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1 Type II fatty acid synthesis pathway and cyclopropane ring formation are dispensable during
2 *Enterococcus faecalis* systemic infection

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15

16 Running head: *E. faecalis* FASII and *cfa* are dispensable in infection

17

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24 **Abstract**

25 *Enterococcus faecalis*, a multi-antibiotic-resistant Gram-positive bacterium, has emerged as a
26 serious nosocomial pathogen. Here, we used a genetic approach to characterize the strategies used
27 by *E. faecalis* to fulfill its requirements for endogenous fatty acid (FA) synthesis *in vitro* and *in*
28 *vivo*. The FA synthesis (FASII) pathway is encoded by two operons and two monocistronic genes.
29 Expression of all these genes is repressed by exogenous FAs, which are incorporated in the *E.*
30 *faecalis* membrane and modify its composition. Deletion of nine genes of the 12-gene operon
31 abolished growth in a FA-free medium. Addition of serum, which is lipid-rich, restored growth.
32 Interestingly, the *E. faecalis* membrane contains cyclic fatty acids that modify membrane
33 properties, but are unavailable in host serum. The *cfa* gene that encodes the cyclopropanation
34 process, is located in a locus independent of the FASII genes. Its deletion did not alter growth under
35 the conditions tested, but yielded bacteria devoid of cyclic FAs. No differences were observed
36 between mice infected with wild-type, or FASII or cyclopropanation mutant strains, in terms of
37 bacterial loads in blood, liver, spleen or kidneys. We conclude that in *E. faecalis*, neither FASII
38 nor cyclopropanation enzymes are suitable antibiotic targets.

39

40 **Importance**

41 Membrane lipid homeostasis is crucial for bacterial physiology, adaptation, and virulence. Fatty
42 acids are constituents of the phospholipids that are essential membrane components. Most bacteria
43 incorporate exogenous fatty acids into their membranes. *Enterococcus faecalis* has emerged as a
44 serious nosocomial pathogen, which is responsible for urinary tract infections, bacteremia and
45 endocarditis, and is intrinsically resistant to numerous antibiotics. *E. faecalis* synthesizes saturated
46 and unsaturated fatty acids, but also cyclic fatty acids that are not found in the human host. We

47 characterized mutant strains deficient in fatty acid synthesis and modification using genetic,
48 biochemical, and *in vivo* approaches. We conclude that neither the fatty acid synthesis pathway nor
49 the cyclopropanation enzyme are suitable targets for *E. faecalis* antibiotic development.

50 INTRODUCTION

51 Bacterial fatty acids (FAs) are usually synthesized by the type II Fatty Acid Synthesis
52 (FASII) pathway. FAs constitute the two hydrophobic tails which, together with a hydrophilic head,
53 comprise phospholipids (PL) that are essential components of cell membranes. *Enterococcus*
54 *faecalis* synthesizes both saturated and unsaturated fatty acids (SFA and UFA, respectively), as do
55 numerous streptococci (1–3). Additionally, *E. faecalis* and numerous other bacteria encode a
56 cyclopropane fatty acid synthase (Cfa), which catalyzes the transfer of a methyl group from S-
57 adenosyl-L-methionine to the double bond of a lipid chain, thereby forming a cyclopropane ring
58 (4). Recent studies uncovered that *E. faecalis* also synthesizes trans-UFA, further expanding the
59 variety of fatty acids produced by this bacterium (5). Both cyclopropane and trans fatty acids are
60 more rigid than their non-modified substrates, and contribute to *E. faecalis* membrane homeostasis
61 in starvation and stress conditions (6–8).

62 FASII pathway defects prevent bacterial growth *in vitro* in FA-non-supplemented media;
63 this suggested that FASII enzymes may be essential for bacterial viability and therefore feasible
64 targets for antibiotic design (9–15). However, most bacteria incorporate exogenous FA into their
65 membranes. FA abundance in host compartments and in serum, led us to hypothesize, and then
66 prove that FA incorporation can bypass FASII-targeted antibiotics in the neonatal pathogen
67 *Streptococcus agalactiae*, as well as other Streptococcaceae (1). Nevertheless, antibiotic bypass
68 may vary according to whether bacteria can survive on exclusively exogenous FAs; for example,
69 *Mycobacterium tuberculosis* remains sensitive to the anti-FASII isoniazid because of its complex
70 lipid requirement (16). In contrast, *Staphylococcus aureus*, which synthesizes FAs not produced
71 by humans, can nevertheless bypass FASII inhibitors with host-derived fatty acids (17–19).

72 *Enterococcus faecalis* is a Gram-positive bacterium that inhabits the gastrointestinal tract
73 of humans and animals (20) and has long been associated with food fermentations. *E. faecalis* has

74 emerged as a serious nosocomial pathogen, and is the second most frequently isolated Gram-
75 positive pathogen isolated in hospitals after *S. aureus* (21, 22). *E. faecalis* is responsible for urinary
76 tract infections, bacteremia and endocarditis (23). It is intrinsically resistant to many antibiotics,
77 and its acquisition of antibiotic resistance genes has made it a public health concern worldwide
78 (23). This situation prompts the search for new antibiotic targets, notably within the FASII pathway
79 (24, 25).

