging to the same transcriptional unit, not significantly expressed, or with a fold change below the imposed cutoff are also indicated. Values with a grey background correspond to a *P*-value above 0.05; Comparisons with a *P*-value below 0.05 and $|\log_2\text{-ratios}| > 5.0$ are indicated in bold type; the locus, gene name, JCVI common name and JCVI cellular role with a *P*-value below 0.05 and $|\log_2\text{-ratios}| > 5.0$ in at least one comparison are indicated in bold type.

| locus | gene name | JCVI Common Name | gene orientation | VE14089 vs VE14089Δ*luxS* | | VE14089 vs *VE14089ΔluxS* + DPD [10µM] | | JCVI Cellular Role: Mainrole |
|---|---|---|---|---|---|---|---|---|
| | | | | $\log_2$ ratio | *p* value | $\log_2$ ratio | *p* value | |
| **EF0019** | ***mptB*** | **PTS system, IIB component** | ▼ | **7.308** | 0.001 | **7.168** | 0.002 | **Transport and binding proteins / Signal transduction** |
| **EF0020** | ***mptA*** | **PTS system, mannose-specific IIAB components** | ▼ | **6.038** | 0.001 | **5.946** | 0.002 | **Transport and binding proteins / Signal transduction** |
| **EF0021** | ***mptC*** | **PTS system, mannose-specific IIC component** | ▼ | **5.526** | 0.001 | 4.705 | 0.002 | **Transport and binding proteins / Signal transduction** |
| **EF0022** | ***mptD*** | **PTS system, mannose-specific IID component** | ▼ | **13.266** | 0.001 | **10.708** | 0.002 | **Transport and binding proteins / Signal transduction** |
| EF0037 | *proA* | gamma-glutamyl phosphate reductase | ▲ | -2.757 | 0.002 | -2.564 | 0.006 | Amino acid biosynthesis |
| **EF0038** | ***proB*** | **glutamate 5-kinase** | ▲ | **-5.989** | 0.001 | **-5.156** | 0.002 | **Amino acid biosynthesis** |
| **EF0066** | ***ruvA*** | **Holliday junction DNA helicase RuvA** | ▼ | **-19.760** | 0.001 | **-14.016** | 0.002 | **DNA metabolism** |
| EF0067 | *ruvB* | Holliday junction DNA helicase RuvB | ▼ | -3.537 | 0.001 | -2.998 | 0.002 | DNA metabolism |
| EF0080 | | gls24 protein | ▼ | 2.296 | 0.002 | 2.111 | 0.004 | Cellular processes |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **EF0081** | | **membrane protein, putative** | ▼ | **5.539** | 0.002 | 4.404 | 0.002 | **Cell envelope** |
| **EF0095** | | **lipoprotein, putative** | ▼ | **6.307** | 0.001 | **5.758** | 0.002 | **Cell envelope** |
| EF0112 | | conserved domain protein | ▼ | -2.329 | 0.004 | -2.351 | 0.005 | Hypothetical proteins |
| **EF0113** | | **hypothetical protein** | ▼ | **-6.083** | 0.001 | -4.649 | 0.002 | **Hypothetical proteins** |
| **EF0237** | ***cbiO*** | **ABC transporter, ATP-binding protein** | ▼ | **-5.381** | 0.001 | -4.471 | 0.002 | **Transport and binding proteins** |
| EF0238 | *cbiO* | ABC transporter, ATP-binding protein | ▼ | -2.590 | 0.002 | -2.309 | 0.009 | Transport and binding proteins |
| EF0239 | | cobalt transport family protein | ▼ | -1.841 | 0.013 | -1.629 | 0.015 | Transport and binding proteins |
| **EF0244** | | **acetyltransferase, GNAT family** | ▲ | **-6.924** | 0.001 | **-5.854** | 0.003 | **Unknown function** |
| **EF0266** | ***hslO*** | **chaperonin, 33 kDa** | ▼ | **-18.690** | 0.001 | **-13.848** | 0.002 | **Protein fate** |
| EF0267 | | zinc-binding TIM-barrel protein, nifR3 family, putative | ▼ | -3.131 | 0.004 | -3.015 | 0.009 | Unknown function |
| EF0291 | *celA* | glycosyl hydrolase, family 1 | ▲ | 4.437 | 0.002 | 3.854 | 0.006 | Energy metabolism |
| **EF0292** | ***celB*** | **PTS system, IIC component** | ▲ | **11.025** | 0.001 | **10.087** | 0.002 | **Transport and binding proteins / Signal transduction** |
| **EF0359** | ***sugE-1*** | **sugE protein** | ▼ | 4.743 | 0.001 | **5.110** | 0.002 | **Protein fate** |
| EF0360 | *sugE-2* | sugE protein | ▼ | 2.559 | 0.003 | 2.764 | 0.003 | Protein fate |
| **EF0371** | | **aminotransferase, class V** | ▼ | **-14.020** | 0.001 | **-13.395** | 0.002 | **Unknown function** |
| **EF0411** | | **PTS system, mannitol-specfic IIBC components** | ▼ | **10.444** | 0.001 | **9.749** | 0.002 | **Transport and binding proteins / Signal transduction** |
| **EF0412** | ***mltF*** | **PTS system, mannitol-specific IIA component** | ▼ | **13.857** | 0.001 | **11.865** | 0.001 | **Transport and binding proteins / Signal transduction** |
| **EF0413** | ***mtlD*** | **mannitol-1-phosphate 5-dehydrogenase** | ▼ | **7.626** | 0.001 | **6.403** | 0.002 | **Energy metabolism** |
| **EF0453** | | **OsmC/Ohr family protein** | ▼ | **9.507** | 0.001 | **9.149** | 0.002 | **Unknown function** |
| **EF0468** | | **LemA family protein** | ▼ | 4.435 | 0.001 | **5.065** | 0.002 | **Unknown function** |
| **EF0475** | ***feoA*** | **ferrous iron transport protein A** | ▼ | **5.429** | 0.001 | 4.249 | 0.002 | **Transport and binding proteins** |
| EF0476 | *feoB* | ferrous iron transport protein B | ▼ | 2.089 | 0.006 | 1.930 | 0.008 | Transport and binding proteins |
| **EF0534** | | **site-specific recombinase, resolvase family** | ▲ | **-5.978** | 0.001 | -3.906 | 0.002 | **DNA metabolism** |
| EF0542 | | conserved hypothetical protein | ▲ | -4.498 | 0.001 | -3.747 | 0.009 | Hypothetical proteins |
| **EF0543** | | **membrane protein, putative** | ▲ | **-6.406** | 0.002 | -4.652 | 0.007 | **Cell envelope** |

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|---|---|---|---|---|---|---|---|---|
| **EF0566** | | **hypothetical protein** | ▼ | **-5.537** | 0.001 | **-5.189** | 0.002 | **Hypothetical proteins** |
| **EF0637** | | **hypothetical protein** | ▼ | **5.393** | 0.001 | 4.474 | 0.002 | **Hypothetical proteins** |
| **EF0638** | | **conserved hypothetical protein** | ▲ | 4.634 | 0.001 | **5.076** | 0.002 | **Hypothetical proteins - conserved** |
| **EF0693** | ***fruK-1*** | **1-phosphofructokinase** | ▼ | **11.755** | 0.001 | **9.193** | 0.002 | **Energy metabolism** |
| **EF0694** | ***fruA*** | **PTS system, fructose-specific family, IIBC components** | ▼ | **7.811** | 0.001 | **6.460** | 0.002 | **Transport and binding proteins / Signal transduction** |
| **EF0695** | ***fruB*** | **PTS system, IIA component** | ▼ | **7.093** | 0.001 | **5.148** | 0.002 | **Transport and binding proteins / Signal transduction** |
| **EF0696** | ***lacD-1*** | **tagatose 1,6-diphosphate aldolase** | ▼ | **6.267** | 0.001 | 4.603 | 0.002 | **Energy metabolism** |
| **EF0819** | | **conserved hypothetical protein** | ▲ | **5.087** | 0.002 | 4.048 | 0.010 | **Hypothetical proteins - conserved** |
| **EF0848** | ***acpS*** | **holo-(acyl-carrier-protein) synthase** | ▼ | **-5.407** | 0.001 | -4.387 | 0.002 | **Fatty acid and phospholipid metabolism** |
| EF0849 | *alr* | alanine racemase | ▼ | -3.754 | 0.001 | -3.143 | 0.002 | Cell envelope |
| EF0850 | | transcriptional regulator, PemK family | ▼ | -3.750 | 0.002 | -3.631 | 0.002 | Regulatory functions |
| **EF0873** | | **transcriptional regulator, Cro/CI family** | ▲ | **-6.325** | 0.001 | **-5.738** | 0.002 | **Regulatory functions** |
| EF0901 | | isopentenyl diphosphate delta isomerase, putative | ▲ | -1.184 | 0.163 | -1.180 | 0.159 | Biosynthesis of cofactors, prosthetic groups, and carriers |
| EF0902 | | phosphomevalonate kinase | ▲ | -1.639 | 0.010 | -1.624 | 0.008 | Biosynthesis of cofactors, prosthetic groups, and carriers / Central intermediary metabolism |
| EF0903 | *mvaD* | mevalonate diphosphate decarboxylase | ▲ | -2.854 | 0.001 | -2.491 | 0.003 | Biosynthesis of cofactors, prosthetic groups, and carriers / Central intermediary metabolism |
| **EF0904** | ***mvk*** | **mevalonate kinase** | ▲ | **-5.716** | 0.001 | -4.700 | 0.002 | **Biosynthesis of cofactors, prosthetic groups, and carriers / Central intermediary metabolism** |
| EF0928 | | glucose uptake protein | ▼ | -3.157 | 0.001 | -2.704 | 0.002 | Transport and binding proteins |
| **EF0929** | | **amino acid permease family protein** | ▼ | **-17.819** | 0.001 | **-16.002** | 0.001 | **Transport and binding proteins** |
| **EF0953** | | **hypothetical protein** | ▼ | **-6.052** | 0.001 | -3.828 | 0.004 | **Hypothetical proteins** |
| **EF1006** | | **conserved hypothetical protein** | ▲ | **6.204** | 0.002 | 4.966 | 0.004 | **Hypothetical proteins - conserved** |
| **EF1012** | | **PTS system, IIB component** | ▼ | **22.983** | 0.001 | **21.558** | 0.001 | **Transport and binding proteins / Signal transduction** |
| **EF1013** | | **PTS system, IIC component** | ▼ | **75.895** | 0.001 | **67.113** | 0.001 | **Transport and binding proteins / Signal transduction** |
| **EF1014** | | **hypothetical protein** | ▼ | **50.548** | 0.001 | **44.472** | 0.001 | **Hypothetical proteins** |
| **EF1016** | | **conserved hypothetical protein** | ▼ | **45.073** | 0.001 | **37.947** | 0.002 | **Hypothetical proteins - conserved** |
| **EF1017** | | **PTS system, IIB component** | ▼ | **17.994** | 0.001 | **17.405** | 0.001 | **Transport and binding proteins / Signal transduction** |

