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Fitness determinants of vancomycin-resistant *Enterococcus faecium* during growth in human serum

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25 **Abstract**

26

27 *Enterococcus faecium* is a commensal of the human gastrointestinal tract and a frequent cause
28 of bloodstream infections in hospitalized patients. Here, we identify genes that contribute to
29 growth of *E. faecium* in human serum. We first sequenced the genome of *E. faecium* E745, a
30 vancomycin-resistant clinical isolate, to completion and then compared its transcriptome
31 during exponential growth in rich medium and in human serum by RNA-seq. This analysis
32 revealed that 27.8% of genes on the *E. faecium* E745 genome were differentially expressed in
33 these two conditions. A gene cluster with a role in purine biosynthesis was among the most
34 upregulated genes in *E. faecium* E745 upon growth in serum. A high-throughput transposon
35 sequencing (Tn-seq) approach was used to identify conditionally essential genes in *E. faecium*
36 E745 during growth in serum. Genes involved in *de novo* nucleotide biosynthesis (including
37 *pyrK_2*, *pyrF*, *purD*, *purH*) and a gene encoding a phosphotransferase system subunit
38 (*manY_2*) were thus identified to be contributing to *E. faecium* growth in human serum.
39 Transposon mutants in *pyrK_2*, *pyrF*, *purD*, *purH* and *manY_2* were isolated from the library
40 and their impaired growth in human serum was confirmed. In addition, the *pyrK_2* and
41 *manY_2* mutants also exhibited significantly attenuated virulence in an intravenous zebrafish
42 infection model. We conclude that genes involved in carbohydrate and nucleotide metabolism
43 of *E. faecium* are essential for growth in human serum and contribute to the pathogenesis of
44 this organism.

45

46 **Introduction**

47

48 Enterococci are commensals of the gastrointestinal tract of humans and animals, but some
49 enterococcal species, particularly *E. faecium* and *E. faecalis*, are also common causes of
50 hospital-acquired infections in immunocompromised patients (1). While *E. faecalis* has been
51 recognized as an important nosocomial pathogen for over a century, *E. faecium* has emerged
52 as a prominent cause of hospital-acquired infections over the last two decades (2). Since the
53 1980s, *E. faecium* acquired resistance to multiple antibiotics, including β -lactams,
54 aminoglycosides and finally, to the glycopeptide vancomycin (3). Nosocomial infections are
55 almost exclusively caused by a specific sub-population of *E. faecium*, termed clade A-1,
56 which has emerged from a background of human commensal and animal *E. faecium* strains
57 (4). Strains in clade A-1 carry genetic elements that are absent from animal or human
58 commensal isolates and which contribute to gut colonization or pathogenicity (5–9).
59 *E. faecium* bloodstream infections frequently develop in patients undergoing
60 immunosuppressive therapy, and can result from translocation of strains from the intestinal
61 tract to the bloodstream (10). In addition, the use of intravenous catheters in hospitalized
62 patients, is another risk factor for the introduction of *E. faecium* into the bloodstream (3, 11,
63 12). Currently, *E. faecium* causes approximately 40% of enterococcal bacteremias. Due to the
64 accumulation of antibiotic resistance determinants in clade A-1 strains, *E. faecium* infections
65 are more difficult to treat than infections caused by *E. faecalis* or other enterococci (13–15).
66 To cause bloodstream infections, *E. faecium* needs to be able to survive and multiply in blood,
67 but the mechanisms by which it can do so, have not yet been studied. To thrive in the
68 bloodstream, an opportunistic pathogen has to evade host immune mechanisms and to adjust
69 its metabolism to an environment that is relatively poor in nutrients (16).

70 To identify genes that are conditionally essential in bacteria, high-throughput screening
71 methods for transposon mutant libraries have been developed and optimized for many
72 different bacterial species (17, 18). To perform high-throughput functional genomics in
73 ampicillin-resistant, vancomycin-susceptible clinical *E. faecium* strains, we previously
74 developed a microarray-based transposon mutagenesis screening method which was used to
75 identify genes involved in the development of endocarditis (7), resistance to ampicillin (19),
76 bile (20) and disinfectants (Guzman Prieto *et al.*, submitted for publication). However,
77 microarray-based methods for transposon mutant library screening are limited in their
78 accuracy and can only be used in strains for which the microarray was designed. To address
79 these limitations, several methods, including Tn-seq (21) and TraDIS (22), which are based
80 on high-throughput sequencing of the junctions of the transposon insertion sites and genomic
81 DNA, have been developed (23).