80 Whether targeting FASII pathway or cyclopropanation enzymes could lead to treatment of
81 *E. faecalis* systemic infection is not resolved. *E. faecalis* was shown to use environmental fatty
82 acids (26). In the presence of 15 % human serum, *E. faecalis* membrane comprises about 10%
83 vaccenic acid (VA; C18:1 Δ 11), an FA not present in serum (26). Thus, the *E. faecalis* FASII
84 pathway is likely active even in the presence of exogenous FAs. The *E. faecalis* FASII pathway is
85 encoded by the twelve gene cluster, *fabT* - *accA*, whose genes are present in numerous Gram-
86 positive species, and a set of three genes, *fabO* - *fabN* and the divergent *fabI* gene, encoding
87 enzymes involved in UFA and SFA synthesis, respectively (14, 27). Finally, *cfa* encodes the
88 cyclopropanation reaction. In one study, deletion of *fabN* resulted in *E. faecalis* UFA auxotrophy
89 (28). In *E. faecalis*, expression of FASII genes is controlled by FabT, the transcriptional repressor.
90 An auxiliary acyl-carrier-protein carrying long chain FAs binds to FabT, and facilitates repression
91 of a consensus sequence that regulates FASII gene expression (14, 29, 30).

92 Although *E. faecalis* incorporates FA from its environment, its FA synthesis requirement
93 in an FA-rich environment cannot be deduced (31, 32). In this study we characterized the repressive
94 effect of serum on expression of FASII and FA-related genes. We also tested whether FASII
95 pathway and/or cyclopropanation functions are possible antibiotic targets in a murine model of *E.*
96 *faecalis* systemic infection.

97 **MATERIALS AND METHODS**

98 **Bacterial strains, media and culture conditions.** *E. faecalis* V583 vancomycin resistant
99 strain was isolated from a bloodstream infection and sequenced. It was used for the *in silico*
100 *analysis*. VE14089, a plasmid-cured derivative of the V583 strain, was the wild type (WT)
101 reference strain used for the construction of mutant and back to the wild type (BWT) strains (33,
102 34). Two mutants were constructed, Ef Δ FASII and Ef Δ cfa corresponding to the in-frame deletion
103 of nine genes from *fabK* to *accA* (7863 bp) and of *cfa* (1176 bp), respectively; the isogenic BWT
104 strains were obtained, BWT-FASII and BWT-cfa, respectively. *Escherichia coli* TG1 (Invitrogen)
105 was used for cloning experiments. *E. faecalis* was cultured in Todd-Hewitt (TH) broth or agar
106 (Difco Laboratories, Detroit, MI) or in RPMI⁺ (RPMI-1640; Life technologies, Gibco)
107 supplemented with amino acids 1X (Gibco®, Life Technologies), vitamins 1X (Sigma Aldrich),
108 glucose 1% and Hepes 0.1mM (Gibco®, Life Technologies). *E. coli* was cultured in Trypticase-
109 Soy (TS) medium (Difco Laboratories, Detroit, MI). When needed, antibiotics were used at the
110 following concentrations: for *E. coli*, ticarcillin, 100 μ g/ml, and erythromycin, 160 μ g/ml; for *E.*
111 *faecalis*, erythromycin, 50 μ g/ml. Cerulenin (Sigma) was added at 8 μ g/ml final concentration.

112 ***In silico* analysis.** The AGTTTGATAATCAAATT sequence was used to seek potential
113 FabT binding sites in the regulatory regions or within the FASII genes (27).

114 **General DNA techniques.** *E. coli* plasmid DNA was prepared by rapid alkaline lysis using
115 the QIAprep Spin Miniprep kit (Qiagen). Genomic DNA from *E. faecalis* was prepared using the
116 DNeasy Blood and Tissue Kit (Qiagen) with pretreatment for Gram-positive bacteria as
117 recommended by the manufacturer. PCR was carried out with the *AmpliTaq* Gold polymerase as
118 described by the manufacturer (Applied Biosystems). The molecular weight standard used in
119 agarose gels is the 1kb-plus DNA ladder (Life Technologies). Amplification products were purified
120 on QIAquick PCR purification kit (Qiagen). PCR products and plasmids used were sequenced with

121 an ABI 310 automated DNA sequencer, using the ABI PRISM dyes terminator cycle sequencing
122 kit (Applied Biosystem). To determine the genetic and transcriptional organization of VE14089
123 FASII genes, primer pairs were designed to flank neighboring FASII genes. Primers are listed in
124 Table S1.

125 **Strain construction.** The primers used for the construction of the in-frame Ef Δ FASII and
126 Ef Δ cfa mutant strains are listed in Table S1. Primers used are based on the published genome
127 sequence of *E. faecalis* V583 (GenBank accession number NC004668.1) (33). The Δ FASII
128 deletion was constructed by using splicing-by-overlap extension PCR as described (35). The
129 corresponding PCR fragment was cloned into the thermosensitive shuttle plasmid pG1. The *Acfa*
130 (*ef0203*, NCBI reference sequence NC_004668.1) deletion was constructed using In-Fusion PCR
131 Cloning Kit (Clontech), cloning the two PCR-amplified fragments into pG1 previously linearized
132 in a one-step reaction following manufacturer's recommendations. The resulting intermediate
133 plasmids, pG1 Δ FASII and pG1 Δ cfa, were checked by sequencing the inserts. Electroporation of
134 VE14089 and allelic exchange were performed as described with minor modifications (35, 36). For
135 the Ef Δ FASII construction Tween 80 0.1% (Sigma), a rich source of FA containing 70% oleic acid
136 (OA; C18:1 Δ 9), was added in all steps of the experiment after the electroporation. The in-frame
137 deletion mutants and BWT strains were confirmed by PCR and sequence analysis.

138 **Growth curves.** *E. faecalis* strains were inoculated into RPMI⁺ supplemented with Tween
139 80 (0.1%) and cultured overnight at 37°C. Overnight cultures were washed twice in PBS and sub-
140 cultured (1:100) in RPMI⁺ and in RPMI⁺ supplemented with 40% human serum. Growth was
141 determined by measuring absorbance at 600 nm (OD₆₀₀). Experiments were repeated three times.

