Homologous Recombination within Large Chromosomal Regions Facilitates Acquisition of ?-Lactam and Vancomycin Resistance in Enterococcus faecium

1 Title: **Homologous recombination within large chromosomal regions facilitates acquisition**

2 **of beta-lactam and vancomycin resistance in *Enterococcus faecium*.**

3

4 Running title: Transfer of Vancomycin resistance

5

6 Mónica García-Solache [a*]

7 Francois Lebreton [b]

8 Robert E. McLaughlin [c]

9 James D. Whiteaker [c]

10 Michael S. Gilmore [b]

11 Louis B. Rice [a]

12

13 [a] Department of Medicine, Rhode Island Hospital, Warren Alpert Medical School of Brown

14 University, Providence, RI 02903, USA

15 [b] Departments of Ophthalmology, Microbiology and Immunology, Massachusetts Eye and Ear

16 Infirmary, Harvard Medical School, Boston, MA, 02114 USA

17 [c] Infection Bioscience, AstraZeneca R&D Boston, Waltham, MA 02451, USA

18 [*]Author for correspondence: monica.garciasolache@cantab.net

19

20

21 Abstract word count: 216

22 Word Count: 8,333

ABSTRACT

23

24 The transfer of DNA between *Enterococcus faecium* strains has been characterized by both the

25 movement of well-defined genetic elements and by the large-scale transfer of genomic DNA

26 fragments. In this work we report on the whole genome analysis of transconjugants resulting

27 from mating events between the vancomycin-resistant *E. faecium* C68 strain and vancomycin

28 susceptible D344RRF to discern the mechanism by which the transferred regions enter the

29 recipient chromosome. Vancomycin-resistant transconjugants from five independent matings

30 were analysed by whole genome sequencing. In all cases but one, the penicillin binding protein

31 5 gene (*pbp5*) and the Tn*5382*-vancomycin resistance transposon were transferred together and

32 replaced the corresponding *pbp5* region of D344RRF. In one instance, Tn*5382* inserted

33 independently downstream of the D344RRF *pbp5*. Single nucleotide variants (SNV) analysis

34 suggests that entry of donor DNA into the recipient chromosome occurred by recombination

35 across regions of homology between donor and recipient chromosomes, rather than through

36 insertion sequence-mediated transposition. Transfer of genomic DNA was also associated with

37 transfer of C68 plasmid pLRM23 and another putative plasmid. Our data are consistent with

38 transfer initiated by a cointegration of a transferable plasmid with the donor chromosome, with

39 subsequent circularization of the plasmid/chromosome cointegrate in the donor prior to transfer.

40 Entry into the recipient chromosome occurs most commonly across regions of homology

41 between donor and recipient chromosomes.

42 **INTRODUCTION**

43 *Enterococcus faecium* has emerged as one of the leading causes of healthcare-associated

44 infections due to a combination of high intrinsic levels of resistance to commonly used

45 antibiotics, a remarkable genome plasticity that favours the ability to acquire *de novo* resistance

46 when challenged with new antibiotics and its ability to survive in diverse environments (1-4).

47 This high prevalence of antibiotic resistance, including widespread high-level resistance to the

48 first-line antibiotics ampicillin and vancomycin (3, 5), presents a challenge for effective

49 treatment of *E. faecium* infections.

50 Acquisition of resistance determinants by enterococci is mediated by a variety of mobile genetic

51 elements, including transferable plasmids, insertion sequences (IS), transposons and a high

52 degree of recombination between strains (4, 6-8). In vancomycin-resistant *E. faecalis* strain

53 V583, it has been estimated that as much as 25% of the genome has been acquired via horizontal

54 gene transfer (9). The spread of vancomycin-resistant enterococci (VRE) is one of the major

55 concerns in hospital settings worldwide. Vancomycin resistance results from acquisition of

56 transposon-associated complex operons that enable the bacteria to use modified cell wall

57 pentapeptide precursors that bind the glycopeptide antibiotics with lower affinity (10). There are

58 several *van* operons that vary in the type of enzymes encoded (11). The most widely distributed

59 in clinical strains worldwide are *vanA* and *vanB* (12). Tn*1546*, a Tn*3*-family transposon, most

60 commonly harbours the *vanA* operon while Tn*5382* most commonly carries *vanB*, which is

61 similar to conjugative transposons (13).

62 The *vanB*/Tn*5382* transposon was first reported in *E. faecium* C68, a multi-resistant clinical

63 isolate in which it was located immediately downstream of a penicillin binding protein 5 (*pbp5*)

64 allele conferring high-level of resistance to ampicillin. The genetic linkage of *pbp5* and *vanB* has

65 been identified in different strains of *E. faecium* isolated from different geographical regions (12,

66 14). This association is not universal and the *vanB* element can insert in other regions of the

67 chromosome or be plasmid-borne (15). C68 is a clinical isolate with broad antibiotic resistance

68 and a large plasmid (pLRM23) implicated in increased gastrointestinal colonization (16).

69   Transfer of the *vanB* transposon and *pbp5*, were observed in association with substantial

70   quantities of C68 genomic DNA (13, 17). Those recipient strains however, lacked *pbp5* due to a

71   large chromosomal deletion involving Tn*916* (18).

72

73   The precise mechanism of transfer of the *vanB* element in *E. faecium* has not been completely

74   resolved. In the literature both *bona fide* conjugative transposition and mobilization of large

75   fragments of chromosomal DNA often in association with plasmids have been observed (10, 13,

76   15, 19, 20). Mechanisms of transfer are better elucidated in *E. faecalis* than in *E. faecium* in a

77   large measure because of the fact that *E. faecalis* is considerably more amenable to genetic

78   manipulation. Yet the clinical problem of resistance, especially resistance to ampicillin, is far

79   greater in *E. faecium* than *E. faecalis*, making understanding the specifics of *E. faecium*

80   resistance transfer a high priority.   In *E. faecalis* the transfer of large segments of chromosomal

81   DNA from V583 to recipient cells has been attributed to the involvement of transferable

82   plasmids in donor cell that recombine with the donor chromosome across common IS elements,

83   followed by an F-like transfer of plasmid and chromosomal DNA using the plasmid origin of

84   transfer (21). These investigators were able to show that virtually any segment of donor

85   chromosome could be mobilized.

86   In the current work we were interested in analysing the mechanism of transfer of Tn*5382* and

87   *pbp5* from C68 to *E. faecium* D344RRF, whose chromosome contains a distinct *pbp5* allele but

88   does not contain Tn*5382* or vancomycin resistance. Our studies were designed to address four

89   specific questions: 1) Does transfer of the *vanB* operon result in the exchange of the D344RRF

90   *pbp5* allele with the high-level ampicillin resistance conferring *pbp5* allele from the donor C68?

91   2) Does transfer of vancomycin resistance involve transposition of Tn*5382* itself? 3) Does

4

92 integration of C68 DNA into the recipient chromosome occur by homologous recombination or

93 by IS-mediated transposition? and 4) Is C68 plasmid pLRM23 associated with the genomic

94 transfer?

95 **MATERIALS AND METHODS**

96 **Strains and media**

97 *E. faecium* strain C68 is a vancomycin-resistant strain carrying a *vanB2* resistance element

98 integrated into the chromosome, and was originally isolated from a faecal sample of a

99 hospitalized patient (13). *E. faecium* strain D344RRF is a rifampicin/fusidic acid-resistant variant

100 of clinical isolate D344R (22). Bacteria were grown on Brain Heart Infusion (BHI) broth or agar

101 (Fluka, St. Louis, MO).