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|---|---|---|---|---|---|---|---|---|
| **EF1018** | | **PTS system, IIA component** | ▼ | **11.656** | 0.001 | **11.943** | 0.001 | **Transport and binding proteins / Signal transduction** |
| **EF1019** | | **PTS system, IIC component** | ▼ | **14.575** | 0.001 | **12.676** | 0.002 | **Transport and binding proteins / Signal transduction** |
| **EF1022** | | **hypothetical protein** | ▼ | **8.216** | 0.002 | **6.892** | 0.003 | **Hypothetical proteins** |
| EF1057 | | Mn2+/Fe2+ transporter, NRAMP family | ▼ | 3.497 | 0.004 | 2.718 | 0.007 | Transport and binding proteins |
| **EF1058** | | **universal stress protein family** | ▼ | **6.579** | 0.002 | 3.762 | 0.020 | **Cellular processes** |
| **EF1149** | ***recU*** | **recombination protein U** | ▲ | **-5.460** | 0.001 | -4.483 | 0.002 | **DNA metabolism** |
| **EF1172** | | **teichoic acid biosynthesis protein B, putative** | ▼ | **-8.166** | 0.001 | **-7.073** | 0.003 | **Cell envelope** |
| **EF1173** | | **glycosyl transferase, WecB/TagA/CpsF family** | ▼ | **-6.272** | 0.001 | **-5.254** | 0.002 | **Cell envelope** |
| **EF1174** | | **hypothetical protein** | ▼ | **-5.808** | 0.001 | **-5.454** | 0.002 | **Hypothetical proteins** |
| **EF1182** | ***luxS*** | **autoinducer-2 production protein LuxS** | ▲ | **-119.061** | 0.001 | **-110.475** | 0.002 | **Cellular processes** |
| **EF1206** | | **malate dehydrogenase, decarboxylating** | ▲ | 4.634 | 0.001 | **5.109** | 0.002 | **Energy metabolism** |
| EF1207 | | citrate carrier protein, CCS family | ▲ | 3.679 | 0.002 | 4.151 | 0.002 | Transport and binding proteins |
| **EF1351** | | **hypothetical protein** | ▼ | **6.632** | 0.001 | **5.161** | 0.003 | **Hypothetical proteins** |
| EF1352 | | magnesium-translocating P-type ATPase | ▼ | 3.338 | 0.002 | 3.059 | 0.003 | Transport and binding proteins |
| **EF1368** | | **conserved hypothetical protein** | ▲ | **6.427** | 0.001 | **5.521** | 0.002 | **Hypothetical proteins - conserved** |
| **EF1582** | | **major facilitator family transporter** | ▲ | **-7.998** | 0.002 | **-7.075** | 0.002 | **Transport and binding proteins** |
| **EF1591** | | **transcriptional regulator, AraC family** | ▲ | **5.424** | 0.001 | 4.693 | 0.002 | **Regulatory functions** |
| **EF1599** | | **TPR domain transcriptional regulator, Cro/CI family** | ▲ | **-9.853** | 0.001 | **-7.473** | 0.002 | **Regulatory functions** |
| **EF1655** | | **2-dehydropantoate 2-reductase, putative** | ▲ | **8.948** | 0.003 | **7.608** | 0.002 | **Biosynthesis of cofactors, prosthetic groups, and carriers** |
| **EF1658** | ***bkdC*** | **branched-chain alpha-keto acid, E2 component, dihydrolipoamide acetyltransferase** | ▲ | **11.589** | 0.001 | **11.484** | 0.003 | **Energy metabolism** |
| **EF1659** | ***bkdB*** | **branched-chain alpha-keto acid dehydrogenase, E1 component, beta subunit** | ▲ | **30.492** | 0.001 | **29.337** | 0.002 | **Energy metabolism** |
| **EF1660** | ***bkdA*** | **branched-chain alpha-keto acid dehydrogenase, E1 component, alpha subunit** | ▲ | **43.955** | 0.001 | **44.740** | 0.002 | **Energy metabolism** |
| **EF1661** | ***bkdD*** | **branched-chain alpha-keto acid** | ▲ | **34.790** | 0.001 | **34.804** | 0.002 | **Energy metabolism** |

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|---|---|---|---|---|---|---|---|---|
| | | **dehydrogenase, E3 component, dihydrolipoamide dehydrogenase** | | | | | | |
| **EF1662** | ***buk*** | **butyrate kinase** | ▲ | **18.434** | 0.001 | **18.639** | 0.001 | **Energy metabolism** |
| **EF1663** | ***ptb*** | **branched-chain phosphotransacylase** | ▲ | **33.405** | 0.001 | **33.734** | 0.001 | **Fatty acid and phospholipid metabolism** |
| **EF1667** | | **short chain dehydrogenase family protein** | ▼ | **8.323** | 0.001 | **6.537** | 0.005 | **Unknown function** |
| **EF1671** | | **oxidoreductase, zinc-binding** | ▲ | **6.522** | 0.001 | **5.730** | 0.002 | **Unknown function** |
| **EF1677** | | **lipoprotein, putative** | ▲ | **5.133** | 0.001 | 4.663 | 0.002 | **Cell envelope** |
| **EF1751** | | **membrane protein, putative** | ▲ | **5.541** | 0.001 | 4.002 | 0.004 | **Cell envelope** |
| EF1752 | | conserved hypothetical protein | ▲ | 3.054 | 0.003 | 2.416 | 0.008 | Hypothetical proteins |
| **EF1771** | | **conserved hypothetical protein TIGR00257** | ▼ | **-8.339** | 0.001 | **-6.515** | 0.002 | **Hypothetical proteins - conserved** |
| EF1772 | | conserved hypothetical protein | ▼ | -3.528 | 0.030 | -3.115 | 0.031 | Hypothetical proteins |
| EF1863 | *hk08* | sensor histidine kinase | ▲ | -1.839 | 0.073 | -1.591 | 0.144 | Signal transduction |
| **EF1864** | ***rr08*** | **DNA-binding response regulator** | ▲ | **-5.792** | 0.002 | -2.975 | 0.004 | **Regulatory functions / Signal transduction** |
| EF1899 | *trmD* | tRNA (guanine-N1)-methyltransferase | ▲ | -2.167 | 0.004 | -2.006 | 0.005 | Protein synthesis |
| **EF1900** | ***rimM*** | **16S rRNA processing protein RimM** | ▲ | **-5.311** | 0.001 | -4.109 | 0.002 | **Transcription** |
| EF1927 | *glpF* | glycerol uptake facilitator protein | ▲ | 4.946 | 0.002 | 4.892 | 0.007 | Transport and binding proteins |
| EF1928 | | alpha-glycerophosphate oxidase | ▲ | 4.200 | 0.002 | 4.321 | 0.002 | Energy metabolism |
| **EF1929** | ***glpK*** | **glycerol kinase** | ▲ | **6.049** | 0.001 | **6.243** | 0.002 | **Energy metabolism** |
| **EF1981** | | **hypothetical protein** | ▼ | **12.818** | 0.001 | **8.752** | 0.003 | **Hypothetical proteins** |
| **EF1982** | | **universal stress protein family** | ▼ | **7.390** | 0.002 | **6.391** | 0.005 | **Cellular processes** |
| **EF2048** | ***rlmN*** | **conserved hypothetical protein TIGR00048** | ▲ | **-5.701** | 0.001 | -4.669 | 0.003 | **Hypothetical proteins - conserved** |
| EF2175 | | licD-related protein | ▲ | -2.328 | 0.002 | -2.090 | 0.004 | Unknown function |
| **EF2176** | | **glycosyl transferase, group 2 family protein** | ▲ | **-6.691** | 0.001 | **-5.057** | 0.002 | **Cell envelope** |
| EF2195 | | glycosyl transferase, group 2 family protein | ▲ | -1.685 | 0.005 | -1.516 | 0.011 | Cell envelope |
| EF2196 | | glycosyl transferase, group 2 family protein | ▲ | -1.917 | 0.007 | -1.822 | 0.016 | Cell envelope |
| **EF2197** | ***epa locus*** | **glycosyl transferase, group 2 family protein** | ▲ | **-5.537** | 0.001 | -4.516 | 0.003 | **Cell envelope** |

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|---|---|---|---|---|---|---|---|---|
| **EF2209** | | **hypothetical protein** | ▲ | **7.022** | 0.002 | **5.124** | 0.009 | **Hypothetical proteins** |
| EF2252 | | hypothetical protein | ▲ | -4.210 | 0.001 | -3.641 | 0.003 | hypothetical protein |
| **EF2253** | | **conserved hypothetical protein** | ▲ | **-5.458** | 0.001 | -4.620 | 0.002 | **Hypothetical proteins - conserved** |
| **EF2275** | | **hypothetical protein** | ▼ | **-5.332** | 0.002 | -4.186 | 0.006 | **Hypothetical proteins** |
| EF2276 | | hypothetical protein | ▼ | -2.536 | 0.001 | -2.362 | 0.002 | hypothetical protein |
| EF2364 | | xanthine permease | ▲ | -3.613 | 0.001 | -3.335 | 0.002 | Transport and binding proteins |
| **EF2365** | ***xpt*** | **xanthine phosphoribosyltransferase** | ▲ | **-5.891** | 0.001 | **-5.170** | 0.002 | **Purines, pyrimidines, nucleosides, and nucleotides** |
| **EF2483** | | **hypothetical protein** | ▲ | **11.234** | 0.002 | **8.391** | 0.004 | **Hypothetical proteins** |
| EF2604 | | conserved hypothetical protein | ▲ | -1.393 | 0.149 | -1.646 | 0.014 | Hypothetical proteins |
| **EF2605** | ***murAA*** | **UDP-N-acetylglucosamine 1-carboxyvinyltransferase 1** | ▲ | **-5.961** | 0.002 | **-5.390** | 0.002 | **Cell envelope** |
| **EF2606** | | **conserved hypothetical protein** | ▲ | **-11.823** | 0.001 | **-9.890** | 0.004 | **Hypothetical proteins - conserved** |
| **EF2627** | | **teichoic acid glycosylation protein, putative** | ▲ | **-9.440** | 0.001 | **-6.234** | 0.005 | **Cell envelope** |
| EF2628 | | N-acetylmuramoyl-L-alanine amidase, family 4 | ▲ | -2.921 | 0.002 | -2.755 | 0.004 | Cell envelope |
| EF2629 | | hypothetical protein | ▲ | -2.230 | 0.004 | -2.063 | 0.005 | hypothetical protein |
| **EF2630** | | **transcriptional regulator** | ▲ | **-5.874** | 0.002 | -4.687 | 0.005 | **Regulatory functions** |
| EF2631 | | hypothetical protein | ▲ | -3.555 | 0.002 | -2.925 | 0.025 | hypothetical protein |
| **EF2643** | | **conserved hypothetical protein** | ▲ | **5.015** | 0.001 | 4.158 | 0.002 | **Hypothetical proteins - conserved** |
| **EF2646** | | **glycerate kinase, putative** | ▲ | **7.921** | 0.001 | **7.128** | 0.002 | **Energy metabolism** |
| **EF2647** | | **permease, GntP family** | ▲ | **7.510** | 0.002 | **8.288** | 0.002 | **Transport and binding proteins** |
| **EF2797** | | **hypothetical protein** | ▲ | **5.364** | 0.001 | 3.686 | 0.004 | **Hypothetical proteins** |
| **EF2801** | | **hypothetical protein** | ▲ | **-7.244** | 0.001 | **-6.395** | 0.003 | **Hypothetical proteins** |
| EF2871 | *nadD* | nicotinate-nucleotide adenylyltransferase | ▲ | -1.605 | 0.009 | -1.651 | 0.008 | Biosynthesis of cofactors, prosthetic groups, and carriers |
| EF2872 | | conserved hypothetical protein TIGR00253 | ▲ | -1.718 | 0.020 | -1.651 | 0.018 | Hypothetical proteins |
| EF2873 | | GTPase of unknown function | ▲ | -2.431 | 0.005 | -2.456 | 0.005 | Unknown function |
| **EF2874** | | **hydrolase, HAD subfamily IIIA** | ▲ | **-5.860** | 0.001 | -4.737 | 0.002 | **Unknown function** |