142 **RNA extraction and RT-PCR.** RNA was extracted from bacteria by the phenol
143 chloroform technique as described (37). RNA (2-5 μ g) was treated with DNase I (Promega)

144 according to manufacturer's recommendations. RNA was quantified by absorbance at 260 and 280
145 nm. RNA purity and integrity were controlled and RNA was stored at -80°C until use.

146 Reverse-transcription was carried out using the Superscript II kit (Invitrogen) and Random
147 Hexamer Primers (Fermentas), according to manufacturers' instructions. PCRs were carried out on
148 cDNA, as well as on gDNA and RNA as positive and negative controls, using primer pairs flanking
149 neighboring FASII genes (Table S1). PCR Amplicons were examined on a 1% agarose gel.

150 **Real-time quantitative PCRs.** Overnight VE14089 RPMI⁺ cultures were then subcultured
151 (1:100) in RPMI⁺ with or without addition of 40% human serum to mid-log phase (OD₆₀₀ = 0.3).
152 RNAs were extracted and cDNA samples were diluted to 50 ng/μl. qPCR was performed using the
153 LightCycler (Roche) and the SYBR Green PCR kits (Applied Biosystems) using primers listed in
154 Table S1. Each assay was performed in duplicate, with two independently prepared total RNA
155 samples. The relative quantification in gene expression was determined by the 2^{-ΔΔCt} method, using
156 *rpoB* as the reference gene (38).

157 **Fatty acid analysis.** Strains were inoculated into RPMI⁺ supplemented with Tween 80
158 (0.1%) and grown overnight at 37°C. Cells were washed three times in RPMI⁺, diluted 100-fold in
159 fresh RPMI⁺ cultures with or without 40% human serum. Cells were harvested after 14 h of growth
160 (stationary phase) in static conditions at 37 °C, and washed once in NaCl 0.9% containing 0.01%
161 Triton X-100 and twice in NaCl 0.9%. Cell pellets were stored at - 20°C until analysis. The FA
162 composition of serum used for experiments was also determined. Whole cell esterified fatty acid
163 determinations were done on an AutoSystem XL Gas Chromatograph (Perkin-Elmer) equipped
164 with a DB-Wax (Agilent, XXX; for all analyses except those shown in Fig. 3D) or ZB-Wax
165 capillary column (30 m x 0.25 mm x 0.25mm; Phenomenex, France; for Fig. 3D) as described (1,
166 17). Bacterial fatty acid composition of each sample was analyzed in three independent
167 experiments.

168 **Ethics statement.** All animal experiments described in this study were conducted in
169 accordance with guidelines of Paris Descartes University, in compliance with the European animal
170 welfare regulation (http://ec.europa.eu/environment/chemicals/lab_animals/home_en.html) and
171 were approved by the University Paris Descartes animal care and use committee and by the
172 Ministère de l'Education Nationale, l'Enseignement Supérieur et la Recherche
173 (2015032714098562_v1, APAFIS #390).

174 **Intravenous infection of mice.** All *in vivo* infections in this study were performed with 6-
175 week-old female BALB/c mice (Charles River, L'Arbresle cedex, France). Bacterial suspensions
176 used for intravenous (i.v.) infection were prepared as follows. Overnight broth cultures of mutant
177 and isogenic BWT strains, grown in TH supplemented with 0.1 % Tween 80, were diluted 1:100
178 into 50 ml of TH-Tween 80 medium and grown until $OD_{600} = 0.6$. The bacterial suspensions were
179 washed twice in NaCl 0.9% and resuspended in NaCl 0.9% to obtain from 6×10^8 to 8×10^8 colony
180 forming units (CFU)/ml final concentration in 500 μ l for intravenous (iv) injections. Mice were
181 injected with a 28-gauge, 0.5-in. needle in the tail vein.

182 **Dissemination assay and organ analysis.** Randomized groups of 9-21 mice or of 6 mice,
183 BWT-FASII and Ef Δ FASII, and BWT-cfa and Ef Δ cfa, respectively, were infected and mice
184 were sacrificed by cervical dislocation at days 1, 7 and 14 or day 7, post infection (pi); blood, liver,
185 spleen and kidneys were harvested. All organs were weighed prior to further experimentation.
186 Liver, spleen and kidneys were homogenized in 0.9% NaCl. Serial dilutions were immediately
187 plated on TH agar plates containing 0.1 % Tween80 (TH-Tween agar). CFU were enumerated after
188 24 h incubation at 37°C.

189 **Statistical analysis.** Statistical analyses were performed using a 2-way ANOVA and a
190 Bonferroni post-test or a Mann-Whitney test (PRISM 8). A *p* value ≤ 0.05 was considered
191 statistically significant; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.0001$.

192 RESULTS and DISCUSSION

193 **FASII pathway genetic organization and regulation of expression.** In *E. faecalis*, a
194 total of 15 genes belonging to the FASII pathway were previously identified (14, 27, 39). However,
195 a 16th gene, *efl773* (NCBI reference sequence for the protein, WP_010706667.1), named *fabG*, is
196 present in the annotated V583 genome. This gene is conserved among *E. faecalis* isolates. As two
197 genes were assigned the same annotation we suggest renaming *ef2881*, which belongs to the 12
198 gene cluster, as *fabGa*, and *efl773*, *fabGb*. FabGa and FabGb proteins share 32% identity and 65%
199 similarity. Physiological and enzymatic properties of the two FabG homologues remain unknown
200 and we hypothesized that they may catalyze the same 3-oxoacyl-ACP reduction step but with
201 different substrate preferences or conditions of expression. *In silico* analysis using the *E. faecalis*
202 FabT consensus sequence indicated the presence of a putative FabT binding site,
203 **AATTTGAAACAAAACG** (palindromic nucleotides in bold), 10 bp downstream of the *fabGb*
204 translation start codon (Fig. 1A) (39). This further suggests that FabGb is involved in FA synthesis.
205 Genetic organization of the 12 gene cluster was initially defined *in silico* on the basis of an upstream
206 FabT consensus binding sequence (27). Depending on the species, an organization in two or three
207 operons was suggested (14, 27, 39). In *E. faecalis*, FabT consensus binding sequences are present
208 upstream of *fabT* and *fabK* (Fig. 1A), suggesting a two-operon organization. Also, the UFA
209 synthesis genes, *fabO* and *fabN*, may constitute another operon (14, 27, 39). This organization was
210 tested by overlapping RT-PCR mapping (Fig. 1A,1B). Results show that the 12-gene cluster is
211 organized as a single operon, which ends before *ef2874*. A transcription reinitiation site may be
212 linked to the FabT consensus binding sequence upstream of *fabK*. Furthermore, respectively
213 intense and faint bands were obtained for *fabO-fabN* and *fabN-pyrD-1* (= *ef0285*), suggesting that
214 *fabO* and *fabN* constitute an operon and that there may be some read-through downstream of *fabN*.