102

103 **Conjugation experiments**

104 Conjugation experiments were performed as previously described either in (23) or by cross-

105 streak technique (24). Briefly, overnight cultures of both donor and recipient were mixed in 15

106 ml conical tubes at a 1:1 ratio (200µl each culture). After 1h, the tubes were spun down, 200 µl

107 of media kept and plated onto non-selective BHI-agar plates. Mating plates were incubated at

108 37ºC overnight. The mixed bacteria were recovered with a loop and resuspended in 3 ml of

109 sterile PBS/2mM EDTA, centrifuged, most of the supernatant removed and the cells were plated

110 onto selective BHI agar plates with vancomycin 25µg/ml, fusidic acid 25 µg/ml and rifampicin

111 50 µg/ml. The plates were incubated for 3 days at 37°C. Colonies were re-streaked onto identical

112 plates to confirm resistance and isolate single colonies. Single colonies were inoculated into BHI

113   broth with vancomycin 25µg/ml, fusidic acid 25 µg/ml and rifampicin 50 µg/ml and used to

114   make glycerol stocks and prepare genomic DNA.

115   To evaluate if extracellular DNA could be implicated in the transfer the donor and recipient cells

116   were mixed and incubated in minimal media salts supplemented with 0.5% glucose, 0.2 mM

117   $MgSO_4$, 0.1 mM $CaCl_2$ with or without 300µg/ml of Bovine pancreas Dnase I (Roche) for 1 h at

118   room temperature, after incubation the mixed cells were plated onto BHI agar. To test if

119   bacteriophages played a role in DNA transfer, we followed the method described by (21), briefly

120   25 ml of C68 overnight growth was pelleted and the supernatant was filter-sterilized using a

121   0.45-µm filter. The cell-free supernatant was diluted 2-fold with fresh BHI medium, and the

122   conditioned medium was inoculated with D344RRF. After an overnight incubation, cells were

123   pelleted, resuspended in 200 µl of fresh BHI, and plated onto selection plates with vancomycin

124   25µg/ml, fusidic acid 25 µg/ml and rifampicin 50 µg/ml.

125   Microbroth MICs for vancomycin, ampicillin, fusidic acid and rifampicin were determined in

126   BHI broth according to previously published method (13).

127

128   **Serial passaging**

129   Transconjugants TC-A and TC-B, obtained from the first mating were subjected to serial passage

130   either with vancomycin 10 µg/ml, ampicillin 12.5 µg/ml or no selection for about 400

131   generations in BHI broth to evaluate if continuous selection with antibiotic had an impact on the

132   resistance levels. The original transconjugants, and the final passage for each condition were

133   subjected to whole genome sequence for comparison.

134

135 **Gene expression studies**

136 D344RRF, C68 and transconjugant TC-A before (P0) and after passaging (P9 and P13) frozen

137 stocks were used to inoculate an overnight BHI culture, the next morning the cells were diluted

138 1:1000 and were grown with shaking at 37°C to an OD_{600} of 0.2. At that point, cultures were

139 treated either with ampicillin, vancomycin or no antibiotic to a final concentration of half the

140 MIC value and were grown up to an OD_{600} of 0.6 (around four hours) with shaking. Cells were

141 broken open with glass beads (Lysing Matrix B, MP Biomedical) using a mini-BeadBeater

142 (BioSpec), and the RNA was purified using the Qiagen RNeasy minikit. Complementary DNA

143 (cDNA) was synthesized using the Bio-Rad iScript gDNA Clear cDNA Synthesis kit.

144 Quantitative PCR was carried out using the Bio-Rad iTaq universal Probes kit in a multiplexed

145 reaction in the CFX98 real-time PCR cycler. Relative gene expression was calculated using the

146 ΔΔCq method and normalized relative to expression of 16S rRNA (25). To compare expression

147 levels we did a one-way ANOVA analysis using Prism 7 (GraphPad Software Inc). The primers

148 and probes used for the experiment are listed in supplemental table 4.

149

150 **Whole genome sequencing**

151 Illumina MiSeq. Total DNA was extracted using the Qiagen genomic tip-100 (Qiagen, Valencia,

152 CA) according to the kit manual, with minor modifications. Briefly, 6 ml of overnight culture

153 with vancomycin 25µg/ml, fusidic acid 25 µg/ml and rifampicin 50 µg/ml was used for each

154 DNA sample. To break up the cells 80 µl of 100 mg/ml lysozyme was used with 2h incubation at

155 37ºC. The DNA samples were diluted to 0.3 ng/µl, and 5µl were used for library generation

156 using the Nextera XT DNA sample preparation kit and Nextera XT index primers (Illumina, San

157 Diego, CA). Sufficient sample was diluted to 600µl to provide a 15-20 pmol multiplexed library,

158 and sequenced on an Illumina MiSeq V2 instrument as 2X150 paired-end reads.

159 Pacific Biosciences single molecule sequencing. DNA was isolated as for Illumina sequencing

160 and 10µg of high quality DNA was used to make large insert libraries (10kb) to sequence using

161 Pacific Biosciences RS II sequencing (Pacific Biosciences, Menlo Park, CA) technology. For

162 each sample we used one PacBio RS II SMRT cell.

163

164 **Bioinformatics**

165 Illumina assemblies were performed off-instrument using CLCBio Genomics Workbench v6.5

166 (Cambridge, MA). Fastq files were trimmed for quality and minimum length (50 bp) and reads

167 were *de novo* assembled at high stringency (Length fraction= 0.9; Similarity fraction = 0.99)

168 using default mismatch/insertion/deletion costs. A summary of the genome assemblies is

169 provided in supplemental table 2.

170 PacBio genome assemblies were done using the HGAPv3 assembly platform with a minimum

171 read length set to 5000kb and a 4% error rate allowed by the Genomic Analysis and

172 Bioinformatics Shared Resource, Duke University Center for Genomic and Computational

173 Biology, Durham, NC, USA.

174 Gene synteny and inferred contig order were analysed by comparing the parental genomes

175 (D344RRF and C68) versus the fully closed genomes of *E. faecium* DO and *E. faecium* Au0004

176 (Accession numbers ASM17439v2 and GCA_000250945.1) with MAUVE 2.3.1 (26). Genome

177 annotation was done using RAST (27, 28) and particular genes manually curated.

178 Detection of SNVs/indels was accomplished by mapping reads to parents (donor and recipient)

179 reference assembly using the same parameters. Quality based SNVs were detected at a

8

180 minimum frequency of 95% with a minimum of 30-fold coverage using default criteria. SNVs

181 were obtained with Geneious 8.1.7 (Biomatters Ltd, New Zealand) (supplemental table 3). The

182 crossover regions for donor to recipient DNA integration were identified by SNV and validated

183 by PCR using Phusion high fidelity polymerase (NEB, Ipswich, MA) and Sanger sequencing, the

184 primers used are listed in supplemental table 4.

