

Contents

ABSTRACT	i
I. INTRODUCTION	1
1. <i>ENTEROCOCCUS</i>	3
1.1 General characteristics	3
1.2 Enterococci taxonomy	3
1.3 Habitat	3
1.4 Nosocomial pathogen	4
1.4.1 Type of infections caused by <i>Enterococcus</i>	6
1.5 Antibiotic resistance	7
1.6 Epidemiology and genomics	12
1.7 Nutritional requirements	15
1.8 Virulence factors of enterococci	18
1.8.1 Surface proteins	18
1.8.2 Virulence-associated genes	20
1.8.3 Carbohydrate metabolism	20
1.8.4 Transcriptional regulator genes involved in pathogenicity	23
2. INTESTINAL COLONIZATION BY ENTEROCOCCI	24
2.1 The microbiota and their functions	24
2.2 Colonization resistance	26
2.2.1 Indirect mechanisms	26
2.2.1.1 Innate immune system	26
2.2.1.2 Adaptive immune system	27
2.2.2 Direct mechanisms	27
2.2.3 Approaches to restore colonization resistance	28
II. OBJECTIVES	37
III. MATERIAL AND METHODS	41
1. Bacterial strains, plasmids and growth conditions	43
2. Bacterial cells transformation	44
2.1 Electrocompetent cells transformation	44
2.2 Heat shock transformation	45
3. Phenol-Chloroform-Isoamyl Alcohol DNA extraction	45
4. Plasmid extraction	46
5. PCR using KAPA HiFi polymerase	46
6. DNA digestion using restriction enzymes	47
7. Ligation	47
8. TOPO® Ta Cloning® Kit and transformation by electroporation	48
9. DNA clean-up and size selection by magnetic beads	49
10. DNA Clean-up from agarose gel	49
11. Transposon mutant library construction and evaluation	49
12. High-throughput sequencing of the Tn-seq library	52

12.1 High-throughput sequencing preparation, 1 st strategy, using pZXL5 plasmid for library construction	52
12.2 Tn-seq sample preparation, 2 nd strategy, using pGPA plasmid for library construction	54
13.Tn-seq data analysis	56
13.1 Determination of the abundance of Tn-Seq mutants	56
13.2 Statistical analysis of the Tn-Seq mutants and visualization of significant differences	56
14.Targeted mutagenesis.....	57
14.1 Construction of mutant strains of the candidate genes.....	57
14.2 Construction of control strain for competition experiments by introducing the gentamicin resistance gene in an intergenic region of the genome	61
15.Growth curves determination.....	62
16.Competition assays	62
16.1 Competition assays <i>in vitro</i>	62
16.2 Competition assays <i>ex vivo</i>	63
17.Characterization of chelator-sensitive growth	63
18.Growth characterization under phosphate starvation conditions	64
19.Characterization of the carbon sources used by VRE for growing	64
20.Confirmation of VRE growth in the presence of individual sugars	66
21.Gene complementation of VRE mutants	66
22.Comparative genomic analysis and phylogenetic tree construction.....	67
23.RNA extraction protocol and VRE <i>in vivo</i> transcriptome analysis	67
24.Transcriptome analysis of the VRE mutant for the LacI family DNA-binding transcriptional regulator	68
25.Transcriptomic analysis to identify a mechanism by which ProBac confers protection against VRE.....	69
26.Metabolomic analysis to analyse fructose levels.....	71
27. <i>ex vivo</i> nutrient competition experiments with ProBac	72
28.Mice experiments	73
28.1 Antibiotic treatment set-up for the study of VRE genes involved in gut colonization.....	73
28.2 Mutant transposon library oral inoculation to identify genes required for gut colonization.....	75
28.3 <i>In vivo</i> competition assays	75
28.4 <i>In vivo</i> model adding mannose to the drinking water.....	76
28.5 Mouse model with probiotics administration	77
28.6 Mouse model with probiotics administration adding fructose.....	78
28.7 Mouse model with <i>Olsenella</i> administration after VRE inoculation	80
28.8 Mouse model used to collect sample for <i>ex vivo</i> experiments	81
29.Protective bacteria preparation to be administered to mice.....	81
30.Statistical tests utilized to analysed different set of data.....	82
31.Composition of prepared media used	83
32.Straains and plasmids	86

