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

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

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Abstract

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

Importance

Membrane lipid homeostasis is crucial for bacterial physiology, adaptation, and virulence. Fatty acids are constituents of the phospholipids that are essential membrane components. Most bacteria incorporate exogenous fatty acids into their membranes. *Enterococcus faecalis* has emerged as a serious nosocomial pathogen, which is responsible for urinary tract infections, bacteremia and endocarditis, and is intrinsically resistant to numerous antibiotics. *E. faecalis* synthesizes saturated 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.

INTRODUCTION

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

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

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

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

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

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

MATERIALS AND METHODS

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

RESULTS and DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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444

FIGURE LEGENDS

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

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

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

Figure 4. Ef Δ FASII and BWT-FASII infection in a mouse septicemic model. **(A)** CFUs shown by scattergraph plots at days 1, 7 and 14 pi of bacteria recovered from BWT-FASII and Ef Δ FASII infected mice. Nine to 20 mice were intravenously infected with 6.10⁸ to 8.10⁸ *E. faecalis* BWT-FASII (closed squares) or Ef Δ FASII (open squares). **(B)** Organ weights shown by scattergraph plots at days 1, 7 and 14 pi of 3 to 9 mice infected by Ef Δ FASII or BWT-FASII. Symbols are as in 'A'. **(C)** CFUs shown by scattergraph plots at day 7 pi of viable bacteria recovered Ef Δ cfa and BWT-cfa infected mice. Six mice were intravenously infected with 6-8.10⁸ *E. faecalis* BWT-cfa (closed circles) or Ef Δ cfa (open circles) strains. Upper panels, liver; middle panels, spleen; lower

490 panels, kidneys. Each symbol represents the number of CFUs per organ of one infected mouse or
491 the weight of one organ. Median ranges are represented. Statistical analysis, A, B, 2-way ANOVA
492 and a Bonferroni post-test; C, Mann-Whitney test. * $p<0.05$; ** $p<0.01$, *** $p<0.005$ ****
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