185

186 **Plasmid identification**

187 The identification of the putative pRLM23 was done using the PacBio assembly of C68 and TC-

188 A and looking for a contig with the presence of the *hyaluronidase* gene. We were able to retrieve

189 a single 217 kb contig containing the predicted full length pLRM23 from the C68 PacBio

190 assembly. After the identification of the canonical pLRM23 sequence using the PacBio

191 assemblies we searched for the presence of genes of plasmid origin in the transconjugants by

192 mapping the Fastq reads from D344RRF and the transconjugants vs. the PacBio assembly of C68

193 (supplemental table 5C) and then by comparing the regions that were unique to C68 and the

194 transconjugants but not to D344RRF. The *hyaluronidase* (*hyl*) gene was used as an experimental

195 pLRM23 marker. The *hyl* gene was detected by PCR and Sanger sequencing (supplemental

196 figure 2 and supplemental table 3).

197

198 **RESULTS**

199 **Selection of transconjugants and whole genome sequencing**

200 We performed five independent matings between C68 and D344RRF to select for

201 transconjugants (TC) that acquired vancomycin resistance. The resistance phenotypes of the

202 parents and the obtained transconjugants are listed on table 1. Our transfer frequencies ranged

203 from 10^{-9} to 10^{-8} per recipient CFU, in agreement of what was previously reported for similar

204 experiments (13, 17). Fourteen individual transconjugants (representing about 5% of all colonies

205 obtained after the mating) resistant to vancomycin, rifampicin and fusidic acid were randomly

206 selected for whole genome sequencing and further characterisation. Vancomycin MICs for the

207 different transconjugants ranged from 12.5 to 400 µg/ml and ampicillin MICs ranged from 6.3 to

208 400 µg/ml (table 2). For both antibiotics the MICs were lower than those observed in the donor

209 C68, as has been reported previously (29). Interestingly the MICs of TC-A and TC-B

210 transconjugants increased to comparable levels of those observed in the parental strains after

211 continuous passaging for about ~400 generations, the MICs increase was observed in passaged

212 cells regardless the passaging conditions (BHI only, ampicillin or vancomycin) (table 2). To look

213 for possible causes for the lower observed MICs and their further increase after passaging, cells

214 from the final passage for each condition were collected and subjected to whole genome

215 sequencing and their genomes were compared to the corresponding un-passaged parental

216 transconjugant to look for SNV or other differences. In all passaged transconjugants (BHI,

217 ampicillin or vancomycin) we did not identify differences that suggest that the increased

218 vancomycin and ampicillin MICs are due to genomic changes.

219 To determine if the expression levels of *pbp5* and *vanB ligase* could be implicated in the MIC

220 differences observed between the transconjugants and the parental strains we used the naïve TC-

221 A and its passaged derivatives, TC-A Van P13 and TC-A Amp P9, to study *pbp5* and *vanB*

222 *ligase* expression in the presence of ampicillin or vancomycin. We found that *pbp5* expression

223 was not significantly modified in the presence of ampicillin compared to the corresponding

224 untreated samples. In contrast when cells were grown in the presence of vancomycin, *pbp5*

225   expression was induced in all groups tested (figure 1 A). The *vanB ligase* gene was minimally

226   expressed in the absence of vancomycin and was highly induced if the cells were grown in the

227   presence of the antibiotic. The expression levels of *vanB ligase* were significantly lower in the

228   naïve TC-A compared to the expression of C68, after passaging both TC-A Amp P9 and TC-

229   Amp P13 shown increased *vanB ligase* expression that were comparable to C68 (figure 1B).

230   To perform in depth analysis of the transferred DNA we analysed the whole genome sequences

231   of the fourteen transconjugants (Accession numbers LRAR00000000-LRBE00000000), the

232   recipient strain D344RRF (Accession number LOQQ00000000) and our laboratory stock of the

233   donor strain C68 (Accession number LRAQ00000000 and LPUE00000000).

234   We did not recover transconjugants from D344RRF cells incubated with cell-free C68

235   supernatant, suggesting that phage-mediated transduction is not the mechanisms for DNA

236   transfer in our system. We did recover the same proportion of transconjugants from mating

237   reactions treated with DNaseI cells compared with non-treated ones, suggesting that extracellular

238   DNA do not play a major role in DNA transfer between our two strains.

239

240   **Vancomycin resistance acquisition is associated with *pbp5* allelic replacement in *E. faecium***

241   *E. faecium pbp5* operon consists of three genes: *ftsW, psr* (penicillin-binding protein synthesis

242   repressor) and *pbp5* (29). C68 and D344RRF *pbp5* operon differ in four positions (figure 2A).

243   The first difference is the insertion of a C 153 bp downstream the ribosomal binding site in the

244   *psr* gene in C68 causing a frame shift of the open reading frame and introducing a premature

245   stop codon 309 bp downstream the start of the gene, possibly generating a truncated protein. The

246   second difference is the presence of an extra codon (AGT) in C68 *pbp5* gene at position 1399,

247   which introduces an additional serine. The other two differences are two non-synonymous SNVs

11

248 on position 1456A>G and 1494T>G of C68 *pbp5* gene (figure 2B). The presence of the extra

249 serine and the two non-conservative amino acid substitutions decreases the affinity of C68-Pbp5

250 to penicillin (30).

251 Whole genome sequencing revealed that the 14 selected transconjugants incorporated the full 34

252 kb region corresponding to the *vanB* resistance element Tn*5382* from the donor strain in the

253 vicinity of *pbp5*. The ends of Tn*5382* were clearly identifiable and were conserved in all

254 transconjugants. In none of the transconjugants did we identify the retention of *pbp5* from both

255 C68 and D344RRF. TC-D was the only transconjugant that maintained the *pbp5* allele from the

256 recipient strain D344RRF, but acquired *vanB*/Tn*5382*. The sequence comparison between TC-D

257 and the donor and recipient strains shows that Tn*5382* integrated into D344RRF chromosome

258 downstream of the *pbp5* gene in a location indistinguishable from that in C68. In this region, the

259 first SNV that distinguishes D344RRF from C68 occurs 546 bp upstream of the *pbp5* stop codon.

260 On the opposite end of the transposon, the first SNV that distinguishes D344RRF from C68

261 occurs ca. 2 kb from the transposon end. The SNV in this location corresponds to sequence from

262 C68. The next SNV occurs roughly 2.4 kb from the first SNV and corresponds to sequence from

263 D344RRF. Interestingly, we identified an additional independent recombination event 13.8 kb

264 further to the left of the transposon insertion that replaced approximately 9.1 kb of the recipient

265 genome (figure 2C, table 3). We identified another transconjugant (TC-M) that also had an

266 additional recombination upstream of *vanB*/Tn*5382*.

267

268 **Transposition of Tn*5382* itself is not necessary for acquisition of vancomycin resistance**

269 We found that that unlike the case of typical conjugative transposons (31, 32) *vanB* insertion did

270 not occur at random/semi-random in the recipient's genome but in association with the *pbp5*

271 locus. In all transconjugants but one (93%), variable quantities of contiguous DNA, including the

272 C68 *pbp5* allele, were also transferred, which is not in tune with conjugative transposition. The

273 amount of chromosomal DNA transferred along with Tn*5382* varied in each transconjugant,

274 ranging from 37 kb to 185 kb (table 3). We did not find evidence of mutations in Tn*5382* that

275 might be responsible for defective conjugative transposition.

276

277 **Integration of acquired DNA into the recipient chromosome occurs by recombination along**

278 **homologous regions**

279 The crossover regions between donor and recipient were identified by SNV analysis in our

280 genome assemblies and were confirmed by PCR and Sanger sequencing.