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|---|---|---|---|---|---|---|---|---|
| EF2875 | *accA* | acetyl-CoA carboxylase, carboxyl transferase alpha subunit | ▲ | -1.516 | 0.009 | -1.489 | 0.011 | Fatty acid and phospholipid metabolism |
| EF2876 | *accD* | acetyl-CoA carboxylase, carboxyl transferase beta subunit | ▲ | -1.539 | 0.011 | -1.503 | 0.011 | Fatty acid and phospholipid metabolism |
| EF2878 | *fabZ-2* | (3R)-hydroxymyristoyl-(acyl-carrier-protein) dehydratase | ▲ | -1.187 | 0.206 | -1.322 | 0.028 | Fatty acid and phospholipid metabolism |
| EF2879 | *accB* | acetyl-CoA carboxylase, biotin carboxyl carrier protein | ▲ | -1.274 | 0.047 | -1.274 | 0.040 | Fatty acid and phospholipid metabolism |
| EF2880 | *fabF-2* | 3-oxoacyl-(acyl-carrier-protein) synthase II | ▲ | -1.386 | 0.019 | -1.352 | 0.027 | Fatty acid and phospholipid metabolism |
| EF2881 | *fabG* | 3-oxoacyl-(acyl-carrier-protein) reductase | ▲ | -1.379 | 0.018 | -1.353 | 0.026 | Fatty acid and phospholipid metabolism |
| EF2882 | *fabD* | malonyl CoA-acyl carrier protein transacylase | ▲ | -2.159 | 0.006 | -2.116 | 0.003 | Fatty acid and phospholipid metabolism |
| **EF2883** | ***fabK*** | **enoyl-(acyl-carrier-protein) reductase II** | ▲ | **-5.130** | 0.001 | -4.588 | 0.002 | **Fatty acid and phospholipid metabolism** |
| **EF2891** | | **glycosyl transferase, group 1 family protein** | ▲ | **-6.088** | 0.001 | -3.983 | 0.002 | **Cell envelope** |
| EF2964 | *ulaA* | putative transport protein, SgaT family | ▲ | 4.302 | 0.001 | 3.964 | 0.002 | Transport and binding proteins |
| EF2965 | | conserved hypothetical protein | ▲ | 4.622 | 0.003 | 4.582 | 0.002 | Hypothetical proteins |
| **EF2966** | | **transcriptional antiterminator, bglG family** | ▲ | **6.427** | 0.001 | **5.989** | 0.002 | **Regulatory functions** |
| **EF3021** | | **conserved hypothetical protein** | ▲ | **6.801** | 0.001 | **5.922** | 0.003 | **Hypothetical proteins - conserved** |
| **EF3101** | | **conserved domain protein** | ▲ | **-5.807** | 0.001 | -4.163 | 0.016 | **Hypothetical proteins - conserved** |
| EF3102 | | hypothetical protein | ▲ | -2.791 | 0.002 | -2.223 | 0.003 | hypothetical protein |
| EF3103 | | membrane protein, putative | ▲ | -3.172 | 0.002 | -2.895 | 0.017 | Cell envelope |
| EF3137 | | PTS system, IIB component | ▲ | 3.498 | 0.003 | 3.176 | 0.003 | Transport and binding proteins / Signal transduction |
| EF3138 | | PTS system, IID component | ▲ | 3.842 | 0.003 | 3.446 | 0.005 | Transport and binding proteins / Signal transduction |
| **EF3139** | | **PTS system, IIC component** | ▲ | **6.164** | 0.001 | **5.166** | 0.002 | **Transport and binding proteins / Signal transduction** |
| **EF3140** | | **alcohol dehydrogenase, iron-containing** | ▲ | **7.712** | 0.001 | **6.707** | 0.003 | **Energy metabolism** |
| **EF3141** | | **D-isomer specific 2-hydroxyacid dehydrogenase family protein** | ▲ | **8.649** | 0.001 | **7.284** | 0.002 | **Unknown function** |
| **EF3142** | | **6-phosphogluconate dehydrogenase family protein** | ▲ | **10.428** | 0.001 | **8.679** | 0.002 | **Energy metabolism** |
| **EF3145** | | **hypothetical protein** | ▲ | **-6.528** | 0.002 | **-5.164** | 0.002 | **Hypothetical proteins** |
| **EF3193** | ***lrgB*** | **LrgB family protein** | ▲ | **27.551** | 0.001 | **18.507** | 0.002 | **Unknown function** |

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|---|---|---|---|---|---|---|---|---|
| **EF3194** | ***lrgA*** | **LrgA family protein** | ▲ | **57.609** | 0.001 | **39.002** | 0.002 | **Unknown function** |
| **EF3223** | | **hypothetical protein** | ▲ | **-5.519** | 0.001 | -4.125 | 0.005 | **Hypothetical proteins** |
| EF3298 | | chromosome partitioning protein ParB family | ▲ | -2.086 | 0.004 | -1.976 | 0.004 | Cellular processes |
| EF3299 | | ATPase, ParA family | ▲ | -3.615 | 0.002 | -3.478 | 0.002 | Cellular processes |
| **EF3300** | ***gidB*** | **glucose-inhibited division protein B** | ▲ | **-5.130** | 0.001 | **-5.104** | 0.002 | **Unknown function** |
| **EF3304** | ***mipB*** | **transaldolase-like protein MIPB** | ▲ | **9.690** | 0.001 | **8.656** | 0.002 | **Energy metabolism** |
| **EF3305** | ***srlA*** | **PTS system, sorbitol-specific IIA component** | ▲ | **7.590** | 0.001 | **7.590** | 0.002 | **Transport and binding proteins / Signal transduction** |
| **EF3306** | ***srlB*** | **PTS system, sorbitol-specific IIBC components** | ▲ | **6.999** | 0.001 | **7.374** | 0.002 | **Transport and binding proteins / Signal transduction** |
| **EF3307** | ***srlE*** | **PTS system, sorbitol-specific IIC component** | ▲ | **6.969** | 0.002 | **7.379** | 0.002 | **Transport and binding proteins / Signal transduction** |
| **EF3308** | ***srlR*** | **transcriptional regulator SrlR** | ▲ | **13.347** | 0.001 | **12.900** | 0.002 | **Regulatory functions** |
| **EF3309** | ***srlM*** | **putative transcriptional activator SrlM** | ▲ | **17.406** | 0.001 | **16.503** | 0.002 | **Regulatory functions** |
| **EF3310** | ***srlD*** | **oxidoreductase, short chain dehydrogenase/reductase family** | ▲ | **10.285** | 0.001 | **11.530** | 0.001 | **Unknown function** |

- $\log_2$-ratios were calculated as [VE14089Δ*luxS*]/[ VE14089] and [VE14089Δ*luxS* supplemented with 10 µM DPD]/[VE14089], with a minus signs representing a down-regulation.

The full 113 differentially regulated genes, 70 upregulated and 43 downregulated, were found to be uniquely affected by the *luxS* mutation, independently of DPD addition, when compared to the parental strain. Almost 40 % of the down-regulated genes and more than 30 % of the up-regulated genes in the *luxS* mutant, when compared to VE14089, are annotated as hypothetical proteins or proteins of unknown function. Genes significantly expressed but with a fold-change in both comparisons below 5 were recovered in order to check if they follow the same regulatory trend as the transcriptional unit they belong to, as predicted by http://biocyc.org (Karp et al., 2005). Consistent transcriptional changes in complete operons and clusters were detected each time for every transcriptional unit (Table 5.4).

The *luxS* mutation resulted in changes in nearly every cellular process (Figure 5.7), and the affected genes were distributed throughout the genome (Table 5.4). Unknown function or hypothetical protein genes contributed the largest fraction, more than 35 %, followed by genes required for transport and binding, signal transduction, energy metabolism, cell envelope, and regulatory functions. Important effects were also observed for DNA metabolism, fatty acid and phospholipid metabolism, and biosynthesis of cofactors. The *luxS* mutation induced deep changes in the transcription of many genes, and extracellular addition of AI-2 did not significantly alter gene expression in VE14089Δ*luxS*. Below, we will therefore discuss several genes that were differentially expressed in the *luxS* mutant, independently of extracellular added DPD.

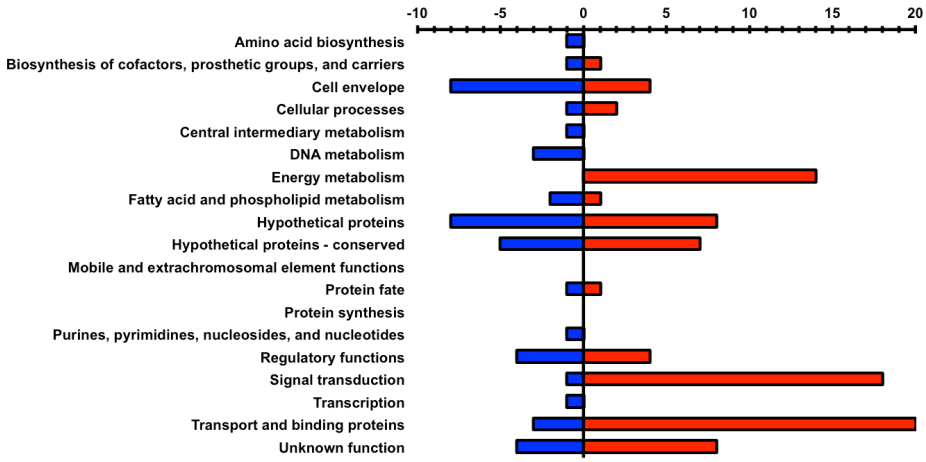


**Figure 5.7.** Distribution by number of differentially expressed genes, up-regulated (in red and shown as positive) and down-regulated (in blue and shown as negative), and by JCVI (http://cmr.jcvi.org) cellular mainrole found to be affected by the *luxS* mutation, which was independent of DPD addition, when compared to the parental strain VE14089. The genes with more than one cellular mainrole were counted twice. Percentage of differentially expressed genes with | log2-ratios | > 5.0 in each JCVI cellular mainrole: Cellular processes (33 %), DNA metabolism (33 %), Energy metabolism (50 %), Fatty acid and phospholipid metabolism (33 %), Hypothetical proteins (19 %), Hypothetical proteins – conserved (17 %), Protein fate (50 %), Regulatory functions (25 %), Signal transduction (47 %), Transport and binding proteins (43 %), and Unknown function (33 %).", should be replaced by: "Percentage of differentially expressed genes with | log2-ratios | > 5.0 in each JCVI cellular mainrole: Transport and binding proteins (20 %), Signal transduction (17 %), Hypothetical proteins (14 %), Energy metabolism (12 %), Cell envelope (11 %), Hypothetical proteins - conserved (11 %), Unknown function (11 %), Regulatory functions (7 %), Cellular processes (3 %), DNA metabolism (3 %), Fatty acid and phospholipid metabolism (3 %), Biosynthesis of cofactors, prosthetic groups, and carriers (2 %), Protein fate (2 %), Amino acid biosynthesis (1 %), Central intermediary metabolism (1 %), Purines, pyrimidines, nucleosides, and nucleotides (1 %), Transcription (1 %).