215 Altogether our data show that *E. faecalis* FASII genes are organized in two operons and two
216 monocistronic transcriptional units (Fig. 1A,1B).

217 The FASII pathway can be strongly repressed by exogenous fatty acids, such that
218 phospholipid synthesis relies on exogenous FA substrates (1, 40). Human serum is rich in lipids
219 comprising long acyl-chain fatty acids such as 18:1 (15%) and 16:0 (25%), which can act as FabT
220 corepressors (29). To our knowledge, the regulation of *E. faecalis* FASII gene expression elicited
221 by addition of FAs has not been studied. To address this question, and after checking that there was
222 no growth difference in the presence or absence of added human serum (data not shown), we used
223 qRT-PCR to analyze the effect of 40% human serum on *E. faecalis* FASII gene expression (Fig.
224 1A, 1C). Serum addition resulted in decreased expression of all FASII genes varying from 2.5- to
225 14-fold depending on the gene. Interestingly, a putative FabT binding site was found close to the
226 most strongly repressed genes, *fabGb*, *fabO* and *fabK*. This suggests that a FabT-controlled
227 transcription initiation site or a transcription repression site exists upstream of these genes.
228 Altogether, our data show that the 12 gene operon (from *fabT* to *accA*), as defined above, is strongly
229 repressed in the presence of a physiological concentration of serum.

230 ***E. faecalis* FASII pathway and cyclopropanation are dispensable for growth in the**
231 **presence of serum.** Supplementation with serum or unsaturated FA overcomes growth inhibition
232 mediated by cerulenin, a FASII inhibitor (1, 17, 41). We confirmed these results with the WT strain
233 VE14089 (data not shown). The consequences of a non-functional FASII pathway were then
234 examined by constructing Ef Δ FASII, in which 9 genes of the 12-gene operon, from *fabK* to *accA*
235 (*ef2883* to *ef2875*) were deleted. The genes that were removed encode products implicated in the
236 initiation and elongation modules (Fig. 1A). Growth of the WT, Ef Δ FASII mutant and back-to-
237 the-wild-type (BWT-FASII) (see Materials and Methods) strains was first compared in RPMI⁺
238 (Fig. 2A). The WT and the BWT-FASII strains behaved similarly. In contrast, Ef Δ FASII growth

239 was essentially arrested in RPMI⁺; slight observed growth is likely due to residual Tween 80 used
240 in precultures. These results confirm that an active FASII pathway is required for growth in FA-
241 free medium. Addition of 40% human serum to growth medium led to equivalent growth of all
242 strains (Fig. 2A), indicating that serum is sufficient to fully restore growth of the EfΔFASII mutant
243 strain. Altogether, these results indicate that the exogenous FAs present in serum are necessary and
244 sufficient to restore growth when *E. faecalis* FASII pathway is abolished, either *via* the addition of
245 a FASII inhibitor (1) or by deletion of FASII genes.

246 The role of cyclopropanation on *E. faecalis* growth in the presence of exogenous FAs
247 remained an open question. *E. faecalis* produces cyclopropane FAs, which are produced from both
248 endogenous and exogenous substrates, presumably to mediate membrane fluidity adjustment by
249 modifying UFA (8). A *cfa* in-frame deletion mutant was constructed to assess the role of
250 cyclopropane FA synthesis in *E. faecalis* growth in medium without or with host serum. The mutant
251 strain grew like the WT and BWT-*cfa* strains in both media, indicating that the presence or absence
252 of cyclic FA does not impact *E. faecalis* growth *in vitro* (Fig 2B).

253 ***E. faecalis* WT and Ef ΔFASII strains show identical FA composition upon growth in**
254 **serum.** Strong repression of *E. faecalis* FASII genes in the presence of human serum correlates
255 with incorporation of FAs, as previously observed in the same culture condition (26). We
256 determined FA compositions of WT VE14089 and BWT derivative (BWT-FASII) grown in the
257 absence or presence of 40% serum (Table S2, S3, Fig. 3A). BWT-FASII profiles were similar to
258 those of the WT; the slight differences probably arose from growth variations that could alter the
259 efficiency of conversion between the UFA and cyclo forms. As previously described, FA of cells
260 grown in FA-free medium (RPMI⁺) comprised mainly palmitic acid (16:0; PA) (40%), vaccenic
261 acid (18:1Δ¹¹; VA) (20%) and cyclopropanated-VA form (C19Δ¹¹) (20%) (Fig. 3A). In contrast,

262 membrane FA composition of bacteria grown in serum resembled profiles of serum alone (Table
263 S3, Fig. 3A) (14, 26, 31). Thus, the proportion of endogenously produced 18:1 Δ^{11} dropped to 1.7%,
264 and that of serum-provided oleic acid (18:1 Δ^9 ; OA) rose to 13.5%. These latter percentages are
265 different from previously reported ones (26, 31); this could be due to the use here of 40% rather
266 than 15% serum. Thus, VE14089, like other *E. faecalis* isolates, incorporates serum-supplied
267 exogenous FA (6). Another notable change in the presence of serum was a decrease in the amount
268 of C19 Δ^{11} , as reported (26), while cyclopropanated-OA (C19 Δ^9), which is not found in serum, rose
269 to 15%, indicating that *E. faecalis* CFA synthase cyclizes both 18:1 Δ^9 and 18:1 Δ^{11} . This indicates
270 that *E. faecalis* CFA synthase is active on different substrates which is in agreement with the fact
271 that bacterial CFAs display lax requirements for positioning of the *cis* double bond, except for *cis*
272 double-bond positions at FA extremities, or *trans* double bonds, which are generally not converted
273 (4).