82 In this study, we set-up Tn-seq in the clinical *E. faecium* isolate E745 to identify genes that
83 contribute to survival and growth in human serum. In addition, we determined the
84 transcriptional response of *E. faecium* E745 in that same environment. Finally, we
85 substantiated the role of several *E. faecium* genes that contribute to growth in serum and in
86 virulence, in a high-throughput zebrafish infection model. Collectively, our findings show that
87 metabolic adaptations are key to *E. faecium* growth in serum and contribute to virulence.

88

89 **Materials and Methods**

90 **Bacterial strains, plasmids, and growth conditions**

91 The vancomycin-resistant *E. faecium* strain E745 was used throughout this study. This strain
92 was isolated from feces of a hospitalized patient, during a VRE outbreak in a Dutch hospital
93 (24, 25). Unless otherwise mentioned, *E. faecium* was grown in brain heart infusion broth
94 (BHI; Oxoid) at 37°C. The *E. coli* strains DH5 α (Invitrogen) was grown in Luria-Bertani
95 (LB) medium. When necessary, antibiotics were used at the following concentrations:
96 chloramphenicol 4 $\mu\text{g ml}^{-1}$ for *E. faecium* and 10 $\mu\text{g ml}^{-1}$ for *E. coli*, and gentamicin 300 μg
97 ml^{-1} for *E. faecium* and 25 $\mu\text{g ml}^{-1}$ for *E. coli*. All antibiotics were obtained from Sigma-
98 Aldrich. Growth was determined by measuring the optical density at 660 nm (OD₆₆₀).

99 **Genome sequencing, assembly and bioinformatic analysis**

100 *E. faecium* E745 was sequenced using a combination of Illumina HiSeq 100 bp paired-end
101 sequencing, long-read sequencing using the Pacific Biosciences RS II SMRT technology and
102 the MinION system with R7 flowcell chemistry (Oxford Nanopore Technologies). Corrected
103 PacBio reads were assembled using the Celera assembler (version 8.1) (26) and assembled
104 contigs were then corrected by aligning Illumina reads using BWA (version 0.7.9a), with
105 default parameters for index creation and BWA-MEM algorithm with *-M* option for the
106 alignment (27). This approach resulted in 15 contigs, including one contig covering the entire
107 2.77 Mbp chromosome. After discarding contigs with low-coverage, the remaining contigs
108 constituted 5 circular plasmid sequences and 5 non-overlapping contigs. These 5 contigs were
109 aligned against the NCBI Genbank database and all were found to be part of the *E. faecium*
110 plasmid pMG1 (28). Based on this alignment the presumed order of contigs was determined
111 and confirmed by gap-spanning PCRs and sequencing of the products. A single gap between
112 two contigs, could not be closed by PCR. Thus, we assembled Illumina reads together with

113 MinION 2D reads using the SPAdes assembler (version 3.0) (29), which produced a contig
114 that closed the gap, resulting in a complete assembly of this plasmid. Sequence coverage of
115 chromosomal and plasmid sequences was determined with SAMtools (version 0.1.18) using
116 short read alignments to the assembly, which were generated using BWA (version 0.7.9a).
117 SAMtools was also used to identify possible base-calling and assembly errors, by aligning
118 short reads to assembled contigs. A base was corrected using the consensus of aligned reads
119 (30). The corrected sequences were annotated using Prokka (version 1.10) (31). The
120 annotated genome of *E. faecium* E745 is available from NCBI Genbank database under
121 accession numbers CP014529 – CP014535.

122

123 **Transcriptome profiling**

124 Approximately 3×10^7 cfu of *E. faecium* E745 were inoculated into 14 ml of BHI broth and
125 heat-inactivated serum, and grown at 37°C until log phase. Cultures were centrifuged at room
126 temperature (15 s; 21.380 g), and pellets were flash frozen in liquid N₂ prior to RNA
127 extraction, which was performed as described previously (19). The ScriptSeq Complete Kit
128 (Bacteria) (Epicentre Biotechnologies, WI) was used for rRNA removal and strand-specific
129 library construction. Briefly, rRNA was removed from 2.5 µg of total RNA. To generate
130 strand specific RNA-seq data, approximately 100 ng of rRNA-depleted RNA was fragmented
131 and reverse transcribed using random primers containing a 5' tagging sequence, followed by
132 3' end tagging with a terminal-tagging oligo to yield di-tagged, single-stranded cDNA.
133 Following magnetic-bead based purification, the di-tagged cDNA was amplified by PCR (15
134 cycles) using ScriptSeq Index PCR Primers (Epicentre Biotechnologies, WI). Amplified
135 RNA-seq libraries were purified using AMPure XP System (Beckman Coulter) and
136 sequenced by a 100 bp paired end reads sequencing run using the Illumina HiSeq 2500

137 platform (University of Edinburgh, United Kingdom). Data analysis was performed using
138 Rockhopper (32) using the default settings for strand specific analysis.