281 In *ca.* 64% of cases (TC-A, TC-D, TC-G, TC-H and TC-I [downstream] being the exceptions)

282 the crossovers occurred in regions devoid of putative transposable elements, suggesting that the

283 DNA integration from the donor into the recipient occurred by recombination across regions of

284 homology and not by IS element-mediated transposition. The upstream crossover point of TC-G

285 occurred in the region of transposon Tn*916* (18) in the D344RRF chromosome, disrupting the

286 genes coding for the transposase and the conjugation proteins. In the case of TC-H we were

287 unable to confirm by PCR the crossover regions as they are presumably within an IS element that

288 prevented amplification.

289 Upstream of the *vanB*/Tn*5382* the crossover regions in 10 transconjugants and the two secondary

290 integrations occurred in a 17 kb region beginning at 10,646 bp left of Tn*5382*. Within this region

291 we identified three groups of transconjugants that share the same or very similar crossovers:

292 Group 1 (TC-B, F, K, M), Group 2 (TC-C, I, J, L) and Group 3 (TC-E and N) (figure 3A).

293   Downstream of *vanB*/Tn*5382* we only found two shared crossover sites in TC-C and TC-G. In

294   other transconjugants we identified crossovers that occurred in the same region but were not

295   flanked by the same SNV, these cases were TC-B and TC-F, TC-H, TC-J and TC-K (figure 3A).

296   Only TC-A did not share any crossover region with other transconjugants. None of the

297   transconjugants shared both crossover regions. DNA integration into the recipient's chromosome

298   was not completely at random as several transconjugants shared crossover regions. In particular

299   the 17 kb region upstream of *vanB*/Tn*5382* appeared to be a hotspot for DNA integration. The

300   average GC content for this region is 36.2%, which is not different for the next 17 kb upstream

301   of it or between the donor and the recipient. Interestingly in the regions surrounding the SNVs

302   that mark the crossover regions we did identified sudden changes in GC content with the

303   presence of AT-rich strings. However, local changes of GC content are not a particular feature of

304   the 17 kb region that could fully explain why it was preferentially targeted for recombination

305   (figure 3B).

306   By analysing the SNV density of the recipient strain D344RRF and the transconjugants along the

307   C68 chromosome we identified that the region flanking *vanB/*Tn*5382* has fewer SNVs compared

308   with the surrounding chromosome (figure S1). Upstream of *vanB*/Tn*5382* we found only 17

309   SNV in 33 kb compared to 67 SNVs in the next 33 kb; and 31 SNV in 56 kb downstream of

310   *vanB*/Tn*5382* compared to 253 SNVs in the next 56 kb. The crossovers for 12 transconjugants

311   (TC-A and TC-I being the exception for the downstream crossover) occurred within this region

312   of very low polymorphisms.

313   The presence of a highly homologous stretch of DNA with local AT-rich regions might explain

314   the preference for recombination in this chromosomal region.

315   The SNV analysis did not suggest additional crossover regions in other parts of the D344RRF

316   chromosome.

317   **pLRM23 associates with genomic DNA transfer**

318   C68 plasmid pLRM23 was previously identified as an important participant in the co transfer of

319   antibiotic resistance (24). To get a better insight into the pLRM23 plasmid sequence and other

320   putative transferable elements we analysed the C68 PacBio assembly (LPUE00000000) for the

321   presence of the *hyaluronidase* (*hyl*) gene, which was previously identified as part of pLRM23.

322   We identified *hyl* residing in a 217,169 bp contig. The contig size is comparable to what was

323   previously experimentally determined for pLRM23 based on caesium chloride purification and

324   agarose gel analysis (16). The putative pLRM23 sequence contains a high abundance of genes

325   for mobile element proteins, transposases and integrases/recombinases (Supplemental table 5A).

326   It also contains putative replication initiation proteins A (*repA*) and B (*rep*B) genes and a

327   putative conjugation protein gene from the *traG/traD* family often identified in conjugative

328   plasmids (33). Unlike other *E. faecium* large plasmids (34), pLRM23 does not carry genes

329   involved in antibiotic resistance but contains a high abundance of genes from the

330   phosphoenolpyruvate-dependent phosphotransferase system (PTS) family involved in

331   carbohydrate metabolism and putative regulation of virulence factors in bacteria (35). pLRM23

332   from C68 exhibits some similarity to other *E. faecium* plasmids, sharing 43.7 % sequence

333   identity with *E. faecium* DO plasmid 3, including the replication and conjugation proteins.

334

335   To investigate if pLRM23 was consistently co-transferred during conjugation, we first compared

336   the canonical pLRM23 sequence (obtained from the C68 PacBio sequencing) to the PacBio

337   sequencing data for TC-A (LRHK00000000). We identified a single TC-A matching contig,

338   suggesting that the full-length pLRM23 was transferred to TC-A. Then, by mapping the Illumina

339   sequencing reads of all transconjugants versus the canonical pLRM23 plasmid sequence, we

340   were able to determine if the plasmid, or regions of it, co-transferred with the acquisition of

341   vancomycin resistance. Transconjugants TC-A and TC-B harboured the same amount of putative

342   plasmid sequences as C68, 217 kb. Transconjugants TC-G and TC-H have 185 and 166 kb,

343   respectively, including in all a previously characterised 16.4 kb region containing the putative

344   *hyl* (16, 36). The presence of the *hyl* gene was confirmed by PCR using the same DNA samples

345   that were used for whole genome sequencing in all transconjugants. The presence of pLRM23 in

346   transconjugants TC-A and TC-B was stable over ~400 generations as the two were subjected to

347   serial passaging and after that their whole genome were sequenced, showing that the canonical

348   pLRM23 sequence did not change during the course of the passaging.

349   Interestingly, the *hyl* gene was also amplified in TC-C, TC-E, TC-F and very faintly in TC-I,

350   whose genome sequences did not have the gene (or other pLRM23 fragments) (figure S2A,

351   supplemental table 1, supplemental table 5C), suggesting that colonies of those transconjugants

352   might constituted a mixed population with cells still retaining fragments of the plasmid. To

353   confirm the PCR finding and to determine if the amplification of plasmid genes was stable in

354   these transconjugants, we performed serial passages for 5 days from the original glycerol stocks

355   and then repeated the PCR amplification of *hyl* DNA. *hyl* was detected weakly in TC-D, TC-F

356   and TC-I after an overnight growth but not after 5 days of continuous culture (figure S2B),

357   suggesting that if a small population containing pLRM23 derived-sequences was present in the

358   original stocks this population did not persist. These data also suggest that pLRM23 or parts of it

359   were likely co-transferred and just did not get fixed in these colonies.

360

16

361   In addition to the transfer of putative pLRM23 we identified a further 15 kb region that

362   transferred from C68 to transconjugants TC-A to TC-E and TC-G to TC-I that may be an

363   additional plasmid. This second plasmid, named pRIH77 has two bacteriocin-related genes,

364   mobilization and replication genes but does not have putative conjugation genes (Supplemental

365   table 5B), suggesting that is a mobilisable but non-conjugative plasmid.