## AMC

In *E. faecalis* V583, LuxS occupies the same position in the AMC as that of most AI-2 type QS system possessing bacteria, which allows the production of AI-2 and can contribute to the recycling of the building blocks needed for methionine biosynthesis. However, *in silico* genomic analysis showed that there is not a complete AMC (Figure 5.8). According to the Sri International Pathway Tools (v15.5) (Karp et al., 2010), that predicts current and filled pathway holes, three pathway holes were identified in *E. faecalis* V583 methionine biosynthesis pathway, including the AMC.

In a complete AMC, SAM is regenerated from SAH via homocysteine and methionine, allowing another round of methylation/ transmethylation to occur. The conversion of homocysteine to methionine can be carried out by either MetE or MetH (Doherty et al., 2010). Despite, in *E. faecalis* V583 genome, *ef0395* being annotated as a putative methionine synthase, a protein BLAST (Altschul et al., 1997; 2005), against the microbial protein database from NCBI, reveals a high identity of EF0395 to MetE (2.1.1.14), an enzyme that could catalyse the last step in the production of methionine, and to the putative MetE that can be found in *E. faecalis* OG1RF, previously described as being auxotrophic for methionine with an OG1 DNA library unable to complement a methionine auxotrophic *E. coli* strain (Murray et al., 1993). CGH-results indicate that V583, the parental strain of VE14089, have similar requirements for amino acids as OG1RF, which was shown to be auxotrophic for amino acids like histidine, isoleucine, methionine, and tryptophan (Murray et al., 1993; Vebø et al., 2009). This methionine auxotrophy reinforces the effective presence of a pathway hole in the biosynthesis pathway for this amino acid in V583. Bacteria lacking an intact AMC lose the ability to recycle methionine and, thus, are dependent on its uptake or synthesis from environmental sulphur

sources. This metabolic gap could be filled by methionine-specific transport systems detected in the genome (Figure 5.8), like *metNPQ* (*ef2082-0*, *ef2498-6* and *ef3200-198*) and *mstTUV* (*ef2154-2*), or by methionine-related ABC transport system involved in the transport of oligopeptides or methionine, *tomA* (*ef3081*) gene that belongs to the *opp* family (Rodionov et al., 2004; Vitreschak et al., 2008).

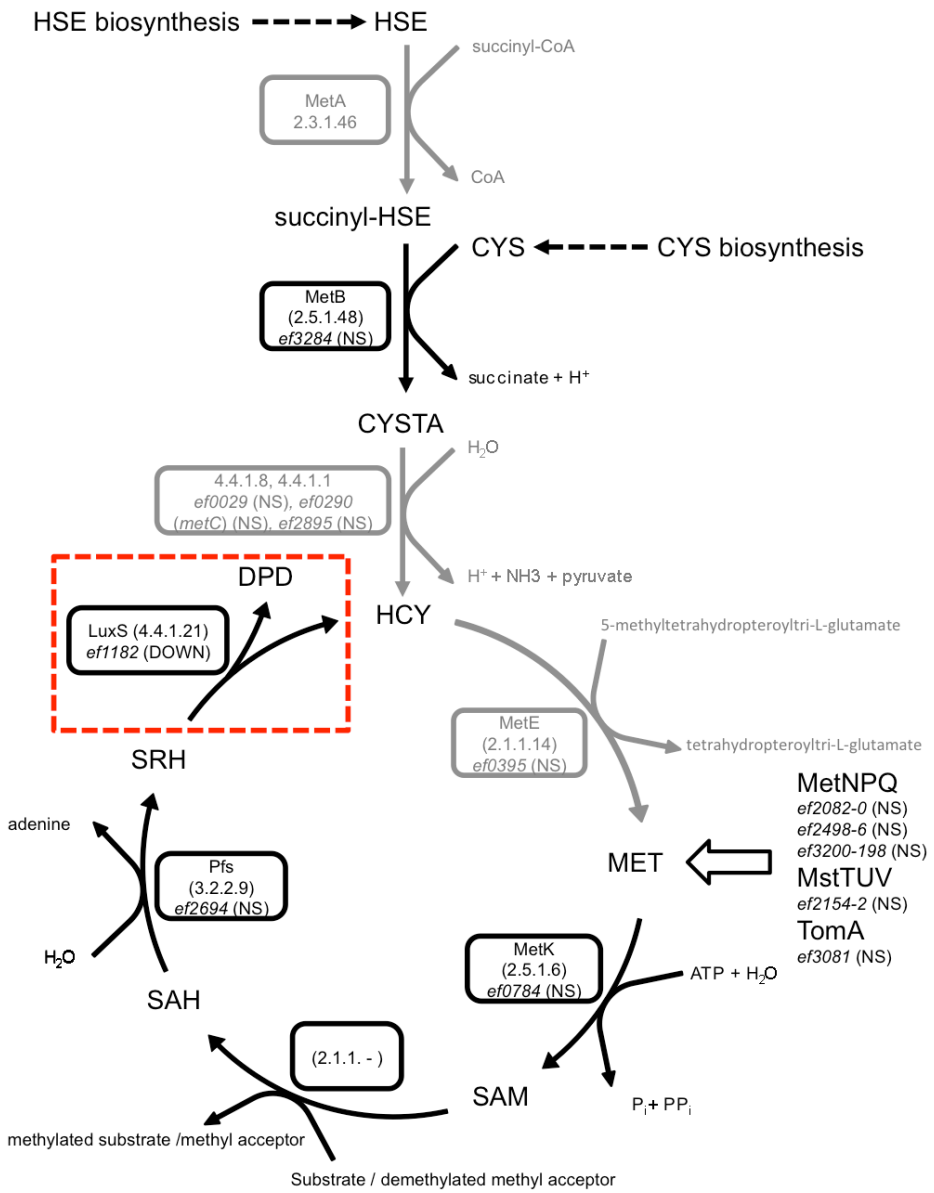


**Figure 5.8.** AMC in V583. Possible pathway holes, as identified by Sri International Pathway Tools (v15.5) (Karp et al., 2010), are indicated in faded grey; the reaction that corresponds to LuxS activity, which is absent in the *luxS* mutant, is indicated in a dashed red box; putative methionine uptake transporters are indicated next to the white arrow; differential expression of genes in the VE14089Δ*luxS* mutant when

compared to VE14089, independently of extracellular added DPD, are indicated next to the corresponding gene locus (NS: not significantly expressed (*P*-value > 0.05 or *P*-value < 0.05 and | $\log_2$ ratio | < 5), DOWN: down-regulation (*P*-value < 0.05, $\log_2$ ratio < -5). HSE: L-homoserine; CYS: L-cysteine; CYSTA: L-cystathionine; HCY: L-homocysteine; MET: L-methionine; SAM: S-adenosyl-L-methionine; SAH: S-adenosyl-L-homocysteine; SRH: S-ribosyl-L-homocysteine: DPD: (*S*)-4,5-dihydroxypentan-2,3-dione.

The absence of a full AMC in VE14089, its putative methionine auxotrophy and the indication that Pfs is sufficient for SAH detoxification (Challan Belval et al., 2006), would reduce the metabolic impact of the added *luxS* deletion in VE14089Δ*luxS*, and could explain the lack of apparent growth defects.

The *luxS* mutation did not significantly alter the expression of any of the genes involved in the AMC in VE14089, except for *luxS* (*ef1182*), the deleted gene (Figure 5.8). There were also no significant alterations in the putative methionine transporters expression or the main pathways for methionine biosynthesis, from homoserine (or even the from the homoserine aspartate precursor) or serine precursors (data not shown).

However, the lack of significant differential expression of genes involved in methionine import and the AMC does not necessarily indicate that the metabolite flux on those pathways remains unaltered in the mutant. In *E. faecalis* the regulation of several genes involved in the methionine cell metabolism are regulated by RNA mediated mechanisms, the transport of methionine and methionine precursors by T-boxes, while the conversion of methionine by MetK in the AMC is regulated by SAM availability and its interaction with a S-box (Rodionov et al., 2004; Vitreschak et al., 2008). The activity of DNA methyltransferases, which are often used for silencing and

regulating genes without changing the original DNA sequence as well as protecting DNA from enzymatic cleavage, can be inhibited by a reduction in the availability of the methyl donor SAM (Kozlova et al., 2008) but also by the accumulation of the intermediary SAH, a potent inhibitor of the methyltransferases, making its removal from the cell important for normal physiology (Keller and Surette, 2006). However, whether *luxS* inactivation reduces SAM concentrations sufficiently to influence these processes significantly remains to be seen.

Having no complete cycle for regeneration of methionine raises the question of whether the only function of LuxS in *E. faecalis* is as a QS molecule synthase, with a concomitant homocysteine regeneration, or whether LuxS in *E. faecalis* has another direct or indirect metabolic role, like maintaining the homeostasis of AMC metabolites and ensuring effective methylation (Heurlier et al., 2009).

## Energy metabolism

In our study, most of the observed up-regulated genes in the *luxS* mutant had functions related with energy metabolism. All differentially expressed components of the phosphoenolpyruvate (PEP) transport system (PTS) were strongly upregulated, between 5 and 75-fold. The PTS is a multiprotein phosphorelay system that couples the transport of sugars across the bacterial cell membrane with their simultaneous phosphorylation. The PTS is composed of three distinct proteins: enzyme I (EI), histidine protein (HPr) and enzyme II (EII). This PTS is the main sugar uptake mechanism in *E. faecalis*, just like for various other bacteria, but can also be associated to amino sugar and nucleotide sugar transport.

V583 genome contains 35 probable PTS systems and complete transport and pathways for exploitation of more than 15 different sugars

have been predicted (Paulsen et al., 2003). V583 has been described as having the ability to utilize cellobiose, fructose, lactose, galactose, glucose, glycerol, maltose, mannitol, mannose, N-acetylglucosamine, ribose, sucrose, and trehalose. Although genes from the degradative pathways of arabinose, rhamnose and xylose can be identified in the genome, no specific transporter has been described and no fermentation for those sugars has been observed (Aakra et al., 2007). Energy production from these substrates occurs via glycolysis or the pentose phosphate pathways, with the tricarboxylic acid (TCA) cycle absent (Paulsen et al., 2003).

The up-regulated PTSs are involved in the main glucose and fructose transport (Muraoka et al., 1991), the mannose PTS (Man family) (*mptBACD* operon— *ef0019-22*), and putatively involved in the transport of cellobiose (Lac family) (*ef0292* that belongs to the *ef0292-1* operon, *ef1012*, *ef1013*, *ef1017-8* and *ef1019*), mannitol (Fru family) (*ef0411-2* operon), fructose (Fru family) (*ef0694-5* operon), sorbitol/glucitol (other family) (*srlEBA* operon— *ef3307-5*), and gluconate (*ef3139* of *ef3139-6* operon and *ef2647,* belonging to the GntP family).

Genes involved in the utilization of these transported carbohydrates, like for mannitol (*mtlD* — *ef0413*), fructose (*fruK-1*— *ef0693*), sorbitol (*srlD* — *ef3310*) and gluconate (*ef3140*, *ef3141* and *ef3142* that belong to the *ef3142-34* cluster that facilitates gluconate uptake and catabolism via the mannonate route (Vebø et al., 2009), and *ef2646* a putative glycerate kinase, the last step in the non-phosphorylative Entner-Doudoroff pathway), were also up-regulated in the *luxS* mutant. *glpK* (*ef1929*) from the glycerol catabolic operon *glpKOF* (*ef1929-7*), *mipB* (*ef3304*), a transaldolase important for the balance of metabolites of the non-oxidative phase of the pentose phosphate pathway, and *maeE* (*ef1206*), coding for a malate

oxidative decarboxylating enzyme (Espariz et al., 2011), also had their transcription increased.