IV. RESULTS	95
1. IDENTIFICATION AND CHARACTERIZATION OF GENES ENCODED BY VRE FOR INTESTINAL COLONIZATION	97
1.1 Mutant library construction.....	97
1.2 Verification of E1162 mutant library	99
1.2.1 Plasmid presence.....	99
1.2.2 Checking the random transposon insertion in the genome of the E1162 obtained mutant library	101
1.3 Selection of <i>E. faecium</i> Aus0004 strain as the clinical strain to construct the transposon mutant library	103
1.4 Setting-up a mouse model to identify genes required for VRE gut colonization	106
1.4.1 Clindamycin and vancomycin promote higher levels of VRE colonization ..	106
1.4.2 Clindamycin induce higher and more homogeneous VRE intestinal colonization levels as compare to vancomycin	107
1.5 Sequencing sample preparation	109
1.6 Transposition mutagenesis analysis	111
1.6.1 Selection of candidate genes that could be key for VRE gut colonization...	127
1.7 Targeted mutagenesis.....	129
1.7.1 Construction of a gentamicin resistant strain as control strain for experiments performed with targeted mutants.....	129
1.7.1.1.. The gentamicin resistance marker does not affect the bacterial growth <i>in</i> <i>vitro</i>	129
1.7.1.2 The gentamicin resistance marker does not affect the fitness of the bacteria under <i>in vivo</i> and <i>in vitro</i> conditions	130
1.7.2 Targeted mutagenesis of the candidate genes.....	132
1.7.2.1 Candidate genes deletion does not affect the bacterial growth <i>in vitro</i>	132
1.7.2.2 <i>In vivo and in vitro</i> competition assays show an <i>in vivo</i> growth advantage of the WT over the mutant strains.....	133
1.8 <i>In vivo</i> competition assays under different conditions confirms the previous results.....	137
1.9 Characterization of mutants with putative functions related with the use of nutrients.....	139
1.9.1 Fe-S cluster may be necessary for the growth of VRE under limited conditions of both Fe ²⁺ and Fe ³⁺	139
1.9.2 Determination of a suitable minimal medium to study the phosphate ABC transporter ATP-binding protein mutant strain	144
1.9.3 Characterization of the impact of the LacI family DNA-binding transcriptional regulator on the VRE transcriptome	145
1.9.4 Detection of the carbon sources utilized for the growth of the wild type and the mutant strains	146
1.9.4.1. The <i>manX</i> and <i>murQ</i> genes are necessary for VRE to grow in the presence of mannose and N-acetyl muramic, respectively	149

1.9.4.2. Complementation of mutant strains involved in the use of specific carbon sources confirms the role of specific genes in utilization of specific sugars	151
1.9.4.3. Gene complementation of mutant strains partially restore VRE growth <i>in vivo</i>	153
1.9.4.3.1 Gene complementation in the clindamycin mouse model partially restores mutant growth <i>in vivo</i>	153
1.9.4.3.2 Gene complementation in the vancomycin mouse model restores the growth of the mutant strains progressively	154
1.9.4.4 Administration of mannose to antibiotic treated mice seems to slightly increase the levels of VRE in the intestine	157
1.10 Prevalence of the five selected candidate genes in clinical <i>E. faecium</i> isolates .	158
1.11 Transcriptomic analysis comparing the gene expression from <i>in vivo</i> vs <i>in vitro</i> samples	164
2. STUDY OF COMMENSAL BACTERIA WITH PROTECTIVE CAPACITY AGAINST INTESTINAL COLONIZATION BY VRE.....	165
2.1 Transcriptomic analysis demonstrated that ProBac colonization restores the expression of transporters for the internalization of sugars.....	167
2.2 ProBac diminish the levels of fructose in the large intestine	170
2.3 Detection of main carbon sources required for VRE ATCC 700221 to grow .	171
2.4 Fructose promotes VRE intestinal colonization.....	172
2.5 <i>Ex vivo</i> experiments demonstrated protection by nutrient competition conferred by ProBac consortium against VRE	174
2.6 <i>Olsenella</i> consumes fructose as one of the main carbohydrate sources.....	178
2.7 <i>Olsenella</i> is sufficient to confer resistance against VRE intestinal colonization..	179
V. DISCUSSION.....	181
1. Efficiency of different <i>E. faecium</i> clinical strains to construct the transposon mutant library	183
2. Study of the effect of different antibiotics on the capacity of VRE to colonize the intestinal tract	185
3. Transposition mutagenesis sequencing and analysis.....	187
4. Implication of identified genes in gut colonization.....	192
4.1 Study the phenotypic differences of mutant strains compared to WT <i>in vivo</i> and <i>in vitro</i>	192
4.1.1 Putative role of <i>yyjI</i> in VRE gut colonization.....	194
4.1.2 Putative role of <i>pgt</i> in VRE gut colonization.....	195
4.1.3 Putative role of <i>adhE</i> in VRE gut colonization.....	196
4.1.4 Putative role of <i>dsbA</i> in VRE gut colonization	196
4.1.5 Characterization of <i>pstB2</i> , a gene related to phosphate uptake	197
4.1.6 Characterization of the Fe-S cluster	199
4.1.7 Characterization of genes related to the use of carbon sources present in the gut	201

4.1.7.1 Characterization of <i>manX</i>	201
4.1.7.2 Characterization of the <i>murQ</i> gene	203
4.1.8 Putative role of <i>ItrR</i> in VRE gut colonization	204
5. Implication of selected candidate genes in gut colonization under different antibiotic treatment conditions	206
6. Presence of the studied genes in clinical <i>E. faecium</i> strains.....	206
7. Transcriptomic analysis from VRE grown <i>in vivo</i> vs <i>in vitro</i>	207
8. Protection mechanism against VRE conferred by the administration of a bacteria consortium	208
VI. CONCLUSIONS	211
VII. RESUMEN	215
VIII. BIBLIOGRAPHY	233
IX. APPENDIX	251