366   **DISCUSSION**

367

368   Although individual transposons have been identified for both *vanA* and *vanB* elements (37-39),

369   the transfer of these determinants between *E. faecium* strains has been associated with the

370   movement of large segments of chromosomal DNA (10, 13, 15). The mechanisms for these

371   transfers have never been precisely described, though in some cases they appear to involve

372   association of the donor chromosome with a conjugative plasmid prior to transfer (40, 41). In

373   elegant experiments in *Enterococcus faecalis*, Manson and colleagues (21) described transfer

374   events that involved chromosomal integration of the transferable plasmids pTEF1 and pTEF2

375   across similar IS elements, and proposed a subsequent transfer event in which portions of

376   plasmid and genomic DNA transfer to recipient strains using an Hfr-like mechanism (21), and a

377   similar mechanism is proposed to happen in *E. faecium* (42), however the precise mechanisms by

378   which the transferred DNA entered into the recipient chromosome were not addressed in these

379   studies.

380   Previous work by our group and others have found conflicting evidence regarding the nature of

381   mobilisation of Tn*5382*-associated vancomycin resistance (10, 13, 15, 19, 20). Our present

382   results suggest that in most cases the acquisition of vancomycin resistance is not mediated by

383   direct transposition of Tn*5382*. In one of our studied transconjugants, TC-D, the insertion of

384 Tn*5383* occurred immediately downstream of *pbp5*, and this transconjugant retained the *pbp5*

385 gene from the recipient strain. These data could be explained by three possibilities. The first is

386 direct transposition of Tn*5382* into the location downstream of *pbp5*, accompanied by

387 homologous recombination of a small (less than 4 kb) region immediately downstream. The

388 second would be one-sided transposition on the left side of Tn*5382* and homologous

389 recombination on the right. The final possibility would be entry by homologous recombination

390 across regions flanking Tn*5382* with the left-side recombination occurring across a small region

391 (within 664 bp) of the genome. Our data do not allow us to distinguish between these

392 possibilities, nor is enough known about enterococcal recombination to determine which is most

393 likely.

394 Our data are consistent in two respects with entry of donor chromosomal regions into the

395 recipient chromosome occurring through homologous recombination. The first is that in none of

396 the strains did we identify co-existence of *pbp5* from both donor and recipient in the same cell.

397 In most cases, the donor (C68) *pbp5* was the only one present in the genome sequence,

398 suggesting that the presumptive pLRM23-chromosome cointegrate does not remain as such for

399 long as we were unable to identify this structure in any of our transconjugants, recombining with

400 the recipients chromosome directly involving the regions flanking *pbp5*. The second is that our

401 SNV analysis suggested that the crossovers occurred in different regions, many of which

402 contained no identifiable mobile elements.

403

404 The nature of our experiments did not allow a precise identification of crossover points in these

405 transconjugants, since we relied on naturally occurring SNVs between donor and recipient,

406 which are not evenly distributed throughout the genomes. Despite this, we did identify several

407   transconjugants in which crossover regions appeared to be very similar, suggesting that

408   characteristics of these regions facilitate homologous recombination. Crossover regions for 9 of

409   the transconjugants were in locations devoid of IS elements or other identifiable mobile

410   elements, suggesting that these putative "hotspots" were not based on the presence of

411   transposable elements and in none of the cases did we found the presence of IS elements in the

412   vicinity of both crossover regions. Surrounding the SNVs that mark the crossover regions

413   clustered within the 17 kb region upstream of *vanB*/Tn*5382* we did identify sudden changes in

414   GC content, with the SNV that mark the beginning and the end of the crossover region occurring

415   in the vicinity of AT-rich stretches, these findings suggest that the regions in which

416   recombination occur have local differences in GC, including A/T strings with lower melting

417   points. Another important finding was that the crossovers occurred in a region were both

418   D344RRF and C68 chromosomes are highly homologous with lower density of SNV and other

419   polymorphisms than other regions of the chromosome, suggesting that a long stretch of highly

420   homologous DNA is preferentially selected for recombination. Interestingly it was previously

421   described that the *pbp5* gene can be mobilised from one *E. faecium* strain to other linked to

422   *vanB*/Tn*5382* (13, 17) or as a part of a larger chromosomal region to create hybrid strains (42).

423

424   Earlier work from our laboratory showed evidence for *pbp5* transfer from *E. faecium* C68 into

425   the *pbp5*-deficient strain D344SRF that included an intermediate that was in a closed circular

426   form in the transconjugant (17). This closed circular form was likely a cointegrate between the

427   donor chromosome and C68 transferable plasmid pLRM23 that persisted for a time because of

428   the absence of a homologous *pbp5* region in the recipient chromosome. Eventually, entry into

429   the   chromosome   in   these   circumstances   (which   occurred   independently   in   several

19

430 transconjugants) likely involved crossover between IS elements on the recipient chromosome

431 and plasmid. In the current study we used a recipient strain that has the entire *pbp5* region in its

432 chromosome, presenting extensive regions of homology with the transferred chromosomal

433 region to facilitate crossover, in a model that more closely approximates transfer events as they

434 may occur in nature.

435

436 The pLRM23 plasmid was previously associated with increased gastrointestinal colonization in a

437 mouse infection model (16), however the genes involved were not identified. Here we found that

438 pLRM23 is highly enriched in PTS genes, including the four-gene cluster of the mannose-family

439 PTS previously reported by Zhang and collaborators (43) as to be important for murine

440 gastrointestinal tract colonization after depletion of the endogenous microbiota. Genes from the

441 PTS including the mannose operon were identified in association with mobile elements and

442 possibly implicated in improved human colonization in Clade A1 strains (44). The presence of

443 the mannose PTS genes might help to explain the role of pLRM23 and related plasmids in

444 gastrointestinal colonization, it is interesting however, that both C68 and D344RRF also carry a

445 chromosomally encoded mannose PTS operons and D344RRF lacks the ability to successfully

446 colonize the mouse gastrointestinal tract even with high inoculum, suggesting that this capability

447 is a complex trait (16).

448 It was previously observed that the transfer of vancomycin resistance from *E. faecium* C68 to a

449 susceptible strain was associated in high frequency (70%) with the acquisition of a *hyl* positive

450 plasmid (pLRM23) (24). Our current results showed only 28% frequency of co-transfer of

451 pLRM23 and vancomycin resistance, but this might be an underestimate due to instability of

452 pLRM23 in the D344RRF background or incomplete plasmid transfer. We identified genes

20

453   corresponding to pLRM23 in the genome assemblies of four of fourteen selected

454   transconjugants. Interestingly, by PCR we identified the presence of the *hyl* gene sequence

455   transiently in four additional transconjugants, suggesting that some parts of pLRM23 transferred

456   to the recipient strain but did not integrate stably into the chromosome or persist as a separate

457   replicon, likely in a mixed bacterial population. The four transconjugants in which pLRM23

458   persisted all had large fragments of plasmid DNA in the genome assemblies, including the *repA*

459   gene, suggesting that the plasmid replication machinery was also transferred. In at least TC-A

460   and TC-B, it appears that plasmid transfer was complete. Complete plasmid transfer is

461   inconsistent with transfer by an Hfr-like mechanism. But in our view is compatible with the

462   formation of a covalently closed circular transfer intermediate (plasmid/chromosome

463   cointegrate), which excises from the donor chromosome, carrying a portion of the chromosome

464   and then transfers by conjugation (45). After conjugation the persistence of the plasmid in the

465   transconjugant would then depend upon whether the plasmid origin of replication and replicase

466   genes were included in the transferred package. In the cases were partial plasmid sequences

467   where detected we could not rule out an Hfr-like mechanism. Another possibility is that

468   chromosome mobilisation may be mediated by chromosomally encoded regions without the need

469   of a conjugative plasmid. In this instance chromosomal DNA transfer and plasmid transfer might

470   be independent of each other, however more work would be necessary to test this possibility.