Along with the increased transcription *srlEBA* and *srlD*, the other two regulatory genes of the *srl* operon (Boyd et al., 2000), *srlR* (*ef3308*) that codes for a transcriptional regulator and *srlM* (*ef3309*) that codes for a putative activator, were also up-regulated. *ef2966*, erroneously annotated as a BglG-type antiterminator and lacking an RNA-binding domain (Opsata et al., 2010), has PTS domains and PTS regulation domains, may therefore be a transcriptional regulator, and belongs to a transcriptional unit (*ef2966-4*) with a gene (*ef2964*) encoding a sugar permease putatively involved in the L-ascorbate utilization pathway. This gene was up-regulated as well as *ef1591*, a transcriptional regulator of the AraC family, and *lacD-1* (*ef0696*). In contrast to its paralog LacD-2, which is involved in lactose metabolism, LacD-1 has been adapted to function as a sensor of its substrates, which are the central intermediates of the Embden-Meyerhoff-Parnas glycolytic pathway, and has been described to be involved in a unique form of carbon catabolite repression (Commichau and Stülke, 2008).

In *E. faecalis* the branched-chain alpha-keto acid dehydrogenase (BCKDH) complex, encoded in gene cluster *ptb-buk-bkdDABC* (*ef1663-58*), is devoted to energy generation (Fernández and Zúñiga, 2006). This complex catalyses the oxidative decarboxylation of branched-chained-keto acids, valine, leucine, and isoleucine, with the concomitant reduction of $NAD^+$, supposedly generating the corresponding acyl coenzyme A and ATP via substrate level phosphorylation (Solheim et al., 2007). In the *luxS* mutant the whole gene cluster was up-regulated indicating an increased usage of branched-chain amino acid (BCAA) possibly as an alternative energy source. There is no evidence linking the enterococcal *bkd* gene cluster to the biosynthesis of branched-chain fatty acids, as found in *B.*

*subtilis* (Solheim et al., 2007). The up-regulation of this operon should therefore not be correlated to the observed repression of genes involved in fatty acid synthase II (FASII) in the mutant.

When compared to VE14089, the *luxS* mutant displays an increased transcription of genes involved in the transport and utilization of less preferred carbon sources. In *S. mutans*, the mutation in a methyltransferase was associated with the up-regulation of loci dedicated to carbohydrate transport, increasing the expression of a mannose/fructose PTS that is fructose-inducible, as well as other secondary carbohydrate sugar transport operons such as mannitol/sorbitol, cellobiose, and the multiple-sugar-metabolism operon, each potentially employing multiple mechanisms of regulating expression (Banas et al., 2011). The majority of the changes triggered by the loss of DNA methylation resulted in up-regulation of particular genes, implying that DNA methylation most often has a moderating effect on gene expression (Banas et al., 2011). A change in the availability of SAM could similarly affect the *luxS* mutant methyltransferases activity, therefore corroborating the observed increase of energy metabolism gene transcription. The less common carbohydrates are channelled to the energy production pathways available to *E. faecalis*, such as the Embden-Meyerhoff-Parnas glycolytic pathway, the pentose phosphate pathway, but also the Entner-Doudoroff pathway. The *luxS* mutant is also up-regulating genes that allow the usage of BCAAs, glycerol and malate. This reveals an increased carbon flux from sources other than hexose sugars that are rapidly depleted from 2×YT growth media, where no glucose has been added.

## Cell envelope components

In the *luxS* mutant we can observe the down-regulation of a number of genes associated with the different cell envelope structures. These involve the peptidoglycan (*ef2605 — murAA*), teichoic acids (*ef1172* and *ef1173* genes from the *ef1172-4* operon, and *ef2627*), lipopolysaccharides (*ef2891*), the *epa* cluster (*ef2197* of one of the *epa* clusters, *ef2200-189*), and the FASII pathway (*ef2176* and *ef2883* from the *ef2886-75* cluster, and *ef0848 — acpS*). Teichoic acids, which are covalently bound to the thick peptidoglycan, are believed to be involved in concentrating metal ions from the surroundings and to also direct autolytic enzymes to sites of peptidoglycan digestion (autolysis), one of the steps in cell wall biosynthesis. Regulating the synthesis of these fatty acids, like in the FASII pathway, allows bacteria to modify membrane lipid composition and survive changes in temperature, pH and other environmental factors (Zhang and Rock, 2008). The remodelling of the fatty acid composition of the cell membrane has been reported and is common in response to different stressors (Vebø et al., 2009; Aakra et al., 2010; Vebø et al., 2010). This suggests an adjustment in fatty acid composition and membrane fluidity in the *luxS* mutant, when compared to VE14089.

Other elements associated with the cell envelope are however up-regulated in the *luxS* mutant. The SugE-1 component (EF0359) of multidrug efflux system proteins SugE-1 and SugE-2, encoded in the *ef0359-60* operon, suggested to play an important role in the uptake of chaperone regulatory compounds (Bay et al., 2008), *glsB* (*ef0081*), the last gene of the *gls24* operon (*ef0076-81*), which was shown to be induced under a number of stress conditions (Choudhury et al., 2011), and a putative membrane protein (EF1751), belonging to a gene cluster (*ef1751-3*) coding for cell membrane proteins previously found to be up-regulated in daptomycin-

resistant *E. faecalis* (Steed et al., 2011), were also significantly up-regulated in the *luxS* mutant, when compared to the WT.

## DNA metabolism, transcription and regulation

Homologous recombination (HR) supplies one of the main avenues for repair of DNA damage and the major cellular pathway to restart arrested replication forks. Repair of DNA double-strand breaks involves the establishment of a four-way DNA crossover from the pairing of strands from homologous duplexes, known as the Holliday junction (HJ) (Carrasco et al., 2009). This process relies on the action of translocases, such as RuvAB, and HJ resolvases, such as RecU. In the *luxS* mutant both processes, involving RuvA (EF0066 coded in the *ef0066-7* operon with *ruvB*) and RecU (EF1149), were down-regulated. Genes encoding for a PAI resolvase (*ef0534* — corresponding to PAI gene *ef0061*) as well as a PAI putative membrane protein (*ef0543* — corresponding to PAI gene *ef0070*), were also down-regulated. The genes coding for these proteins showed a decreased transcription in the *luxS* mutant, which may indicate a reduced need for DNA repair than in VE14089 or the lack of the necessary signals for that repair to occur.

The methylation of DNA provides an epigenetic signal that influences and regulates numerous physiological processes in the bacterial cell including chromosome replication, mismatch repair, transposition, and transcription (Heusipp et al., 2007), and alterations in the AMC intermediaries could interfere.

## Other genes

The gene coding for FeoA (*ef0475* belonging to *feoAB* operon — *ef0475-6*), a small soluble SH3-domain protein probably located in the

cytosol of still unknown specific function, was up-regulated in the *luxS* mutant. Bacteria commonly utilize a unique type of transporter, called Feo, to specifically acquire the ferrous ($Fe^{2+}$) form of iron from their environment (Cartron et al., 2006). The Feo system has been associated to increased virulence in *Streptococcus suis* (Aranda et al., 2009) as well as having a potentially important role of iron acquisition and metabolism during growth in urine in *E. faecalis* (Vebø et al., 2010). However, an increased FeoAB-mediated ferrous iron uptake is anticipated to yield hydroxyl radical by the Fenton reaction (Guo et al., 2011) and cause oxidative stress that can damage that damage DNA, proteins, and membranes (Stevens et al., 2010; Cesselin et al., 2011).

Possibly to compensate this and other increases in oxidative stress, the genes coding for the two general stress proteins (Gsps) associated with increased survival against organic hydroperoxide and ethanol (Gsp64 and Ohr) (Auffray et al., 2011) were up-regulated in the *luxS* mutant, as well as two other putative universal stress protection genes (*ef1982* and *ef1058*). *ef1058* has been similarly upregulated after $Zn^{2+}$ metal stress (Coelho Abrantes et al., 2011).

When compared to the WT, the *luxS* mutant displayed a reduced transcription of genes involved in the biosynthesis of osmoprotectant, salvage of purines and transport of metabolites. The gene coding for ProB (EF0038), a glutamate 5-kinase that catalyses the first step in the biosynthesis of proline, was down-regulated when compared to VE14089, indicating a decreased need for that amino acid, which has been described as being able to function as an important osmostress protectant in *B. subtilis* (Brill et al., 2011). The transcription of xanthine phosphoribosyltransferase gene, *xpt* (*ef2365*), from the purine salvage pathway was also down-regulated in the *luxS* mutant. This enzyme can

help to take up xanthine, allowing these compounds to serve as substrates for nucleotide biosynthesis and as sources of carbon and nitrogen, just like described for *B. subtilis* (Belitsky and Sonenshein, 2011). When compared to VE14089, the *luxS* mutant shows a reduced need to activate this salvage pathway.

The transcription of a chaperone and of several of genes involved in the regulatory functions was also down-regulated in the *luxS* mutant. HslO (EF0266), along with other chaperones, is involved in the refolding of denatured proteins to maintain homeostasis (Santos et al., 2010), protecting cells from various stress conditions (Misra et al., 2011). From the 4 down-regulated genes involved in regulatory functions, *ef1864, ef1599*, ef0873 and *ef2630*, the gene coding for a response regulator (RR) (RR08 — EF1864) of a two-component system (Ehk-Err08 — EF1863-4) has already been describes as being involved in stress response against heat (Le Breton et al., 2003), being linked to DNA mobile elements (Hancock and Perego, 2004), and is suggested to be involved in response to oxidative stress, other cellular stresses and sensing changes in cell wall intermediates and components (Ma et al., 2008), while *ef0873* has an increased expression level after copper (Cu) exposure (Reyes-Jara et al., 2010).

The *ef3194-3* operon, that encodes a system made of genes *lrgA* — encoding a putative murein hydrolase regulator holin-like protein — and *lrgB* — encoding an antiholin-like protein, was highly up-regulated. Increased expression of *lrgAB* operon, a negative regulator of autolysis whose product negatively affect peptidoglycan hydrolases (Liu et al., 2011), has already been described in *Staphylococcus aureus* in response to antibacterial compounds (Liu et al., 2011), in *Streptococcus mutans* in response to multiple environmental signals (playing important roles in

biofilm formation, oxidative stress tolerance and regulation of autolysis) (Ahn et al., 2010), but also in *E. faecalis* response to blood (Vebø et al., 2009). It is proposed that the LytSR two-component regulatory system senses decreases in membrane potential caused by proton motive force and responds by inducing *lrgAB* transcription (Bayles, 2007). The exact mechanism of *lrg* genes products in virulence remains however unclear (Ahn et al., 2010). In the *luxS* mutant all the detected differences in gene expression associated with metabolism could cause alteration in the surrounding media, like pH, which could alter the membrane potential therefore serving as a signal for LytSR allowing a responds by inducing *lrgAB* transcription.

