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RESEARCH ARTICLE

Type II Fatty Acid Synthesis Pathway and Cyclopropane Ring Formation Are Dispensable during *Enterococcus faecalis* Systemic Infection

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ABSTRACT *Enterococcus faecalis*, a multiple 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 type II fatty acid synthesis (FASII) pathway is encoded by two operons and two monocistronic genes. Expression of all of these genes is repressed by exogenous FAs, which are incorporated into the *E. faecalis* membrane and modify its composition. Deletion of nine genes of the 12-gene operon abolished growth in an 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 that 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 (WT) or with 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 that is responsible for urinary tract infections, bacteremia, and endocarditis and is intrinsically resistant to numerous antibiotics. *E. faecalis* synthesizes saturated and unsaturated fatty acids, as well as cyclic fatty acids that are not found in the human host. Here, we characterized mutant strains deficient in fatty acid synthesis and modification using genetic, biochemical, and *in vivo* approaches. We conclude that neither the fatty acid synthesis pathway nor the cyclopropanation enzyme are suitable targets for *E. faecalis* antibiotic development.

KEYWORDS *Enterococcus faecalis*, FASII pathway, antibiotic target, cyclopropane ring formation, fatty acids, septicemic infection

Bacterial fatty acids (FAs) are usually synthesized by the type II fatty acid synthesis (FASII) pathway. FAs constitute two hydrophobic tails, which, together with a

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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 nonmodified substrates, and they contribute to *E. faecalis* membrane homeostasis under starvation and stress conditions (6–8).

The FASII pathway defects prevent bacterial growth *in vitro* in medium not supplemented with FA; this suggests that FASII enzymes may be essential for bacterial viability and are 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 in other *Streptococcaceae* taxa (1). Nevertheless, antibiotic bypass may differ 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 of the FASII pathway or cyclopropanation enzymes could lead to treatment of *E. faecalis* systemic infection is not resolved. *E. faecalis* was previously shown to use environmental fatty acids (26). In the presence of 15% human serum, the *E. faecalis* membrane comprises about 10% vaccenic acid (VA; $C_{18:1\Delta11}$), 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 12-gene cluster *fabT* to *accA*, whose genes are present in numerous Gram-positive species, and by a set of three genes, *fabO-fabN* and the divergent *fabI* gene, which encode 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 (ACP) 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, the 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.

RESULTS AND DISCUSSION

FASII pathway genetic organization and regulation of expression. In *E. faecalis*, a total of 15 genes belonging to the FASII pathway have been previously identified (14, 27, 33). However, a 16th gene, *ef1773* (NCBI GenPept accession number WP_010706667), 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