139

140 **Confirmation of RNA-seq data by quantitative real-time RT-PCR (qRT-PCR).**

141 Total RNA isolated as described previously was used to confirm the transcriptome analysis by
142 qRT-PCR. cDNA was synthesized as described above and qRT-PCR on these cDNAs was
143 performed using the Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific,
144 Breda, The Netherlands) and a StepOnePlus instrument (Life Technologies). The expression
145 of *tufA* was used as a housekeeping control. Ct values were calculated using the StepOne
146 analysis software v2.2. Transcript levels, relative to *tufA*, of the assayed genes were calculated
147 using REST 2009 V2.0.13 (Qiagen, Venlo, The Netherlands). This experiment was performed
148 with three biological replicates.

149

150 **Generation of *mariner* transposon mutant library in *E. faecium***

151 To create a transposon mutant library in *E. faecium* E745 suitable for Tn-seq, the *mariner*
152 transposon cassette (carrying a gentamicin resistance gene) in the transposon delivery plasmid
153 pZXL5 (19) was adapted as follows. The transposon from pZXL5 was amplified by PCR
154 using the set of primers: pZXL5_MmeI_SacII_Fw and pZXL5_MmeI_SacII_Rv (primer
155 sequences listed in Supplementary table 7). These primers introduced MmeI restriction sites
156 in the inverted repeats on both sides of the transposon. The modified transposon delivery
157 vector, termed pGPA1, was generated by the digestion of pZXL5 with SacII, followed by the
158 insertion of the SacII-digested *mariner* transposon that contained MmeI restriction sites at its
159 extreme ends. pGPA1 was electroporated into *E. faecium* E745 and the transposon mutant
160 library was generated by selecting for gentamicin-resistant transposon mutants as described
161 previously (19).

162

163 **Tn-seq analysis of conditionally essential genes in *E. faecium* E745**

164 The transposon mutant library created in E745 was prepared for Tn-seq analysis, similar to
165 previously described procedures (33). To identify genes that are essential for the viability of
166 *E. faecium* in BHI, we used ten experimental replicates of the mutant library. Aliquots (20 μ l)
167 of the transposon mutant library, containing approximately 10^7 cfu, were used to inoculate 20
168 ml BHI broth and grown overnight at 37°C. Subsequently, 1 ml aliquots of the cultures were
169 spun down (15 s, 21.380 g) and used for the extraction of genomic DNA (Wizard genomic
170 DNA purification kit, Promega Benelux). 2 μ g of the extracted DNA was digested for 4 hr at
171 37°C using 10U MmeI (New England Biolabs) and immediately dephosphorylated with 1U of
172 calf intestine alkaline phosphatase (Invitrogen) during 30 min at 50°C. DNA was isolated
173 using phenol-chloroform extraction and subsequently precipitated using ethanol. The DNA
174 pellets were then dissolved in 20 μ l water. The samples were barcoded and prepared for Tn-
175 seq sequencing as described previously (33). The sequence reads from all ten experimental
176 replicates were mapped to the genome, and the mapped read-counts were then tallied for the
177 analysis of the essentiality of the genes in the *E. faecium* E745 genome (further described
178 below).

179 To identify genes that are required for growth in human serum, 20 μ l aliquots of the frozen
180 mutant library in E745 were inoculated in BHI broth and grown overnight as described above.
181 Subsequently, bacterial cells were washed with physiological saline solution. Approximately
182 3×10^7 cfu were inoculated into 14 ml BHI broth, and approximately 3×10^6 cfu were
183 inoculated into 14 ml human serum obtained from Sigma (Cat. No. H4522; Sterile filtered
184 type-AB human serum) or heat-inactivated human serum (the same, after incubation for 30
185 min at 56°C). The different inoculum-sized were used in order for a similar number of
186 divisions to occur during the experiment. Cells were incubated at 37°C for 24 hours without

187 shaking and then further processed for Tn-seq (33). This experiment was performed in
188 triplicate.