The *luxS* mutation may have diverse effects in the cell. As a direct effect this mutation can shift the equilibrium of the intracellular concentrations of SRH, DPD, and homocysteine, as previously described in *E. coli* (Halliday et al., 2010). These metabolites may have some signalling roles inside the cell and therefore may globally affect gene expression. SRH, though not toxic, may over-accumulate in the mutant and therefore disturb metabolite concentration (like promoting the accumulation of SAH, a potent inhibitor of the methyltransferases (Keller and Surette, 2006)), which may affect the AMC, the carbon usage metabolism, or even promote biofilm formation, as seen in *L. monocytogenes* (Challan Belval et al., 2006). The intracellular function of DPD, in cells where no internalization has been described, has never been elucidated, and the necessary *de novo* biosynthesis of homocysteine may not be enough to compensate the lack of recycling and therefore also affect the cell's metabolite homeostasis, as previously observed (Halliday et al., 2010). The LuxS protein can also have a dual role and not only function as the enzyme catalysing the last step of the AMC, belonging to the so called moonlighting protein, as it has been

speculated for *Salmonella* Typhimurium LuxS (Kint et al., 2009). Although apparently no effect is detected in the expression of genes involved in the AMC, the *luxS* deletion may nevertheless still alter methionine import (methionine participates in initiating protein biosynthesis as well as being a constituent of proteins) or SAM production (as an universal methylating agent), by altering the homeostasis of intermediary metabolites, as seen in *E. coli* (Halliday et al., 2010). However, *luxS* may also have an indirect effect on global gene expression.

## Global regulators

By searching the *E. faecalis* V583 genome using the catabolite responsive elements (*cre*) query consensus sequence WTGWAARCGYWWWCW developed for *E. faecalis*, like previously performed by (Opsata et al., 2010), the previously described sequence of a *rex*-box, TGTGANNNNNNTCACA (Mehmeti et al., 2011), and the sigma-54 consensus sequence YTGGCACNNNNNTTGCW (Opsata et al., 2010), developed for *B. subtilis*, using Virtual Footprint v3.0 and allowing 1 sequence mismatch, several promoter regions of differentially expressed genes between the WT and the *luxS* mutant were identified (Table 5.5).

Many processes differentially regulated between the *luxS* mutant and the WT belong to pathways with genes that respond to a number of global regulators (Table 5.5). Microorganisms have evolved multiple mechanisms to sense and respond to the availability of specific nutrient types. However, in order to optimize growth, microorganisms must not only sense the availability of a nutrient type (e.g., carbon and energy), but they must also adjust their uptake or production of other nutrient types (e.g., nitrogen-, phosphorus-, and sulphur-containing compounds) according to the availability of carbon. Only in a few cases have such interactions been

investigated (Choi and Saier, 2005). Many pleiotropic regulators have been described in *E. faecalis* as being involved in the regulation of these metabolic processes. These global regulators are involved in sensing carbohydrate availability, the redox turnover in the cell, interaction with environment but also a number of different stresses such as oxidative stress or starvation. The *luxS* mutation impact on these intricate regulatory networks is clear, though a direct or indirect contribution remains to be elucidated.

The TCA cycle being absent in *E. faecalis,* the energy is derived via glycolysis, the pentose phosphate pathway, but also the Entner-Doudoroff pathway. *E. faecalis* has a carbon catabolite repression (CCR) pathway to actively regulate energy metabolism while growing on easily fermentable sugars such as glucose (Manson and Gilmore, 2006a). It has been previously described that Luria-Bertani broth, which has different amounts of the same components as 2×YT broth used in this study, contains <100 µM fermentable sugar equivalents utilizable (free sugars, sugar phosphates, oligosaccharides, nucleotides, etc.) (Sezonov et al., 2007). Such concentrations of glucose is below the threshold for release of CCR, and the cells thus initiate use of less preferred carbon and energy sources (Vebø et al., 2010). In Firmicutes, the global mechanism of CCR is mediated by the pleiotropically acting catabolite control protein A (CcpA) and involving its target sites, the *cre* (Fujita, 2009; Suárez et al., 2011). The *cre*-site is located in the 5'-upstream regulatory region or within the reading frames of CCR-controlled genes. A protein complex involving CcpA inhibits or activates the transcription of CCR-controlled genes or operons (Deutscher et al., 2006). CCR seem to be controlling carbon hierarchy usage in the *luxS* mutant, by up-regulating the transport of less preferred sugars associated with *cre*-sites (Table 5.5), which is in agreement with

previous findings (Opsata et al., 2010). In *E. faecalis* the BCKDH complex, encoded in gene cluster *ptb-buk-bkdDABC* (*ef1663-58*), is devoted to energy generation. The control by CCR of this pathway would be consistent with its catabolic role in the utilization of branched-chain α-oxoacids as an alternative energy source (Fernández and Zúñiga, 2006), with a *cre*-site found in its promoter region. LacD.1 has been adapted to function as a sensor of its substrates, which are the central intermediates of the Embden-Meyerhoff-Parnas glycolytic pathway, implicating LacD.1 as a regulator of global carbon catabolite control (Kietzman and Caparon, 2011).

Other genes are responding to the NADH/NAD+ ratio (Table 5.5). This ratio is an important component of the redox state of a cell, a measurement that reflects both the metabolic activities and the health of cells. Both pyridine nucleotides bind to the Rex protein, but only NADH causes Rex to lose affinity for target DNA. The high turnover of NAD(H) means that a decrease in oxygen availability, due to cells reaching stationary phase, to high cell density, or the inhibition of respiration by other means, and can lead to a rapid increase in the NADH/NAD+ redox balance, by an increase in the NADH pool. Presumably, the increase in the NADH/NAD+ ratio leads to adaptation through the induction of the Rex regulon. *E. faecalis* contains in its genome two ORFs similar to Rex (*ef2638* and *ef2933*), suggesting that one might respond to other signals like NADPH instead of NADH, for instance.

**Table 5.5.** Differential expression of genes associated with the interaction with transcriptional regulators, with the consensus motif localized in the intergenic region (int) or in the coding region (cod) in the VE14089Δ*luxS* mutant when compared to VE14089, independently of extracellular added DPD. DOWN: down-regulation (*P*-value < 0.05, $\log_2$ ratio < -5), UP: up-regulation (*P*-value < 0.05, $\log_2$ ratio > 5).

| | **gene name** | **$\log_2$ ratio** | **motif (localization)** |
|---|---|---|---|
| **response to carbon usage:** | | | |
| fructose usage | *ef00693-6* | UP | cre (int) |
| cellobiose usage | *ef1012* | UP | cre (int) |
| cellobiose usage | *ef1013?* | UP | cre (int) |
| transaldolase important for pentose phosphate pathway | *ef1207-6* | UP | cre (int) |
| *ptb-buk-bkdDABC* BCAA usage | *ef1663-58* | UP | cre (int) |
| *ptb-buk-bkdDABC* BCAA usage | *ef1663-58* | UP | cre (cod) |
| *glpKOF* glycerol usage | *ef1929-7* | UP | cre (int) |
| gluconate usage | *ef3142-37* | UP | cre (int) |
| amino acid permease | *ef0929* | DOWN | cre (cod) |
| teichoic acid biosynthesis | *ef1172-4* | DOWN | cre (int) |
| HP | *ef3145* | DOWN | cre (int) |
| **response to NADH/NAD+ ratio:** | | | |
| *mptBACD ef0019-22* | *ef0020* | UP | rex (int) |
| HP | *ef1671* | UP | rex (int) |
| *proB* - proline biosynthesis | *ef0037* | DOWN | rex (int) |
| hydrolase | *ef2874-1* | DOWN | rex (int) |
| HP | *ef3145* | DOWN | rex (int) |
| **response to the interaction with the environment:** | | | |
| *mptBACD* | *ef0019-22* | UP | sigma-54 (int) |
| *sugE-1* | *ef0359* | UP | sigma-54 (int) |
| *sugE-2* | *ef0360* | UP | sigma-54 (int) |
| cellobiose usage PTS | *ef1012* | UP | sigma-54 (int) |
| cellobiose usage PTS | *ef1017-8* | UP | sigma-54 (int) |
| *murAA* / HP peptidoglycan biosynthesis | *ef2605-4* | DOWN | sigma-54 (cod) |
| FASII pathway | *ef2883-75* | DOWN | sigma-54 (cod) |
| gluconate usage (in *ef3142-37* operon) | *ef3139* | UP | sigma-54 (cod) |

In the *luxS* mutant the transcription of some genes, like PTS operons, can also be initiated at sigma-54-dependent promoters (Table

5.5). The role of sigma-54 has been well documented in a variety of bacterial species and implicated as a central player in the control over the processes that involve the physical interaction of an organism with its environment (Francke et al., 2011), by regulating numerous biological properties including those that relate to virulence, like in the colonization of a host or the formation of biofilm (Francke et al., 2011; Iyer and Hancock, 2012). While not belonging to the alternative sigma factors, the role of *E. faecalis* sigma-54 (RpoN — EF0782) has been limited to observations made regarding its contribution to sensitivity to class IIa bacteriocins through the regulation of sugar PTS systems (Auffray et al., 2011; Iyer and Hancock, 2012).

The *luxS* mutation altered the expression of several genes that are under the regulation of enterococcal pleiotropic regulators. This interconnected regulatory network is apparent when observing the presence of, for example, dually regulated pathways that link two different sensing mechanisms. For example, in the major glucose PTS, the mannose-PTS, in addition to the sigma-54 promoter preceding *ef0019*, a Rex box found in front of *ef0020*, suggesting that this PTS is also regulated by the NADH/NAD ratio (Mehmeti et al., 2011). In *E. faecalis* it is clear that there are many genes that appear to be regulated by a network involving global regulators and energy and redox sensing aimed at maintaining homeostasis. The deletion of *luxS* gene and the global change in gene expression evokes the complexity of the central energy metabolism, suggesting intricate direct and indirect regulation on how these bacteria cope with their changing access to carbon as well as nitrogen sources.

## Virulence associated genes

Besides the already mentioned and described cell component *ef2197* from the *epa* cluster, none of the other potential virulence factors described by Manson et al. (Manson and Gilmore, 2006b) were significantly regulated above the set fold change (Table 5.6). However, significantly regulated genes, below the determined cutoff, in the *luxS* mutant when compared to VE14089, were all down-regulated. The possible reduction in the availability of AMC intermediary SAM in the *luxS* mutant may inhibit Dam (DNA adenine methylase) and Dcm (DNA cytosine methylase)-dependent DNA methylation, which have been associated with bacterial virulence (Kozlova et al., 2008) and play a role in the pathogenesis (Sun et al., 2010).