471

472   In conclusion, transfer and replacement of large regions of genomic DNA between *E. faecium*

473   strains appears to be commonly facilitated by recombination across regions of homology. A

474   better understanding of these transfer events will inform analyses of the molecular epidemiology

475   of resistance and virulence in this species and suggest possible mechanisms by which transfer of

476   these determinants could be interrupted.

482   **REFERENCES**

483

484   1.   **Hollenbeck BL, Rice LB.** 2012. Intrinsic and acquired resistance mechanisms in

485   *Enterococcus*. Virulence **3:**421-433.

486   2.   **Howden BP, Holt KE, Lam MMC, Seemann T, Ballard S, Coombs GW, Tong SYC,**

487   **Grayson ML, Johnson PDR, Stinear TP.** 2013. Genomic insights to control the

488   emergence of vancomycin-resistant enterococci. mBio **4:**e00412-00413.

489   3.   **Rice LB.** 2012. Mechanisms of resistance and clinical relevance of resistance to β-

490   lactams, glycopeptides, and fluoroquinolones. Mayo Clin Proc **87:**198-208.

491   4.   **Palmer KL, Kos VN, Gilmore MS.** 2010. Horizontal gene transfer and the genomics of

492   enterococcal antibiotic resistance. Curr Opin Microbiol **13:**632-639.

493   5.   **Arias CA, Murray BE.** 2008. Emergence and management of drug-resistant

494   enterococcal infections. Expert Review of Anti-infective Therapy **6:**637-655.

495   6.   **Starikova I, Al-Haroni M, Werner G, Roberts AP, Sørum V, Nielsen KM, Johnsen**

496   **PJ.** 2013. Fitness costs of various mobile genetic elements in *Enterococcus faecium* and

497   *Enterococcus faecalis*. J Antimicrob Chemother **68:**2755-2765.

498   7.   **de Been M, van Schaik W, Cheng L, Corander J, Willems RJ.** 2013. Recent

499   recombination events in the core genome are associated with adaptive evolution in

500   *Enterococcus faecium*. Genome Biology and Evolution **5:**1524-1535.

501   8.   **Galloway-Pena J, Roh JH, Latorre M, Qin X, Murray BE.** 2012. Genomic and SNP

502   analyses demonstrate a distant separation of the hospital and community-associated

503   clades of *Enterococcus faecium*. PLoS One **7:**e30187.

504   9.   **Paulsen IT, Banerjei L, Myers GSA, Nelson KE, Seshadri R, Read TD, Fouts DE,**

505   **Eisen JA, Gill SR, Heidelberg JF, Tettelin H, Dodson RJ, Umayam L, Brinkac L,**

506   **Beanan M, Daugherty S, DeBoy RT, Durkin S, Kolonay J, Madupu R, Nelson W,**

507   **Vamathevan J, Tran B, Upton J, Hansen T, Shetty J, Khouri H, Utterback T,**

508   **Radune D, Ketchum KA, Dougherty BA, Fraser CM.** 2003. Role of mobile DNA in

509   the evolution of vancomycin-resistant *Enterococcus faecalis*. Science **299:**2071-2074.

510   10.   **Quintiliani R, Jr., Courvalin P.** 1994. Conjugal transfer of the vancomycin resistance

511   determinant *vanB* between enterococci involves the movement of large genetic elements

512   from chromosome to chromosome. FEMS Microbiol Lett **119:**359-363.

513   11.   **Cetinkaya Y, Falk P, Mayhall CG.** 2000. Vancomycin-resistant enterococci. Clin

514   Microbiol Rev **13:**686-707.

515   12.   **Hanrahan J, Hoyen C, Rice LB.** 2000. Geographic distribution of a large mobile

516   element that transfers ampicillin and vancomycin resistance between *Enterococcus*

517   *faecium* strains. Antimicrob Agents Chemother **44:**1349-1351.

518   13.   **Carias LL, Rudin SD, Donskey CJ, Rice LB.** 1998. Genetic linkage and cotransfer of a
519   novel, *vanB*-containing transposon (Tn*5382*) and a low-affinity penicillin-binding protein
520   5 gene in a clinical vancomycin-resistant *Enterococcus faecium* isolate. J Bacteriol
521   **180:**4426-4434.

522   14.   **Lu J-J, Chang T-Y, Perng C-L, Lee S-Y.** 2005. The *vanB2* gene cluster of the majority
523   of vancomycin-resistant *Enterococcus faecium* isolates from Taiwan is associated with
524   the *pbp5* gene and is carried by Tn*5382* containing a novel insertion sequence.
525   Antimicrob Agents Chemother **49:**3937-3939.

526   15.   **Dahl KH, Røkenes TP, Lundblad EW, Sundsfjord A.** 2003. Nonconjugative
527   transposition of the *vanB*-containing Tn*5382*-like element in *Enterococcus faecium*.
528   Antimicrob Agents Chemother **47:**786-789.

529   16.   **Rice LB, Lakticova V, Carias LL, Rudin S, Hutton R, Marshall SH.** 2009.
530   Transferable capacity for gastrointestinal colonization in *Enterococcus faecium* in a
531   mouse model. J Infect Dis **199:**342-349.

532   17.   **Rice LB, Carias LL, Rudin S, Laktičová V, Wood A, Hutton-Thomas R.** 2005.
533   *Enterococcus faecium* low-affinity *pbp5* is a transferable determinant. Antimicrob Agents
534   Chemother **49:**5007-5012.

535   18.   **Rice LB, Carias LL, Marshall S, Rudin SD, Hutton-Thomas R.** 2005. Tn*5386*, a
536   novel Tn*916*-like mobile element in *Enterococcus faecium* D344R that interacts
537   withTn*916* to yield a large genomic deletion. J Bacteriol **187:**6668-6677.

538   19.   **Launay A, Ballard SA, Johnson PDR, Grayson ML, Lambert T.** 2006. Transfer of
539   vancomycin resistance transposon Tn*1549* from *Clostridium symbiosum* to *Enterococcus*
540   spp. in the gut of gnotobiotic mice. Antimicrob Agents Chemother **50:**1054-1062.

541   20.   **Rice LB, Carias LL, Donskey CL, Rudin SD.** 1998. Transferable, plasmid-mediated

542   *van*B-type glycopeptide resistance in *Enterococcus faecium*. Antimicrob Agents

543   Chemother **42:**963-964.

544   21.   **Manson JM, Hancock LE, Gilmore MS.** 2010. Mechanism of chromosomal transfer of

545   *Enterococcus faecalis* pathogenicity island, capsule, antimicrobial resistance, and other

546   traits. Proc Natl Acad Sci USA **107:**12269-12274.

547   22.   **Williamson R, Le Bouguénec C, Gutmann L, Horaud T.** 1985. One or two low

548   affinity penicillin-binding proteins may be responsible for the range of susceptibility of

549   *Enterococcus faecium* to benzylpenicillin. J Gen Microbiol **131:**1933-1940.

550   23.   **Rice LB, Carias LL.** 1998. Transfer of Tn*5385*, a composite, multiresistance

551   chromosomal element from *Enterococcus faecalis*. J Bacteriol **180:**714-721.