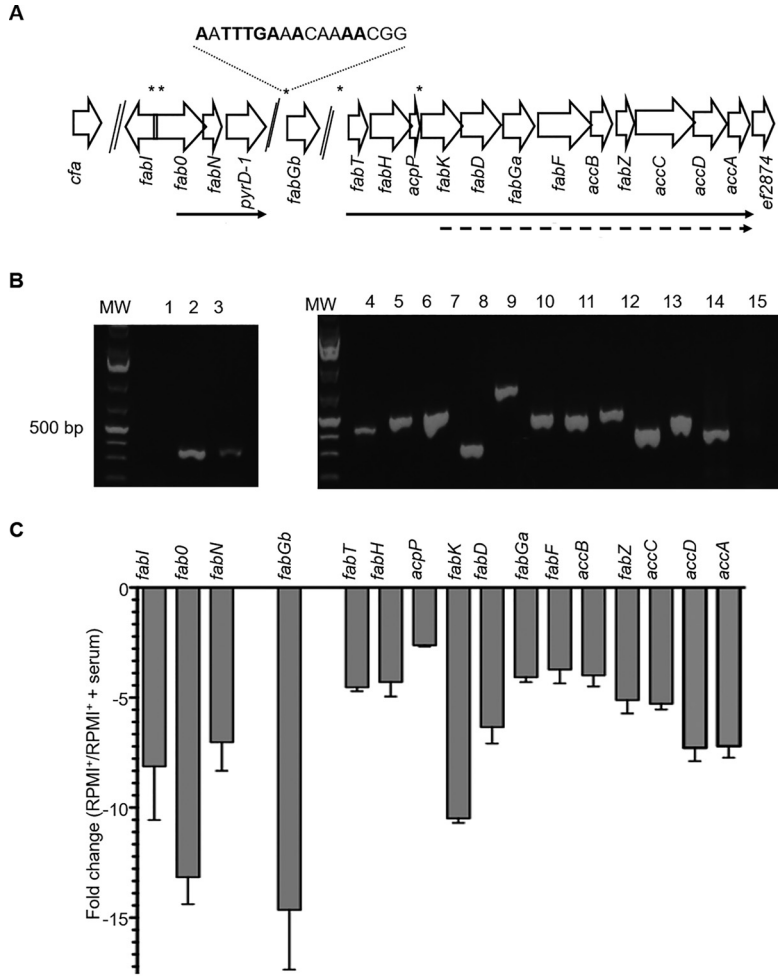

FIG 1 Genetic organization of the *Enterococcus faecalis* type II fatty acid synthesis (FASII) pathway and impact of exogenous fatty acids (FAs) on *E. faecalis* FASII gene expression. (A) Schematic representation of *E. faecalis* VE14089 FASII pathway genes. Gene positions with names below are represented 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, solid arrows indicate transcripts predicted by PCRs, and dotted arrows indicate transcripts identified by predicted FabT binding sequence and expression levels. (B) Agarose gel of the real-time PCR (RT-PCR) amplification products on cDNA using primer pairs from neighboring genes. The order is the same as that in panel A. 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*; and 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 ± standard deviation (SD) from three independent experiments are represented.

ef2881, which belongs to the 12-gene cluster, to *fabGa*, and *ef1773* to *fabGb*. The FabGa and FabGb proteins share 32% identity and 65% similarity. The 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, **A**A**TTTGA**AA**C AAAA**CG (palindromic nucleotides shown in bold) 10 bp downstream of the *fabGb* translation start codon (Fig. 1A) (33). FabT belongs to the family of MarR regulators (34). An HpaR binding site, HpaR also belonging to the MarR family, is centered 47 bp downstream of the *hpaG* to *hpaI* operon start site, and it was suggested that HpaR interferes with transcription elongation (35). Based on this precedent, we suggest that *fabGb* is controlled by FabT and, furthermore, 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, 33). 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, 33). This organization was tested by overlapping real-time PCR (RT-PCR) mapping (Fig. 1A and B). Results show that the 12-gene cluster is organized as a single operon that ends before *ef2874*. A transcription reinitiation site may be linked to the FabT consensus binding sequence upstream of *fabK*. Furthermore, intense and faint bands, respectively, 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*. Together, our data show that *E. faecalis* FASII genes are organized in two operons (*fabT* to *accA* and *fabO-fabN*) and two monocistronic transcriptional units (*fabI* and *fabGb*) (Fig. 1A and B).

The FASII pathway can be strongly repressed by exogenous fatty acids, such that phospholipid synthesis relies on exogenous FA substrates (1, 36). 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 the addition of FAs has not been studied. To address this question, and after checking that there was no growth difference in RPMI+ medium in the presence or absence of added human serum (data not shown), we used real-time quantitative PCR (qRT-PCR) to analyze the effect of 40% human serum on *E. faecalis* FASII gene expression (Fig. 1A and C). Serum addition resulted in decreased expression of all FASII genes, ranging 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. Together, 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.

The *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, 37). We confirmed these results with the wild-type (WT) strain VE14089 (data not shown). The consequences of a nonfunctional FASII pathway were then examined by constructing the EfΔFASII mutant, 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, the 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, growth of the EfΔFASII strain was essentially arrested in RPMI+. To test whether the slight growth observed with the EfΔFASII strain was due to fatty acids carried over from the preculture, we rediluted the cultures to an absorbance at 600 nm (OD_{600}) of 0.01 in RPMI+ when the EfΔFASII culture reached a plateau (Fig. 2B). Although the WT and BWT strains resumed growth, the EfΔFASII strain barely did (OD_{600} change from 0.01 to 0.015). 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. Together, 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 an FASII inhibitor (1) or by deletion of FASII genes.

The role of cyclopropanation in *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*