274 We also established the FA content of the Ef Δ FASII mutant strain grown in 40% human
275 serum (Fig. 3B). The FA profiles of the Ef Δ FASII and BWT-FASII strains were similar (Fig. 3B).
276 Both strains similarly incorporated serum FAs and generated C19 Δ^9 . These data demonstrate that
277 FASII activity has no impact on membrane composition in the presence of human serum.

278 ***E. faecalis* cyclopropane fatty acid synthase impacts the cellular FA composition.**

279 To characterize the impact of the *cfa* deletion on *E. faecalis* membrane FA composition, we
280 compared FA profiles of Ef Δ *cfa*, BWT-*cfa*, and WT strains grown in FA-free medium and in the
281 presence of human serum (Fig. 3C, 3D, Tables S2, S4). The BWT-*cfa* profiles were near identical
282 to those of the WT (Tables S2, S4). Compared to the BWT-*cfa* control strain in RPMI⁺ the Ef Δ *cfa*
283 mutant strain was devoid of C19 Δ^{11} and was enriched in 18:1 Δ^{11} (Fig. 3C). In serum-supplemented
284 medium, the Ef Δ *cfa* strain was devoid of C19 Δ^9 and enriched in 18:1 Δ^9 (Fig. 3D). These data
285 confirm that the Ef Δ *cfa* strain is defective for cyclopropanation of unsaturated fatty acids, mainly

286 18:1 Δ^{11} in RPMI⁺ and 18:1 Δ^9 in the presence of serum. This implies that, *in vivo*, the fatty acid
287 composition of *afa* defective strains may be different from those of wild-type strains and this could
288 have physiological consequences.

289 ***E. faecalis* septicemic infection is independent of both the FASII pathway and fatty**
290 **acid cyclopropanation.** The WT, Ef Δ FASII and BWT-FASII strains grew similarly and displayed
291 the same FA composition when grown in the presence of human serum. During infection however,
292 *E. faecalis* infects organs whose FA contents are variable (42, 43). We therefore asked whether
293 exogenous FAs in the host may compensate the loss of the active FASII pathway *in vivo*.

294 We first followed the infection process, looking at the physiological consequences of
295 intravenous infection with the BWT-FASII strain. CFU counts and organ weights were examined
296 1, 7, and 14 days post infection (pi) (Fig. 4A, 4B). Throughout the course of the experiment, the
297 CFUs in blood were below the detection threshold (data not shown). The CFU counts in the liver
298 diminished 31-fold, at 14 d pi, with no change in the weight of livers throughout the experiment.
299 In the spleen, the bacterial levels strongly decreased over a 2-week period, from approximately 10⁴
300 CFU 24 h pi, to 66% spleens being sterile at the end of the experiment. However, d14-spleens
301 weighed twice as much as d1 spleens (Fig. 4B). Finally, kidney weights increased from d7 to d14
302 (Fig. 4B). This correlated, albeit with a time laps, to the tendency in the CFU counts increase at d7
303 in BWT-FASII-infected mice (15-fold, respectively) and is in agreement with *E. faecalis* tropism
304 for the kidneys (31, 32).

305 We then assessed the consequences of the FASII gene deletion by comparing the BWT-
306 FASII- and Ef Δ FASII-infected mice. Bacterial counts in liver, spleen and kidneys, as well as organ
307 weights, were similar for BWT-FASII- and Ef Δ FASII-infected mice at all-time points (Fig. 4A,
308 4B). This demonstrates that, as in other species, targeting the FASII pathway would be ineffective
309 for the treatment of *E. faecalis* septicemic infection (1).

310 The consequences of a *cfa* deletion were also examined in the mouse septicemia infection
311 model. *Cfa* is reportedly more expressed in stationary phase (44). We reasoned that differences, if
312 any, between *Ef* Δ *cfa* and BWT-*cfa* would be more pronounced after CFUs reached their highest
313 level in the wild-type strain (here BWT) -infected mice, i.e., at d7. No differences were observed
314 in terms of bacterial loads in the different organs between *Ef* Δ *cfa*- and BWT-*cfa*-infected mice
315 (Fig. 4C). This indicates that cyclopropanation activity does not confer an advantage to *E. faecalis*
316 during systemic infection in this model.

317 In conclusion, our data demonstrate that targeting the FASII pathway or the
318 cyclopropanation process would be ineffective for the treatment of *E. faecalis* septicemic infection.