189 Tn-seq samples were sequenced (50 nt, single-end) on one lane of a Illumina Hiseq 2500
190 (Baseclear, Leiden, the Netherlands and Sequencing facility University Medical Center,
191 Utrecht, The Netherlands), generating an average of 15 million high quality reads per sample.
192

193 **Tn-seq data analysis**

194 Raw Illumina sequence reads from Illumina sequencing were split, based on their barcode,
195 using the Galaxy platform (34), and 16-nucleotide fragments of each read that corresponded
196 to E745 sequences, were mapped to the E745 genome using Bowtie 2 (35). The results of the
197 alignment were sorted and counted by IGV (36) using a 25-nucleotide window size and then
198 summed over the gene. Read mapping to the final 10% of a gene were discarded as these
199 insertions may not inactivate gene function. Read counts per gene were then normalized to the
200 total number of reads that mapped to the genome in each replicate, by calculating the
201 normalized read-count RPKM (Reads Per Kilobases per Million input reads) via the following
202 formula: $RPKM = (\text{number of reads mapped to a gene} \times 10^6) / (\text{total mapped input reads in}$
203 $\text{the sample} \times \text{gene length in kbp})$. Statistical analysis of the RPKM-values between the
204 experimental conditions was performed using Cyber-T (37). Genes were determined to be
205 significantly contributing to growth in human serum when the Benjamini-Hochberg corrected
206 P -value was <0.05 and the difference in abundance of the transposon mutant during growth in
207 BHI and serum was >2 . To determine the essentiality of *E. faecium* genes during growth in
208 the rich medium BHI, the normalized read-counts of the ten replicates in BHI were used as
209 data input for the EL-ARTIST analysis, as described in the user manual of the ARTIST
210 pipeline (38).

211

212 **Isolation of mutants from the transposon mutant library pool**

213 To recover a targeted transposon mutant from the complete mutant pool, a PCR-based
214 screening strategy was developed (Supplementary figure 3). 40 µl of the transposon mutant
215 library was inoculated into 40 ml of BHI broth with gentamicin and grown overnight at 37°C
216 with shaking (200 rpm). The overnight culture, containing approximately 10⁹ cfu/ml, was
217 then diluted to approximately 20 cfu/ml in 500 ml of BHI with gentamicin and kept on ice.
218 Subsequently, 200 µl aliquots were transferred to wells of sterile 96 wells plates (n = 12,
219 Corning Inc.). After overnight incubation at 37°C without shaking, aliquots (15 µl) of each
220 one of the 96 wells, were further pooled into a single new 96 well plate, as described in
221 Supplementary figure 3.

222 PCRs were performed on the final plate in which the transposon mutants were pooled, to
223 check for the presence of the Tn-mutants of interest, using the primer
224 ftp_tn_both_ends_MmeI, which is complementary to the repeats flanking the transposon
225 sequence, in combination with a gene-specific primer (Supplementary table 7). When a PCR
226 was found to be positive in one of the wells of this plate, the location of the Tn-mutant was
227 tracked backwards to the wells containing approximately 4 independent transposon mutants,
228 by performing PCRs mapping the presence of the transposon mutant in each step. Cells from
229 the final positive well were plated onto BHI with gentamicin and colony PCR was performed
230 to identify the desired transposon mutant.

231

232 **Growth of *E. faecium* E745 and individual mutants in human serum**

233 Wild-type E745 and the mutant strains were grown overnight at 37°C in BHI broth.
234 Subsequently, bacterial cells were washed with physiological saline and approximately 3 x
235 10⁵ cfu were inoculated into 1.4 ml BHI broth or heat-inactivated serum. Cells were grown in

236 1.5 ml tubes (Eppendorf) in triplicate for each condition and incubated at 37°C for 24 hours
237 without shaking. Bacterial growth was determined by assessing viable counts, for which the
238 cultures were serially diluted using physiological saline solution and plated onto BHI agar
239 followed by overnight incubation at 37°C.

240

241 **Intravenous infection of zebrafish embryos**

242 London wild-type (LWT) inbred zebrafish embryos, provided by the aquarium staff of The
243 Bateson Center (University of Sheffield), were used for infection experiments. The parental
244 E745 strain and its *pyrK_2* and *manY_2* transposon mutants were grown in BHI broth until
245 they reached an optical density at 600 nm of approximately 0.5 and were then harvested by
246 centrifugation (5,500 g, 10 min). Bacteria were microinjected into the circulation of
247 dechorionated zebrafish embryos at 30 hours post fertilization, as previously described (39).
248 Briefly, anesthetized embryos were embedded in 3% (w/v) methylcellulose and injected
249 individually with approximately 1.2×10^4 cfu using microcapillary pipettes. For each strain,
250 29 to 32 infected embryos were observed for survival up to 90 hours post infection (hpi). This
251 experiment was performed in triplicate.