552   24.   **Arias CA, Panesso D, Singh KV, Rice LB, Murray BE.** 2009. Cotransfer of antibiotic

553   resistance genes and a *hylefm*-containing virulence plasmid in *Enterococcus faecium*.

554   Antimicrob Agents Chemother **53:**4240-4246.

555   25.   **Pfaffl MW.** 2001. A new mathematical model for relative quantification in real-time RT–

556   PCR. Nucleic Acids Res **29:**e45.

557   26.   **Darling ACE, Mau B, Blattner FR, Perna NT.** 2004. Mauve: Multiple alignment of

558   conserved genomic sequence with rearrangements. Genome Res **14:**1394-1403.

559   27.   **Aziz R, Bartels D, Best A, DeJongh M, Disz T, Edwards R, Formsma K, Gerdes S,**

560   **Glass E, Kubal M, Meyer F, Olsen G, Olson R, Osterman A, Overbeek R, McNeil L,**

561   **Paarmann D, Paczian T, Parrello B, Pusch G, Reich C, Stevens R, Vassieva O,**

562   **Vonstein V, Wilke A, Zagnitko O.** 2008. The RAST server: Rapid annotations using

563   subsystems technology. BMC Genomics **9:**75.

564   28.   **Overbeek R, Olson R, Pusch GD, Olsen GJ, Davis JJ, Disz T, Edwards RA, Gerdes**

565   **S, Parrello B, Shukla M, Vonstein V, Wattam AR, Xia F, Stevens R.** 2014. The SEED

566   and the rapid annotation of microbial genomes using subsystems technology (RAST).

567   Nucleic Acids Res **42:**D206-D214.

568   29.   **Rice LB, Carias LL, Hutton-Thomas R, Sifaoui F, Gutmann L, Rudin SD.** 2001.

569   Penicillin-binding protein 5 and expression of ampicillin resistance in *Enterococcus*

570   *faecium*. Antimicrob Agents Chemother **45:**1480-1486.

571   30.   **Rice LB, Bellais S, Carias LL, Hutton-Thomas R, Bonomo RA, Caspers P, Page**

572   **MGP, Gutmann L.** 2004. Impact of specific *pbp5* mutations on expression of β-lactam

573   resistance in *Enterococcus faecium*. Antimicrob Agents Chemother **48:**3028-3032.

574   31.   **J R Scott, Churchward GG.** 1995. Conjugative transposition. Annu Rev Microbiol

575   **49:**367-397.

576   32.   **Mark Osborn A, Böltner D.** 2002. When phage, plasmids, and transposons collide:

577   Genomic islands, and conjugative- and mobilizable-transposons as a mosaic continuum.

578   Plasmid **48:**202-212.

579   33.   **Schroder G, Krause S, Zechner EL, Traxler B, Yeo HJ, Lurz R, Waksman G,**

580   **Lanka E.** 2002. Trag-like proteins of DNA transfer systems and of the *Helicobacter*

581   *pylori* type IV secretion system: Inner membrane gate for exported substrates? J Bacteriol

582   **184:**2767-2779.

583   34.   **Laverde Gomez JA, van Schaik W, Freitas AR, Coque TM, Weaver KE, Francia**

584   **MV, Witte W, Werner G.** 2011. A multiresistance megaplasmid pLG1 bearing a *hylefm*

585   genomic island in hospital *Enterococcus faecium* isolates. Int J Med Microbiol **301:**165-

586   175.

587   35.   **Deutscher J, Francke C, Postma PW.** 2006. How phosphotransferase system-related protein phosphorylation regulates carbohydrate metabolism in bacteria. Microbiol Mol Biol Rev **70:**939-1031.

590   36.   **Rice LB, Carias L, Rudin S, Vael C, Goossens H, Konstabel C, Klare I, Nallapareddy SR, Huang W, Murray BE.** 2003. A potential virulence gene, *hylefm*, predominates in *Enterococcus faecium* of clinical origin. J Infect Dis **187:**508-512.

593   37.   **Tsvetkova K, Marvaud J-C, Lambert T.** 2010. Analysis of the mobilization functions of the vancomycin resistance transposon Tn*1549*, a member of a new family of conjugative elements. J Bacteriol **192:**702-713.

596   38.   **Dahl KH, Lundblad EW, Røkenes TP, Olsvik Ø, Sundsfjord A.** 2000. Genetic linkage of the *vanB2* gene cluster to Tn*5382* in vancomycin-resistant enterococci and characterization of two novel insertion sequences. Microbiology **146:**1469-1479.

599   39.   **Hegstad K, Mikalsen T, Coque TM, Werner G, Sundsfjord A.** 2010. Mobile genetic elements and their contribution to the emergence of antimicrobial resistant *Enterococcus faecalis* and *Enterococcus faecium*. Clin Microbiol Infect **16:**541-554.

602   40.   **François B, Charles M, Courvalin P.** 1997. Conjugative transfer of *tet*(s) between strains of *Enterococcus faecalis* is associated with the exchange of large fragments of chromosomal DNA. Microbiology **143:**2145-2154.

605   41.   **Dahl KH.** 2000. Structure and mobility of the *vanB* operon in enterococci. Doctor Scientiarum. University of Tromso, Norway.

607   42.   **Palmer KL, Godfrey P, Griggs A, Kos VN, Zucker J, Desjardins C, Cerqueira G, Gevers D, Walker S, Wortman J, Feldgarden M, Haas B, Birren B, Gilmore MS.** 2012. Comparative genomics of enterococci: Variation in *Enterococcus faecalis*, clade

610   structure in *E. faecium*, and defining characteristics of *E. gallinarum* and *E. casseliflavus*.

611   mBio **3:**e00318-00311.

612 43. **Zhang X, Top J, de Been M, Bierschenk D, Rogers M, Leendertse M, Bonten MJM,**

613   **van der Poll T, Willems RJL, van Schaik W.** 2013. Identification of a genetic

614   determinant in clinical *Enterococcus faecium* strains that contributes to intestinal

615   colonization during antibiotic treatment. J Infect Dis **207:**1780-1786.

616 44. **Lebreton F, van Schaik W, Manson McGuire A, Godfrey P, Griggs A, Mazumdar**

617   **V, Corander J, Cheng L, Saif S, Young S, Zeng Q, Wortman J, Birren B, Willems**

618   **RJL, Earl AM, Gilmore MS.** 2013. Emergence of epidemic multidrug-resistant

619   *Enterococcus faecium* from animal and commensal strains. mBio **4:**e00534-00513.

620 45. **Cabezón E, Ripoll-Rozada J, Peña A, de la Cruz F, Arechaga I.** 2015. Towards an

621   integrated model of bacterial conjugation. FEMS Microbiol Rev **39:**81-95.