319 **ACKNOWLEDGMENTS**

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444

445 **FIGURE LEGENDS**

446 **Figure 1 Genetic organization of *E. faecalis* FASII pathway and impact of exogenous FAs on**
447 ***E. faecalis* FASII gene expression. (A)** Schematic representation of *E. faecalis* VE14089 FASII
448 pathway genes. Gene positions with names below are represented with open arrows. Two genes in
449 the locus, *pyrD-1* (*ef0285*), and *ef2874*, are unrelated to FASII. Above, asterisks indicate putative
450 FabT binding sites; below, transcripts predicted by PCR reactions, straight arrows; transcripts
451 identified by predicted FabT binding sequence and expression levels, dotted arrow. **(B)** Agarose
452 gel of the RT-PCR amplification products on cDNA using primer pairs from neighboring genes.
453 The order is the same as that in Fig. 1A above; lane: 1, *fabI-fabO*; 2, *fabO-fabN*; 3 *fabN-ef0285*; 4,
454 *fabT-fabH*; 5, *fabH-acpP*; 6, *acpP-fabK*; 7, *fabK-fabD*; 8, *fabD-fabGa*; 9, *fabGa-fabF*; 10, *fabF-*
455 *accB*; 11, *accB-fabZ*; 12, *fabZ-accC*; 13, *accC-accD*; 14, *accD-accA*; 15, *accA-ef2874*. MW,
456 molecular weight standard. **(C)** FASII gene expression fold change in *E. faecalis* grown in RPMI⁺
457 medium in the absence or the presence of 40% human serum. The means \pm SD of three independent
458 experiments are represented.

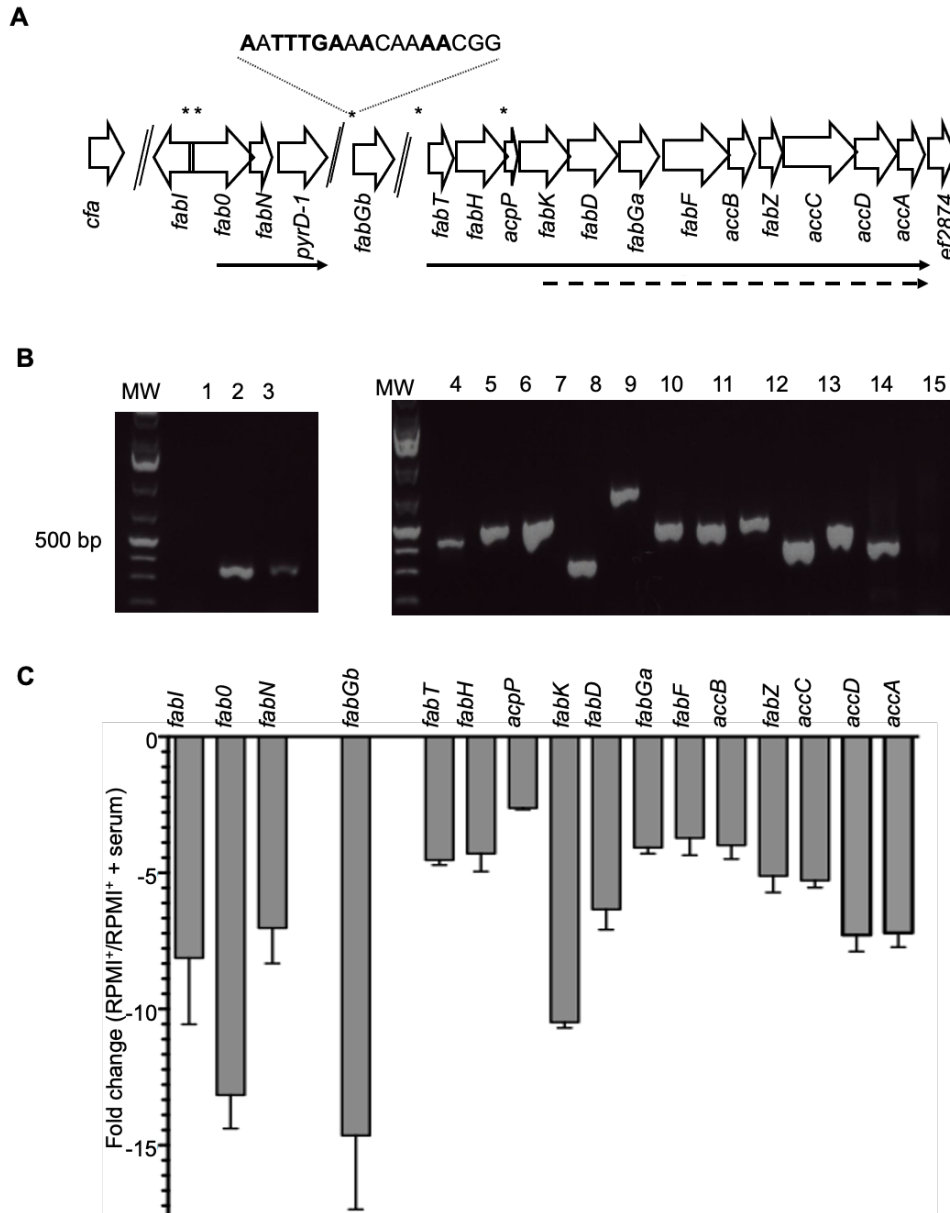
459
460 **Figure 2. Impact of FASII pathway mutations on *E. faecalis* growth. (A)** Growth curves of
461 VE14089 (WT) (●), Ef Δ FASII (▲) and BWT-FASII (■) strains in RPMI⁺ in the absence (empty
462 symbols) or presence (closed symbols) of 40% human serum. **(B)** Growth curves of VE14089 (●),
463 Ef Δ *cfa* (▲) and BWT-*cfa* (■) strains in RPMI⁺ in the absence (empty symbols) or presence (closed
464 symbols) of 40% human serum. Growth curves shown are representative of at least 3 independent
465 experiments.