252

253 **Data availability**

254 Sequence reads generated in this study have been made available at the European Nucleotide
255 Archive under accession number PRJEB19025.

256 **Results**

257

258 **The complete genome sequence of *E. faecium* E745.**

259 In this study, we implemented RNA-seq and Tn-seq analyses in *E. faecium* strain E745, an
260 ampicillin- and vancomycin-resistant clinical isolate that was isolated from faeces of a
261 hospitalized patient during an outbreak of VRE in the nephrology ward of a Dutch hospital in
262 2000 (24, 25). To allow the application of RNA-seq and Tn-seq in *E. faecium* E745, we first
263 determined the complete genome sequence of this strain through a combination of short-read
264 Illumina sequencing and long-read sequencing on the RSII Pacific Biosciences and Oxford
265 NanoPore's MinION systems. This resulted in a circular chromosomal sequence of 2,765,010
266 nt and 6 plasmids, with sizes ranging between 9.3 kbp and 223.7 kbp (Supplementary table 1).
267 Together, the chromosome and plasmids have 3,095 predicted coding sequences.

268

269 **Transcriptome of *E. faecium* E745 during growth in rich medium and in human serum**

270 The transcriptional profile of E745 was determined using RNA-seq during growth in rich
271 medium (BHI) and in heat-inactivated human serum. A total of 99.9 million (15.6 - 17.6
272 million per sample) 100 bp paired-end reads were successfully aligned to the genome,
273 allowing the quantification of rare transcripts (Fig. 1). A total of 3217 transcription units were
274 identified, including 651 predicted multi-gene operons, of which the largest contains 22 genes
275 (Fig. 1A and Supplementary table 2).

276 A comparative analysis of E745 during growth in BHI and in human serum, showed that 860
277 genes exhibited significantly ($q < 0.001$ and a fold change in expression of > 2 or < 0.5 between
278 cultures grown in BHI versus heat-inactivated serum) different expression between these
279 conditions (Supplementary table 3). The large number (27.8% of genes on the *E. faecium*
280 E745 genome) of differentially expressed genes, indicates that growth in human serum leads

281 to a dramatic reprogramming of global *E. faecium* gene expression, involving both
282 chromosomal and plasmid-located genes. Among the genes with the highest difference in
283 expression between growth in serum and in rich medium, we identified a gene cluster with a
284 role in purine biosynthesis (Fig. 1B). In addition, we found a 58.4 kbp prophage-like gene
285 cluster that exhibited higher expression in E745 during growth in serum (Fig. 1C).
286 To confirm the RNA-seq analysis, we independently determined expression levels of eight
287 genes during growth in serum versus growth in BHI by qPCR (Supplementary Fig. 1). RNA-
288 seq and qPCR data were highly concordant ($r^2 = 0.98$).

289

290 **Construction and analysis of a transposon mutant library in *E. faecium* E745.**

291 A *mariner*-based transposon mutant library was generated in *E. faecium* E745 and Tn-seq
292 (21) was performed on ten replicate transposon mutant libraries, that were grown overnight in
293 BHI at 37°C, resulting in an average of 15 million Tn-seq reads for each library. To analyze
294 the Tn-seq data, we divided the E745 genome in 25-nt windows. Of a total of 110,601
295 windows, 49,984 (45%) 25-nt windows contained one or more sequence reads. No positional
296 bias was observed in the transposon insertion sites in the chromosome and plasmids of *E.*
297 *faecium* E745 (Supplementary Fig. 2A). The genome-wide coverage of this transposon
298 mutant library allowed the identification of genes that are conditionally essential in *E.*
299 *faecium*. A total of 455 chromosomal genes were determined to be essential for growth of *E.*
300 *faecium* E745 in BHI (Supplementary Table 4). An example of a gene identified as essential
301 is shown in Supplementary Fig. 2B. Among the genes that were essential for growth of *E.*
302 *faecium* E745 in BHI, 87% were present in all genome sequences of a set of 74 previously
303 sequenced *E. faecium* strains (4) that represented the genetic and ecological diversity of the
304 species. An additional 7% of the essential genes were present in 73 out of 74 *E. faecium*
305 genomes (data not shown). The conserved presence of these essential genes among diverse *E.*

306 *faecium* strains is in line with their crucial role in *E. faecium* viability. The *E. faecium* E745
307 transposon mutant library was then used to identify genes that were specifically required for
308 growth of *E. faecium* in serum.