622

623   **FIGURE LEGENDS AND TABLES**

624   **Figure 1:** Relative expression levels of *pbp5* and *vanB ligase* in the naïve TC-A transconjugant

625   (P0) or in the resulting selected ones (A P9: continuously passaged in the presence of ampicillin

626   for ~400 generations and V P13: continuously passaged in the presence of vancomycin for ~400

627   generations). The parental strains (D344RRF and C68) were included for comparison.   The

628   parents and the three versions of TC-A were grown with either ampicillin (amp) or vancomycin

629   (van) and relative expression was calculated using the corresponding group without antibiotic

630   treatment. Expression levels of 16S rRNA were used as calibrator. Error bars indicate the

631   standard error of the mean for biological triplicates. A) *pbp5* expression is not modified with

632   respect to the control (untreated cells) in the presence of half the MIC of ampicillin, *pbp5*

633   expression was   induced in C68 and the three different TC-A   groups in the presence of

634   vancomycin. B) *vanB ligase* expression was very low in the absence of vancomycin. In the

635   presence of the antibiotic there was a significant induction in *vanB ligase* expression. The un-

636   passaged TC-A (P0) had lower *vanB ligase* expression levels compared to C68 and the passaged

637   groups which correlates with lower vancomycin MIC. White bars: BHI grown cells (calibrator),

638   grey bar: amp grown cells, black bar: van grown cells. ****p <0.0001, ***< 0.0002, *<0.05.

639

640   **Figure 2:** *pbp5* and TC-D insertion site. A) *pbp5* operon cartoon showing the differences

641   between strains D344RRF and C68. In C68 an insertion (C 153) bp downstream the ribosomal

642   binding site causes a frame shift introducing a premature stop codon 309 bp downstream the start

643   of the gene, possibly generating a non-functional gene. B) *pbp5* gene in C68 codes for an extra

644   serine and has two amino acid substitutions compared with D344RRF (bold red). C) Alignment

645   of D344RRF, TC-D and C68 in the region of the *vanB*/Tn*5382* integration. The vancomycin-

646   resistance carrying transposon is shown in green, flanked by the transposon ends (light blue) the

647   segments in which C68 genome replaced that of D344RRF in the transconjugant are shown in

648   light grey. The crossover regions are shown in purple, flanked by the SNV used to identify the

649   region. Note the additional C68 integration in TC-D upstream the *pbp5* operon. *pbp5* from

650   D344RRF was not replaced by that of C68 in this transconjugant.

651

652   **Figure 3:** Crossover regions. A) Cartoon representation of crossover regions in all individual

653   transconjugants. The area of the crossover is measured from the last SNV corresponding to the

654   donor strain, C68, to the first SNV corresponding to the recipient strain, D344RRF.

655   Transconjugants are organised by groups with shared left crossover region. Notice additional

656   crossover regions in transconjugants D and M (light grey). The amount of chromosomal DNA

657   integrated in addition to Tn5382 is measured from the left or right ends of the element up to the

658   last SNV between D344RRF and the each transconjugant. The integrated DNA shown in

659   kilobases. B) GC and AT content plot for C68 (donor) and D344RRF (recipient) in the

660   chromosomal region were crossovers occurred; GC content is shown in blue, AT content is

661   shown in green. The high GC area in C68 corresponding to *vanB*/Tn*5382* element is highlighted

662   between red lines.

663

664

665 TABLE 1: Antibiotic resistance profile of the parental strains and selected transconjugants

666

Strain	Resistance traits	Origin
C68	**Amp[r], Van[r]**, Ery[r], Tet[r], Str[r], Gen[r],	Clinical isolate (13)
D344RRF	Amp[r] [a], **Rif[r], Fus[r]**, Ery[r], Tet[r], Str[r], Kan[r],	Derived from clinical isolate D344R (22)
Transconjugants	**Amp[r], Van[r], Rif[r], Fus[r]**, Ery[r], Tet[r], Str[r], Kan[r]	Transconjugants obtained from mating C68 and D344RRF during this work

667

668 Amp: Ampicillin, Van: Vancomycin, Ery: Erythromycin, Tet: Tetracycline, Str: Streptomycin,

669 Gen: Gentamicin, Rif: Rifampicin, Fus: Fusidic acid, Kan: Kanamycin.

670 [a]Intermediate resistance levels.

671 **TABLE 2: MICs for parental strains and transconjugants**

672

STRAIN	MIC µg/ml			
	Van	**Amp**	**Fus**	**Rif**
C68	>400	>400	1.6	<0.2
D344RRF	1.6	12.5	>400	>400
TC-A P0	100	50	>400	>400
TC-A BHI P13	400	200	>400	>400
TC-A Van P13	400	>400	>400	>400
TC-A Amp P9	400	400	>400	>400
TC-B P0	200	50	>400	>400
TC-B BHI P13	400	>400	>400	>400
TC-B Van P13	400	200	>400	>400
TC-B Amp P9	400	>400	>400	>400
TC-C	25	25	200	>400
TC-D	50	25	400	>400
TC-E	50	50	200	>400
TC-F	50	100	400	>400
TC-G	25	12.5	200	400
TC-H	50	25	200	>400
TC-I	200	25	>400	>400
TC-J	200	25	>400	>400
TC-K	100	100	>400	>400

TC-L	50	6.3	>400	>400
TC-M	100	25	>400	>400
TC-N	12.5	6.3	200	200

673

674

675   **TABLE 3: Integration site and size of integrated DNA in the studied transconjugants**

TC	1st SNV of COR Left of *vanB* [a,b]	1st SNV of COR Right of *vanB* (integrase site)	Minimum DNA transferred[c]	Size of COR between the two delimiting SNVs	Additional integration sites
TC-A	-49.87	+100.8	184.6	Left 574 Right 307	
TC-B	-10.6	+49.9	94.5	Left 3,112 Right 4,315	
TC-C	-16.7	+27.7	78.4	Left 6,229 Right 1,159	
TC-D	0	+2.9	36.9	Left 654 Right 2,466	9,098; -14 left of *vanB*
TC-E	-14.9	+15.1	64	Left 2,005 Right 2171	
TC-F	-10.9	+47.9	92.8	Left 3,112 Right 2,049	
TC-G	-61.8	+27.6	123.4	Left 2,878 Right 1,157	
TC-H	-60.2	+81.3	175.5	Left 593 Right 126	
TC-I	-16.9	+124	174.9	Left 6,182	

34

				Right 11	
TC-J	-16.9	+79.9	130.8	Left 6,182	
				Right 1,240	
TC-K	-10.9	+78.7	123.6	Left 3,112	
				Right 185	
TC-L	-16.9	+48.4	99.3	Left 6,182	
				Right 947	
TC-M	-10.9	+36	80.9	Left 3,113	11,719; -16.9
				Right 7,373	of *vanB*
TC-N	-14.9	+5.9	54.8	Left 2,005	
				Right 371	

676   COR: Crossover region

677   [a] DNA in kilobases

678   [b] Considering the first position of Tn*5382* to the left side (-); and the last position of Tn*5382* to

679   the right side (+).

680   [c] Including the 34 kb *vanB/*Tn*5382* element

A *pbp5* **expression**

B *vanB ligase* **expression**

A

280,000 bp

TC-A   −49.8   +100.8

TC-D   −14   0   +2.9

TC-G   −61.8   +27.6

TC-H   −60.2   +81.3

TC-B   −10.6   +49.9

TC-F   −10.9   +47.9

TC-K   −10.9   +78.7

TC-M   −10.9   +36

TC-C   −16.7   +27.7

TC-I   −16.9   +124

TC-J   −16.9   +79.9

TC-L   −16.9   +48.4

TC-E   −14.9   +15.1

TC-N   −14.9   +5.9

D344RRF   Crossover region   C68 *pbp5*
C68   VanB/Tn*5382*   D344RRF *pbp5*

B

GC Content

C68
100%
50%
0%

D344RRF
100%
50%
0%