466

467 **Figure 3. Fatty acid profiles of *E. faecalis* WT, Δ FASII, and Δ cfa strains grown without and**
468 **with serum.** *E. faecalis* was grown in RPMI⁺ or in RPMI⁺ containing 40% human serum to
469 stationary phase. FA composition was compared to assess the roles of medium FA content and
470 genetic background. The means of the percentages of each FA found in *E. faecalis*, BWT or mutant
471 strains, grown to stationary phase in the presence or absence of 40 % serum of three independent
472 experiments are presented. (see also Table S2, S3, S4). (A) BWT-FASII grown in the absence or
473 presence of serum. (B). BWT-FASII and Ef Δ FASII strains grown in 40% serum. (C) BWT-cfa and
474 Ef Δ cfa strains grown in RPMI⁺. (D) BWT-cfa and Ef Δ cfa strains grown in 40% human serum.
475 Only major fatty acids (>2% of total in at least one profile) are shown. Green stars, fatty acids
476 derived from serum. Green arrows: cyclopropanation in the BWT strains of unsaturated fatty acids,
477 dark green, produced by *E. faecalis* (mainly 18:1 Δ ¹¹) or, light green, incorporated from serum
478 (mainly 18:1 Δ ⁹). Columns: dark green BWT-FASII; dark blue, Ef Δ FASII; light green, BWT-cfa;
479 light blue, Ef Δ cfa; hatched, strains grown in RPMI⁺; empty, strains in RPMI⁺ supplemented with
480 40% human serum.