309

310 ***E. faecium* E745 genes required for growth in human serum**

311 In order to identify genes that contribute to growth of *E. faecium* E745 in human serum, we
312 performed Tn-seq on cultures of the *E. faecium* E745 transposon mutant libraries upon growth
313 in rich medium (BHI) and in human serum. The human serum was either used natively or
314 heat-treated. The serum showed effective complement activity in both classical and
315 alternative pathways, as determined by hemolytic assays (40), while this activity was
316 abolished by heat-inactivation (data not shown). Minor differences were observed among
317 conditionally essential genes between the experiments performed in native human serum or
318 heat-inactivated serum (Supplementary Table 5) and the following results correspond to the
319 experiments obtained with heat-inactivated serum. This condition was chosen because it may
320 be a more reproducible *in vitro* environment, particularly since the interaction between the
321 complement system and Gram-positive bacteria remains to be fully elucidated (41, 42).

322 We identified 37 genes that significantly contributed to growth of E745 in human serum (Fig.
323 2 and Supplementary table 6): twenty-nine genes were located on the chromosome and eight
324 genes were present on plasmids (six genes on pE745-5, two genes on plasmid pE745-6). The
325 relatively large number of genes identified indicates that growth of *E. faecium* in human
326 serum is a multi-factorial process. The genes that conferred the most pronounced effect on
327 growth of *E. faecium* in serum included genes that are part of a phosphotransferase systems
328 (PTS) involved in carbohydrate uptake (*manZ_3*, *manY_2*, *ptsL*), a putative transcriptional
329 regulator (*algB*) and genes involved in the biosynthesis of purine and pyrimidine nucleotides
330 (*guaB*, *purA*, *pyrF*, *pyrK_2*, *purD*, *purH*, *purL*, *purQ*, *purC*) (Fig. 2). Notably, the *purD*,

331 *purH*, and *purL* genes were found to exhibit higher expression upon growth in human serum
332 in the RNA-seq analysis (Fig. 1). Nine genes were identified as negatively contributing to
333 growth in serum, i.e. the transposon mutants in these genes were significantly enriched upon
334 growth in serum. The effects of these mutations were relatively limited, compared to the
335 major effects observed in the transposon mutants discussed above (Supplementary Table 6),
336 but it is notable that five (*clsA_1*, *ddcP*, *ldt_{fm}*, *mgs*, and *lytA_2*) of these genes have roles in
337 cell wall and cytoplasmic membrane biosynthesis.

338

339 ***E. faecium* genes involved in nucleotide biosynthesis or carbohydrate metabolism** 340 **contribute to growth in human serum**

341 We developed a PCR-based method (Supplementary Fig. 3) to selectively isolate five
342 transposon mutants (in the purine metabolism genes *purD* and *purH*, the pyrimidine
343 metabolism genes *pyrF* and *pyrK_2* and the PTS gene *manY_2*) from the transposon library.
344 Growth in rich medium of these transposon insertion mutants was equal to the parental strain.
345 However, all mutants were significantly impaired in their growth in human serum (Fig. 3A),
346 confirming the results of the Tn-seq experiments.

347

348 ***E. faecium pyrK_2* and *manY_2* contribute to intravenous infection of zebrafish**

349 Next, we investigated whether the transposon insertion mutants in the *manY_2* and *pyrK_2*
350 genes were attenuated *in vivo* (Fig. 3B). The mutants in these genes were selected because
351 they represent the mutants in nucleotide and carbohydrate metabolism genes that were
352 previously shown to contribute to the growth of *E. faecium* in human serum. As a model for
353 intravenous infection, we used a recently described model in which *E. faecium* was injected
354 into the circulation of zebrafish embryos to mimic systemic infections (43). We showed that
355 both the *manY_2* and the *pyrK_2* mutant were significantly less virulent than the parental

356 strain. The overall survival at 92 hours post infection (hpi) of zebrafish embryos infected with
357 WT strain was 53%, compared to 88% and 83% respectively, for zebrafish embryos that were
358 infected with the transposon insertion mutants in *manY_2* and *pyrK_2*.