**Table 5.6.** Differential expression of potential virulence factors (according to Manson et al. (Manson and Gilmore, 2006b)) in the VE14089Δ*luxS* mutant when compared to VE14089 with no extracellular addition of DPD (similar data not shown for VE14089Δ*luxS* mutant when compared to VE14089 with extracellular addition of DPD). ns: not significantly expressed (*P*-value > 0.05), bold type: l $\log_2$ ratio l > 5.0.

| gene name/symbol | gene locus | $\log_2$ ratio | function |
|---|---|---|---|
| putative hemolysin | *ef0700* | -3.037 | hemolysin/unknown |
| putative hemolysin | *ef0982* | -1.722 | hemolysin/unknown |
| putative hemolysin | *ef1685* | ns | hemolysin/unknown |
| putative exfoliative toxin A | *ef0645* | ns | toxin/unknown |
| putative xanthan lyase | *ef0818* | ns | breaking glycosidic bonds |
| putative xanthan lyase | *ef3023* | ns | breaking glycosidic bonds |
| FsrA | *ef1822* | -1.930 | *fsr* operon |
| FsrB | *ef1821* | ns | *fsr* operon |
| FsrC | *ef1820* | ns | *fsr* operon |

| | | | |
|---|---|---|---|
| GelE | *ef1818* | ns | zinc metalloprotease |
| SprE | *ef1817* | ns | serine protease |
| Eep | *ef2380* | -3.235 | zinc metalloprotease |
| $CylR_2$ | *ef0523* | -1.555 | cytolysin operon |
| $CylR_1$ | *ef0524* | -1.736 | cytolysin operon |
| $CylL_L$ | *ef0525* | ns | cytolysin operon |
| $CylL_S$ | *ef0526* | ns | cytolysin operon |
| CylM | *ef0527* | -2.943 | cytolysin operon |
| Ace | *ef1099* | -1.892 | conjugation |
| internalin family protein | *ef2686* | ns | |
| aggregation substance | *ef0149* | ns | conjugation |
| aggregation substance, putative | *ef0485* | ns | conjugation |
| protein with homology to FbpA | *ef1249* | ns | unknown |
| BopD | *ef0954* | -1.614 | biofilm |
| *cps* locus | *ef2495-85* | -3.127/-2.225/-2.823/-2.477/-2.061/ns/ns/ns/ns/ns/ns | immune evasion |
| *epa* cluster 1 | *ef2200-189* | -1.458/-2.743/-2.281/-5.537/-1.917/-1.685/-1.390/ns/ns/ns/ns/ns | cell wall immune evasion |
| *epa* cluster 2 | *ef2184-77* | ns/-2.156/-1.376/ns/ns/ns/ns/ns | cell wall immune evasion |
| *dlt* operon | *ef2749-6* | ns | immune evasion |
| homologous to Mprf | *ef0031* | ns | homology to defensins |

## Conclusion

In this study we were able to associate the extracellular production of AI-2 in *E. faecalis* to the presence and expression of *luxS* gene. When compared to the WT, the *luxS* mutant has no apparent phenotype regarding growth, biofilm formation, adhesion to Caco-2 cells, resistance to oxidative stress and survival in a macrophage survival assay. Despite no apparent phenotype alteration, a microarray transcriptional analysis revealed that the *luxS* mutation caused pleiotropic effects in gene expression in the mutant, when compared to its WT. The lack of

extracellular AI-2 was not responsible for the pleiotropic effects observed, since the extracellular addition of DPD, in a similar concentration to the one produced by the WT, had no effect on gene expression. The transcriptional analysis indicates that VE14089 and its isogenic *luxS* mutant are not displaying the same gene expression pattern, in the same moment of the growth phase, and that the effects of the mutation are global. These global differences in gene expression affected all the main gene functional roles like energy, DNA, fatty acid and intermediary metabolites metabolism, transport and regulatory functions, cell envelope, but mainly affected hypothetical and unknown function proteins. No significant differential expression was detectable in the genes involved in the AMC in the mutant when compared to the WT.

Although not able to sense extracellular AI-2 in our study, VE14089 showed to be capable of coping with a *luxS* mutation, displaying no significant difference in the performed phenotypic tests, despite the observed global transcriptional alterations and consequent metabolic rearrangement induced by that mutation. A number of mechanisms have already been described in *E. faecalis* as being able to sense and respond to different stresses and metabolic states, not overruling the possibility that changes in AMC intermediary metabolites may affect gene expression (e.g. SAM influencing DNA methylation). Much more has to be revealed regarding LuxS function in *E. faecalis* and the direct and indirect repercussions in general metabolism, as well as the implication of this enzyme activity and its products in the establishment maturation and composition of multi-species microbial communities. To continue to understand the efficient and complex regulatory network of this bacterium, which promotes survival in response to changes in the environment, like

the most diverse stressors, such as carbon and nitrogen availability or temperature and oxidative stress, is of the utmost importance. It is tempting to speculate that this could allow predicting vulnerabilities in *E. faecalis* main behaviour and therefore attempt control bacterial presence, whether eliminating it in the case of host infection, or promoting its occurrence in some foods.

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# Chapter 6

# General Discussion

## Problem with enterococci

Over the past several decades, enterococci have emerged as one of the most common sources of hospital acquired infections. Modern medical practices using antibiotic therapeutics combined with innate and acquired antibiotic resistances in these bacteria have resulted in a rapid increase in the incidence of enterococcal diseases (Hancock and Gilmore, 2000). Multiple antibiotic resistant enterococci are not only more difficult to treat, but antibiotic resistance also provides enterococci with a selective advantage over competing microbes in the gastrointestinal (GI) tract. These intrinsic and acquired antibiotic resistance mechanisms of the genus can, upon antibiotic therapy with agents inactive against enterococci, lead to an increased enterococcal burden in the gut by eliminating competitors (Jin et al., 2011), or maybe even by decreasing the host's killing ability (Albesharat et al., 2011). This broad-spectrum antibiotic use is a risk factor for developing vancomycin-resistant *Enterococcus* (VRE) (Rice et al., 2004), one of the most disturbing resistances seen in enterococci that is often regarded as the antibiotic of last resort for treating various multi-resistant Gram-positive cocci infections. Colonization with VRE is in turn associated with increased rates of morbidity and mortality (Lin et al., 2012) and with a great concern for the transfer of vancomycin resistance from enterococci to

methicillin-resistant *Staphylococcus aureus* (MRSA) (Sievert et al., 2008; Tenover, 2008).

## Clinical enterococci and infection

Along with multiple intrinsic and acquired antibiotic resistances, enterococcal persistence has also been related with carriage of certain genetic determinants. These putative virulence factors, described in Chapter 1, are relevant to some of the different stages of the bacterial infection process, including adhesion, colonization, invasion, elusion of the immune system and spread through the host's tissues.

## Presence of virulence factors in commensal and food strains

At the time of the beginning of this work researchers were starting to realize that not only nosocomial enterococci but also isolates from diverse other origins were also harbouring putative virulence genes. Based on more work done during these last few years along with the one included in this thesis (Chapters 2 and 4) it is clear that, from the putative virulence factors described until now, there is no virulence trait exclusive to strains isolated from clinical settings. Also, the sole presence of these putative virulence traits does not allow the identification of a strain's habitat. The issues that quickly became imperative to understand switched from the dissemination of virulence factors and their responsibility for the virulent behaviour of clinical strains to the potential ability of food strains harbouring putative virulence factors to behave as pathogenic as clinical strains.

## Expression of virulence traits in food isolates

We showed in this thesis for the first time (Chapter 3) that, using the simple *Galleria mellonella* infection model, gelatinase and Fsr virulence factors of *Enterococcus faecalis* contribute to the virulence of food isolates, as well as of clinical strains. So, the presence of these tested virulence factors in *E. faecalis* strains proved to have an associated pathogenic potential, for the virulence model where they were assayed and independently on the environment where they were isolated from.

But then the next question arose: does the presence of virulence genes be assumed as synonymous of their expression? In this thesis, the mere presence of specific virulence traits in the genome did not compulsory translate into a straightforward pathogenic behaviour. While in the aforementioned *G. mellonella* virulence model one of the tested food isolates QA29b revealed to be virulent, as it killed the larvae as efficiently as a clinical isolate, due to the presence of specific virulent factors (Chapter 3), the same strain, in opposition to a reference clinical strain, failed to express another harboured virulence trait, the *cps* capsule locus, and was readily eliminated in a macrophage survival assay (Chapter 4). The presence of virulence-associated genes in the genome does not necessarily and directly correlate to the expression of that same trait. So, the presence of virulence-associated genes, and therefore an associated virulence potential, does not necessarily translate to a virulent and pathogenic behaviour in *E. faecalis* isolated from food.

However, this already mentioned incongruence between phenotype and genotype assays was evident regarding the *cyl* operon (Chapter 2). Both genotype and phenotype became always correlated for different tested enterococcal species, always independently of environment of

isolation, simply by changing the screening methodology. The pursue of good experimental design and analysis is essential to avoid misleading gene detection that could conceal inactive genes, incomplete operons, and therefore a misleading deduction of phenotype.

## Colonization as a stage of infection

It has been recognized for more than 30 years that commensal bacteria, like nosocomial pathogens, are able to adhere to epithelial cells and survive in mucus therefore colonizing the human's GI tract (Hartley et al., 1979). Since then, they have been described as also being able to adhere and colonize the totality of the exposed surfaces of the host, from skin to the diverse mucosa. Like pathogenic bacteria, commensal bacteria have the ability to follow the same first stages described for the bacterial infection process. They are not only able to follow these same stages but, in the case of probiotics, some bacteria are chosen for those specific characteristics. When colonizing the gut, enterococci are part of a complex microbiota where the ability to communicate with the other bacterial species may influence the structure and function of the community.

In Chapter 5 we showed also for the first time for the genus *Enterococcus* that the LuxS enzyme is responsible for the production of the interspecies communication molecule, AI-2. Though not sensed by *E. faecalis* in our study, we cannot confirm that in other conditions, or for other species, AI-2 could serve as a signalling molecule regulating quorum sensing or other processes. However, the production of this universal communication molecule may confer *E. faecalis* the ability to interfere and alter the communication in the polymicrobial communities where it naturally inhabits, possibly acting indirectly as a virulence factor.

## Putative virulence factors or complete genome responsible for pathogenic and virulent behaviour

Since the presence of putative virulence traits is not synonymous of their expression, and is not a specific feature of enterococcal nosocomial population, does pathogenic and virulent behaviour depend on the genome of the strain? It is suggested that an adaptation to the hospital environment has occurred in *E. faecalis* and in *Enterococcus faecium*. The genetic subset of *E. faecalis* clonal complex 2 (CC2) and CC9, which are vancomycin and gentamicin resistant, produce β-lactamase, and carry pathogenicity islands, and *E. faecium* CC17, which is characterized by quinolone and ampicillin resistance and the presence of a putative pathogenicity island carrying *esp* and *hyl* genes, seems to be responsible for the worldwide emergence of nosocomial infections by these bacteria (Rathnayake et al., 2011). The characterization and study of the population structure of *E. faecalis* and *E. faecium* remains important to investigate how nosocomial enterococcal populations are evolving toward a predominance of highly specialized enterococcal genetic subpopulations that are capable of surviving, spreading, and infecting patients with increasing frequencies in the hospital environment.

Members of these CCs harbour putative virulence traits, antibiotic resistances, and pathogenicity islands (PAIs). Like for putative virulence traits, these other characteristics are not exclusive of nosocomial subpopulation, but are nonetheless accountable for possibly lethal hospital acquired infections. An important question is whether whole genome, gene expression and metabolic patterns, more than just specific gene content among different isolates, is the virulence-defining characteristic of *E. faecalis*. For example, regarding glycerol dissimilation pathways there is an

astonishing diversity in metabolism patterns among different *E. faecalis* isolates, demonstrating that the species could not be represented by only one model of aerobic glycerol catabolism. However, this diversity was based not on different gene contents but rather on differences in gene expression, among strains of the same species (Bizzini et al., 2010). In Chapter 5 it was evident that the deletion of a single gene, the *luxS* gene, could induce a pleiotropic effect on gene expression in vancomycin resistant clinical isolate V583, which was not phenotypically detectable. An important gene regulatory network might be in place allowing the correction and compensation of disturbances in function. The food strain QA29b was also the target of *luxS* gene deletion. While for the clinical strain no phenotype for the mutation could be significantly detected (Chapter 5), for the food strain a significant decrease in survival in the macrophage survival assay, and a significant enhanced survival in the $H_2O_2$ challenge was seen (data not shown). In different strains with different genomic backgrounds of the same species, the deletion of the same gene can have completely divergent outcomes. This reiterates the importance of the whole genome in the orchestration of a response when presented to a specific phenomenon, being gene inactivation or environmental condition. Not only the presence of a specific gene is important to determine the outcome on the persistence and resilience of enterococci but the whole genome also plays a role in the regulatory network, which may directly or indirectly affect global gene expression.