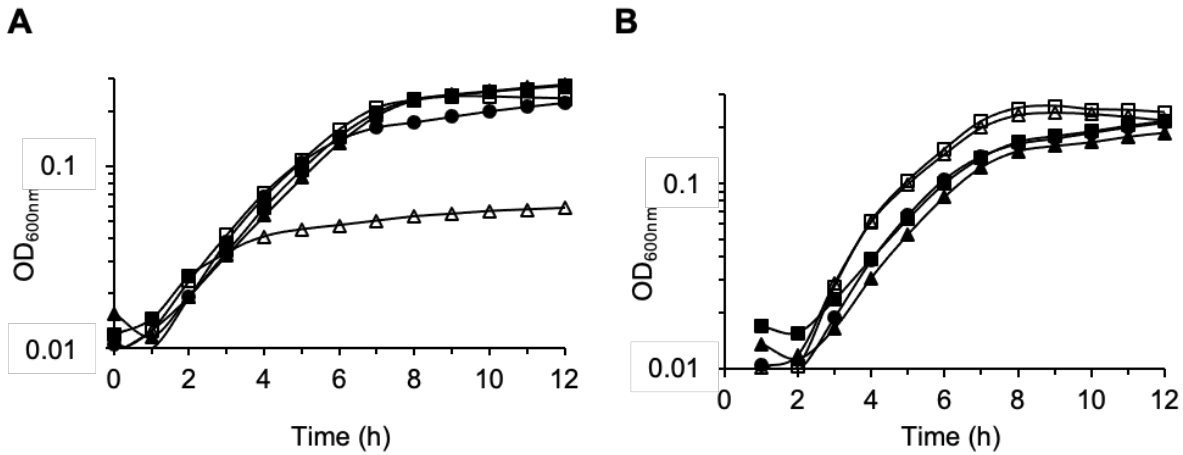
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482 **Figure 4. Ef Δ FASII and BWT-FASII infection in a mouse septicemic model.** (A) CFUs shown
483 by scattergraph plots at days 1, 7 and 14 pi of bacteria recovered from BWT-FASII and Ef Δ FASII
484 infected mice. Nine to 20 mice were intravenously infected with 6.10⁸ to 8.10⁸ *E. faecalis* BWT-
485 FASII (closed squares) or Ef Δ FASII (open squares). (B) Organ weights shown by scattergraph
486 plots at days 1, 7 and 14 pi of 3 to 9 mice infected by Ef Δ FASII or BWT-FASII. Symbols are as
487 in 'A'. (C) CFUs shown by scattergraph plots at day 7 pi of viable bacteria recovered Ef Δ cfa and
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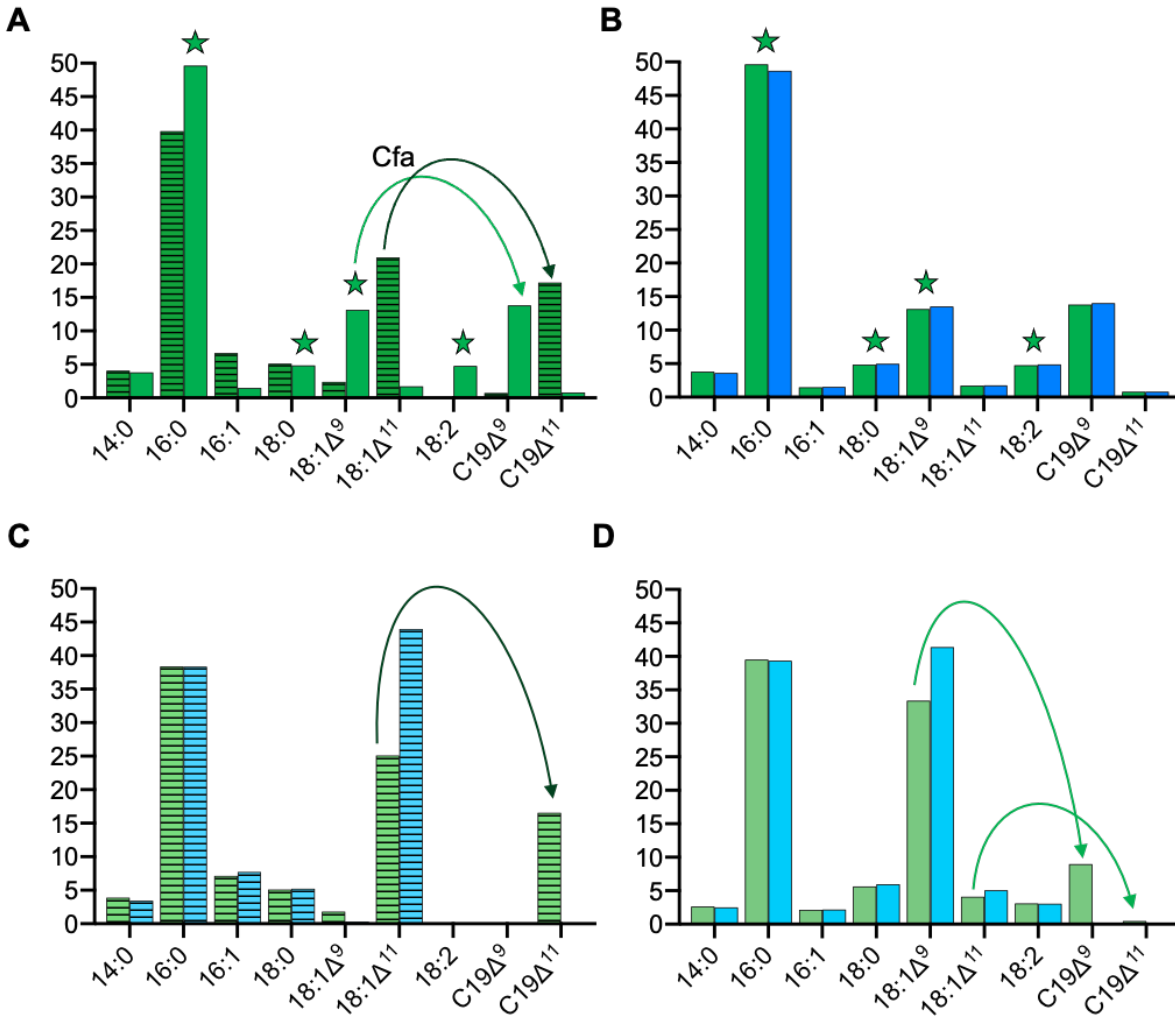
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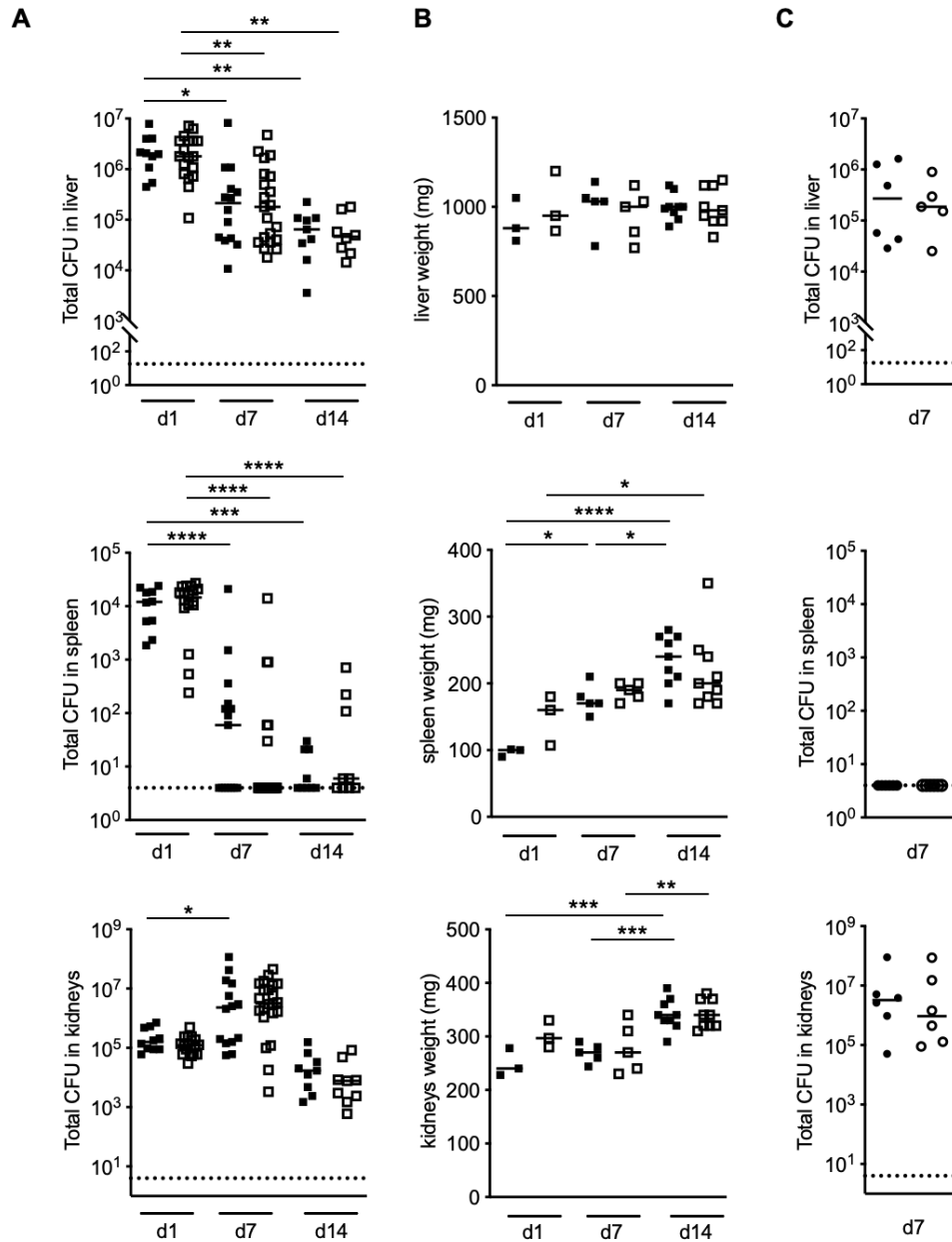


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530

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