359 Discussion

360 *E. faecium* can contaminate the skin and from there colonize indwelling devices such as
361 intravenous catheters, or it can translocate from the gastrointestinal tract in
362 immunosuppressed patients, leading to the development of bacteremia and endocarditis. *E.*
363 *faecium* infections are often difficult to treat, due to the multi-drug resistant character of the
364 strains causing nosocomial infections (3, 4). However, the bloodstream poses challenges for
365 the proliferation and survival of *E. faecium*, including a scarcity of nutrients.

366 In the present study we sequenced the complete genome of a vancomycin-resistant *E. faecium*
367 strain, and identified *E. faecium* genes that were essential for growth in human serum. A total
368 of 37 genes were found to be required for fitness of *E. faecium* E745 in serum, among which
369 genes with roles in carbohydrate uptake and nucleotide biosynthesis. Previously, fitness
370 determinants for growth in human serum have been identified through large-scale screening
371 of mutant libraries in both a Gram-negative (*Escherichia coli*) and a Gram-positive
372 (*Streptococcus pyogenes*) pathogen (44, 45). Notably, these studies have also identified the
373 ability for *de novo* synthesis of purines and pyrimidines as a crucial factor for growth in
374 serum. In addition, in diverse bacteria (including *Burkholderia cepacia*, *Pasteurella*
375 *multocida*, *Acinetobacter baumannii*, *Salmonella enterica* serovar Typhimurium, *Bacillus*
376 *anthracis*, and *Streptococcus pneumoniae*), the ability to synthesize nucleotides contributes
377 importantly to virulence (46–51). The data presented here indicate that *de novo* biosynthesis
378 of nucleotides is also required for *E. faecium* growth in serum and virulence. The nucleotide
379 biosynthesis pathway of *E. faecium* may be a promising target for the development of novel
380 antimicrobials for the treatment of *E. faecium* bloodstream infections. Indeed, compounds that
381 target guanine riboswitches, thereby inhibiting nucleotide biosynthesis, have already shown
382 their efficacy in a *Staphylococcus aureus* infection model (52).

383 Three genes (*ptsL*, *manY_2* and *manZ_3*) encoding subunits of PTSs were found to contribute
384 to growth in serum in our Tn-seq experiments. Previously, PTSs have been associated with
385 gut colonization (5) and endocarditis (7) in another clinical *E. faecium* strain. The PTSs
386 identified in this study are different from these previously characterized systems, suggesting
387 that the remarkably large number of genes encoding for PTSs in a typical *E. faecium* genome
388 (4) provide metabolic flexibility for growth in a wide variety of environments.

389 It is notable that among the nine genes that exhibited increased fitness upon inactivation by
390 transposon insertion, five genes are predicted to have a role in cell wall or cytoplasmic
391 membrane biosynthesis. The protein encoded by *ddcP* was previously characterized as a low-
392 molecular-weight penicillin binding protein with D-alanyl-D-alanine carboxypeptidase
393 activity (19), while *ldt_{fm}* acts as a peptidoglycan L,D transpeptidase (53). The predicted α -
394 monoglucosyldiacylglycerol synthase gene *mgs* is orthologous (73% amino acid identity) to
395 *bgsB* in *E. faecalis*, which is required for the biosynthesis of membrane glycolipids (54). The
396 *clsA_1* gene is predicted to be responsible for the synthesis of cardiolipin
397 (bisphosphatidylglycerol) and its inactivation may modulate the physical properties of the
398 cytoplasmic membrane (55). Finally, *lytA_2* is predicted to encode an autolysin, which may
399 be involved in the turnover of peptidoglycan in the cell wall (56). The transposon mutants in
400 these genes were not further characterized in this study, but our findings suggest that genes
401 involved in cell wall or cytoplasmic membrane remodeling may confer subtle fitness defects
402 to *E. faecium* when growing in serum.

403 Our RNA-seq-based transcriptional profiling of *E. faecium* E745 during mid-exponential
404 growth in serum showed pervasive changes in gene expression compared to exponential
405 growth in rich medium. The purine metabolism genes *purL*, *purH*, *purD*, which were found to
406 be required for growth in serum in our Tn-seq experiments, were among those that were
407 significantly upregulated during growth in serum compared to growth in rich medium.

408 Notably, a single prophage was expressed at higher levels during growth in serum than in rich
409 medium. The abundance of prophage elements in the genome of *E. faecium* has been noted
410 before (4, 57, 58). Interestingly, in the related bacterium *Enterococcus faecalis* prophages
411 encode platelet-binding proteins (59) and may have a role in intestinal colonization (60). The
412 contribution of *E. faecium* prophages to traits that are important for colonization and infection
413 may provide important insights into the success of *E. faecium* as a nosocomial pathogen.