Besides the obvious influence of the whole genome on enterococcal behaviour, the involvement of genes coding for hypothetical and unknown function proteins was also evident in the V583 *luxS* mutant (Chapter 5), indicating the possibility of a specific and effective function in metabolism for these genes. These hypothetical proteins have no significant sequence

identity to other proteins available in the protein databases. In the gene content evaluation of QA29b genome, by comparative genomic hybridization (CGH) using a macroarray, more than 55 % of the missing V583 chromosomal genes probed were hypothetical proteins (Chapter 4). Also, a study aiming to detect promoters, employing recombinase-based *in vivo* expression technology (RIVET), on the *E. faecalis* OG1RF chromosome that were specifically activated during the course of infection identified approximately one-third of both the sense and antisense loci encoding proteins with hypothetical or unknown functions (Frank et al., 2012). What role are these hypothetical proteins playing? Are they specific to certain species, strains, habitat specific isolates, or CCs? Might they be involved in the coordination of gene regulation of pathogenic subpopulations of *E. faecalis* and therefore be one discerning factor from commensal or food isolates?

## Virulence models for an effective virulence assessment

It has been made clear that the effective expression of virulence factors, depending on the virulence model used, results in diverse divergent virulent behaviours (Chapters 3, 4 and 5), which is not unusual. The construction of mutants of putative virulence determinants and their use in specific virulence model remains a well-suited technique to identify host and enterococcal factors critical for pathogenesis, thus providing a better understanding of the underlying mechanisms and function of that trait in bacteria (Chapters 3 and 5). However, most virulence models are dedicated to individual and particular aspects of a putative virulence trait. They are able to reveal the various effects of a given virulence determinant, but might

reveal to be inadequate or insensitive to do the same for others. While comparing a wild type and its corresponding isogenic virulence factor mutant in a discerning virulence model may allow to test the virulence potential of a given bacteria, the comprehensive assessment of the virulence potential of a bacteria in a random virulence model may give a biased result, dependent of the model used and the virulence traits that may be sensed by it.

The transposition of the pathogenicity potential of bacteria determined with a given virulence model to a human host cannot be directly done and requires cautious analysis. As previously mentioned, infection development is not based on a single and unique process. As it is also the case with other nosocomial infections, nosocomial enterococcal disease globally occurs by a two-step process. There is an initial, usually asymptomatic colonization of patients, mainly of the GI tract or occasionally the skin, by strains endemic in a hospital. Once colonized with enterococci, patients may carry them for months or even years. For a number of patients, however, the second step follows, i.e. tissue invasion from the enterococcal reservoir and eventually disease (Vebø et al., 2010). The colonization part of this process is identical for probiotic or commensal bacteria, so the distinction must be generally made when invasion occurs. An efficient bacterial infection is therefore a several stages process for which no virulence model has yet been described that globally enables the multifactorial aspect of enterococcal putative pathogenicity to be assessed. The currently used virulence models do not rely on the monitoring of a natural transition from commensalism to virulence but always accentuate a specific impairment in the response of the model in order to accomplish an effective infection, therefore helping the pathogenesis study. More

comprehensive models of virulence are necessary to allow the study of the enterococcal virulent behaviour.

## Many described virulence associated features are essential for the host's health

As previously mentioned, the first stages necessary for an effective infection are also required in the beneficial interaction of commensal or probiotic enterococci with the host. Their existence in the large intestine and fundamental functions in nutrition and metabolism (fermentation of non-degradable oligosaccharides, metabolism of xenobiotics, and activation or destruction of mutagenic metabolites) make the colonic microbiota a large fermentative organ (Tlaskalová-Hogenová et al., 2011), but also a critical stimulus for the adequate maturation of the immune system, which contributes to reducing infections and aberrant immune responses (Sanz, 2011). Intestinal microbiota contributes also to many aspects of host defence against invading pathogens both through direct microbial antagonism and promotion of maturation of the intestinal immune system. Bacterial metabolism results in the production of several by-products with an antimicrobial effect that not only inhibit the growth of pathogenic microorganisms themselves, but might also potentiate the effectiveness of other antimicrobial substances. In addition, production of biosurfactants by the microbiota and competition for sites of attachment and nutrients prevent the pathogens from establishing themselves within the host (Sekirov and Finlay, 2009). So, virulence factors cannot be collectively grouped as uniquely promoting pathogenesis, since the supposedly virulent associated behaviours supported by them (like adhesion, colonization) can prove essential for the resulting health promotion aspects of human health.

It is even conceivable to consider bacterial translocation (BT), a common event in the healthy host, as a normal and essential process, regulating local and systemic immunity, and tolerance to the innumerable antigens that make contact with the intestinal epithelium (Wiest and Rath, 2003). Translocation of viable and non-viable bacteria and microbial products in low numbers from gut to extra-gut tissues, especially the gut-associated lymphoid tissues, is a normal and a beneficial physiological process associated with immune stimulation by alerting the local immune defence (Wiest and Rath, 2003; Albesharat et al., 2011).

BT in the nursing mother has also been associated to the essential neonatal process of GI tract colonization. For a long time it has been considered that bacteria in breast milk were acquired by skin or faecal contamination (Albesharat et al., 2011). However, even when collected aseptically, breast milk of healthy human mothers contains a low total concentration of up to $10^3$ CFU/ml commensal microbes, including several predominant bacterial species, such as staphylococci, streptococci, micrococci, lactobacilli, lactococci, bifidobacteria, as well as enterococci, such as *E. faecalis*, *E. faecium*, *Enterococcus durans*, *Enterococcus hirae*, and *Enterococcus mundtii* (Donnet-Hughes et al., 2010; Albesharat et al., 2011; Jin et al., 2011).

What are the possible benefits of bacterial populations to harbour and express factors that allow adhesion, colonization, translocation and therefore viability and presence in human milk? Providing not only all the required nutrients but also a myriad of soluble factors (Donnet-Hughes et al., 2010; Jin et al., 2011; Sanz, 2011), mother's milk is also a source of bacteria that influences the initiation, development, and composition of the infant commensal oral cavity and gut microbiota (Albesharat et al., 2011; Jin et al., 2011), that allows the large intestine, that is sterile at birth, to

become rapidly colonized with commensal bacteria, that also arise from the mother's skin or the infant's mouth (Donnet-Hughes et al., 2010; Albesharat et al., 2011). Commensal bacteria in milk may be a natural maternal protective mechanism to promote the health of infants by improving the development of their gut and reducing infection (Jin et al., 2011). This interesting possibility that breast milk is not sterile harbouring a natural bacterial inoculum (Perez et al., 2007; Hunt et al., 2011), without any deleterious effect on maternal health (Perez et al., 2007), imply a novel form of mother–infant communication, but it also highlights a potentially new mechanism of immune regulation in healthy individuals, since the blood of normal healthy subjects contains bacterial components. This may represent an evolutionary strategy of immune surveillance allowing that such bacterial imprinting maintains tolerance to specific bacterial species and alerting distant anatomical sites of changes in local lymphoid tissues (Donnet-Hughes et al., 2010).

Previous studies have demonstrated that germ-free animals do not develop normal lymph node architecture, with extensive defects in the development of gut-associated lymphoid tissue, arrested capillary network development in the gut and reduced antibody production (Murgas Torrazza and Neu, 2011). Exposure to microbes in early life, which largely occurs through the microbial colonization of the newborn baby intestine, has been related to a decreased susceptibility to infections and sensitization to environmental antigens in early and later life. These observations constitute the basis of the "hygiene hypothesis," according to which the lack of microbial exposure due to highly hygienic conditions found in the Western world prevents proper maturation of the immune system and predisposes individuals to diseases such as type 1 diabetes, asthma, allergies, celiac

disease, inflammatory bowel disease, and obesity (Murgas Torrazza and Neu, 2011; Sanz, 2011).

It is therefore clear that genetic features, like some putative virulence factors, which allow enterococci's prevalence in the GI tract from birth, cannot be exclusively linked to a pathogenic potential. Similarly, the presence of enterococci and commensal bacteria counts in milk from dairy cattle and the resulting fermented products cannot be uniquely associated with a poor hygiene, in the milking practice as well as the further fermentation processes. Does BT happen in the dairy cattle? When and how does BT take place? What precautions must be taken regarding the dairy cattle microbiota? Which hygiene indicators must be chosen and how can hygiene levels, which could raise public health concerns, be cost-effectively determined in dairy products?

## Health, disease and the host

Taken together, the data reported in this thesis regarding food enterococcal strains showed a virulence potential in a specific virulence model (Chapter 3), while being avirulent in other models (Chapter 4), indicating that a pathogenic potential in humans cannot be entirely excluded, and therefore reiterating the need to keep a close surveillance on their presence in food. This putatively pathogenic effect of enterococci is nonetheless highly dependent of the host. Pathogenic nosocomial subpopulations have been able to cause disease mainly due to defects in the resistance mechanism of the host to these microorganisms (Kayser, 2003). Enterococci can become pathogenic in patients in intensive care units, in hospitalized patients with severe underlying diseases or an impaired immune system, and in elderly people (Ogier and Serror, 2008),

all of which are more susceptible to infections. Severely ill patients with hematologic malignancies and deep neutropenia are especially at an increased risk of developing enterococcal bacteraemia (Leendertse et al., 2009). These microorganisms are mostly observed as part of a mixed flora causing infections in these patients (Kayser, 2003; Jin et al., 2011), while enterococci alone can only cause sepsis or endocarditis in immunocompromised (Kayser, 2003) and elderly persons (Jin et al., 2011).

Although enterococci rarely cause diseases in healthy individuals (Kayser, 2003; Ogier and Serror, 2008), mainly owed to a globally low intrinsic pathogenicity and virulence potential (Kayser, 2003), colonization with the more virulent nosocomial isolates may have a future impact upon switching from a healthful state to healthless one. When presented to a switch in habitats, enterococci readjust their behaviour by enhancing the many previously mentioned features (Chapter 1) that allow a better chance for survival. All these fitness factors are responsible for a pathogenic behaviour when entering the sterile core of the host upon infection. How may this entrance in the sterile core of the host be avoided or even blocked? May adhesion and therefore proliferation be avoided after host invasion? Since complex regulatory networks orchestrate the response to environmental changes that impact the metabolic state of bacteria, continuing to understand stress response, environmental sensing, and the corresponding signal cascades might allow interfering with the signal transduction and the corresponding adaptation of enterococci to changes and therefore the new environmental conditions.

## Finishing notes

Besides being targeted as pathogenic, the resilient bacteria of the genus *Enterococcus* are key factors contributing to the ripening, flavour, and the organoleptic characteristics of fermented food products, part of the microbiota necessary for the stimulation of the gut immune system (e.g. occupying a niche that could be occupied by obligate pathogenic bacteria), but also, along with other commensal bacteria, thought to be important in contributing to prevent the “hygiene hypothesis” in the neonatal period. In order to prevent possible infections it is essential to understand how to enhance the protection of possibly sensitive human hosts, by continuing to comprehend this bacterium regulatory network that allows sensing and fitness response to changes in environmental conditions after translocation, in order to prevent and control them, therefore avoiding the colonization of the host’s sterile core leading to disease.

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