414 Collectively our data indicates that nucleotide biosynthesis and carbohydrate
415 metabolism are critical metabolic pathways for the proliferation and survival of *E. faecium* in
416 the bloodstream. The proteins encoded by the genes required for growth in human serum that
417 were identified in this study, could serve as candidates for the development of novel anti-
418 infectives for the treatment of bloodstream-infections by multi-drug resistant *E. faecium*.

419

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622

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628

629

630 **Author contributions**

631

632 X.Z, R.J.L.W. and W.v.S. designed the study. X.Z., A.M.G.P., T.K.P., M.B., J.R.B., M.R. and

633 S.M. performed experiments. All authors contributed to data interpretation. The manuscript

634 was written by Z.X., A.M.G.P., M.J.M.B., R.J.L.W. and W.v.S.

635 **Figures**

636

637 **Figure 1: Coverage plots of RNA-seq data aligning to chromosome and plasmid DNA.**

638 The y axis of each track indicates reads coverage and is represented on a log scale, ranging
639 from 0 to 10000. The x axis represents the genomic location. Light blue (BHI) or orange
640 (serum) tracks correspond to sequencing reads aligned to the plus strand of the replicon, and
641 dark blue (BHI) or dark red (serum) tracks correspond to sequencing reads aligned to the
642 minus strand of the replicon. The grey track corresponds to multi-gene operons. The green
643 track corresponds to differentially expressed genes (BHI vs serum), with the height of the
644 green bars indicative of differential expression. In panels B and C, two serum-induced
645 regions are shown, i.e. a gene cluster involved in nucleotide biosynthesis (panel B) and a
646 prophage (panel C). The RNA-seq experiments were performed using three biological
647 replicates.

648

649 **Figure 2: Tn-seq analysis for *E. faecium* genes required for growth in human serum.**

650 Bubbles represent genes, and bubble size corresponds to the fold-changes (for visual reasons,
651 a 100-fold change in transposon mutant abundance is set as a maximum) derived from the
652 read-count ratio of libraries grown in BHI to libraries grown in human serum. On the x axis
653 genes are shown in order of their genomic location and the chromosome and plasmids are
654 indicated. The outcome of statistical analysis of the Tn-seq data is indicated on the y axis.
655 Genes with a significant change ($q < 0.05$) in fitness in serum versus BHI are grouped by
656 function and are labelled with different colors, and the name or locus tag and the change in
657 abundance between the control condition and growth in serum is indicated next to the
658 bubbles. Negative values indicate that mutants in these genes outgrow other mutants in serum,
659 suggesting that these mutants, compared to the wild-type E745, have a higher fitness in
660 serum.

661

662 **Figure 3: *E. faecium* transposon mutants with a growth defect in human serum and an**

663 **attenuated phenotype in a zebrafish model.** (A) Ratios of the viable counts of five mutants

664 compared to wild-type *E. faecium* before (blue bars) and after 24 h of growth in human serum

665 (red bars) or BHI (yellow bars). The viable counts of wild-type *E. faecium* E745 were $(3.52 \pm$

666 $0.07) \times 10^5/\text{ml}$ in the inocula, $(2.92 \pm 0.14) \times 10^8/\text{ml}$ after 24 h of growth in serum and $(1.20 \pm$

667 $0.20) \times 10^9/\text{ml}$ after 24 h of growth in BHI, respectively. Error bars represent the standard

668 deviation of the mean of three independent experiments. Asterisks represent significant

669 difference (*: $p < 10^{-3}$, **: $p < 10^{-5}$ as determined by a two-tailed Student's *t*-test) between the

670 mutant strains and wild-type. (B) Kaplan-Meier survival curves of zebrafish embryos upon

671 infection with *E. faecium*. The virulence of *E. faecium* mutants upon intravenous infection of

672 zebrafish embryos was determined upon injection with 1.2×10^4 cfu of the *manY_2::Gm* and

673 *pyrK::Gm* transposon mutants and wild-type *E. faecium* E745. The experiment was performed

674 three times and the mutants were significantly different ($p < 0.01$) from the wild-type in each

675 experiment as determined by the Log-rank (Mantel-Cox) test with Bonferroni correction for

676 multiple comparisons. This figure represents the combined results of the three replicates for

677 *E. faecium* E745 ($n = 93$ zebrafish embryos), *manY_2::Gm* ($n = 92$) and *pyrK::Gm* ($n = 90$).

678

679