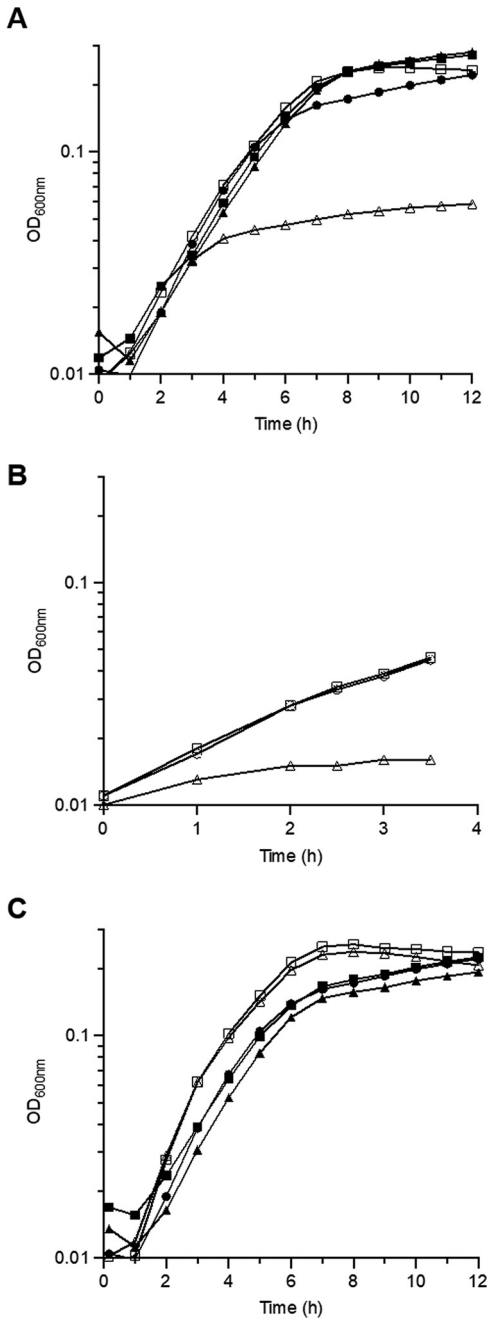

FIG 2 Impact of FASII pathway mutations on *E. faecalis* growth. (A) Growth curves of VE14089 (WT) (●), EfΔFASII (▲), and BWT-FASII (■) strains in RPMI+ in the absence (empty symbols) or presence (closed symbols) of 40% human serum. (B) Growth curves of VE14089 (WT) (○), EfΔFASII (Δ) and BWT-FASII (□) and diluted in RPMI+ to an absorbance at 600 nm (OD_{600}) of 0.01 when EfΔFASII strain growth reached a plateau. (C) Growth curves of VE14089 (●), EfΔ*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. Note change of scales for panel B.

strains in both media, indicating that the presence or absence of cyclic FA does not impact *E. faecalis* growth *in vitro* (Fig. 2C).

***E. faecalis* WT and EfΔFASII 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 under the same culture conditions (26). We determined the FA compositions of WT VE14089 and BWT-derivative (BWT-FASII) strains grown in the absence or presence of 40% serum (Fig. 3A; see

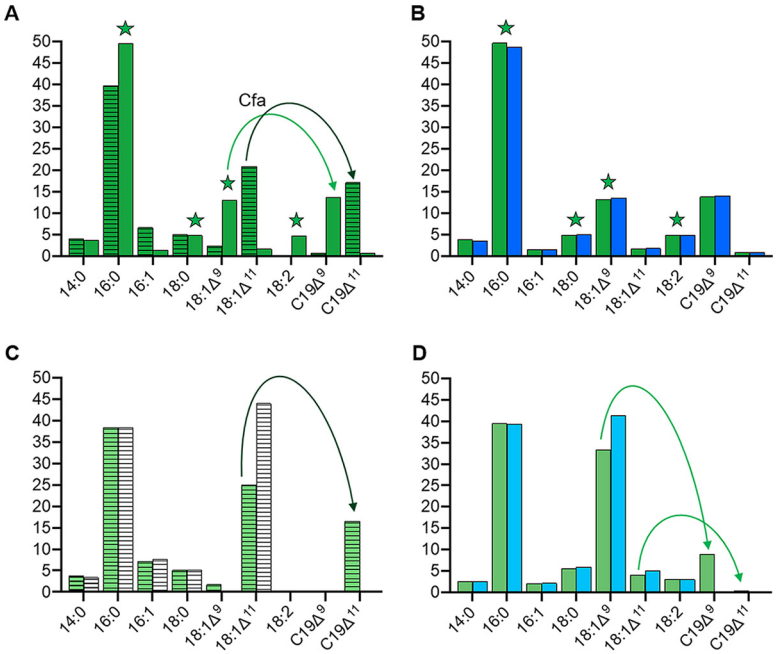

FIG 3 Fatty acid profiles of *E. faecalis* WT, ΔFASII, and Δ*cfa* strains grown without and with serum. *E. faecalis* was grown in RPMI+ or in RPMI+ containing 40% human serum to the stationary phase. FA composition was compared to assess the roles of medium FA content and genetic background. The means of the percentages of each FA found in *E. faecalis* BWT or mutant strains grown to the stationary phase in the presence or absence of 40% serum of three independent experiments are presented (see also Tables S2 to S4 in the supplemental material). (A) BWT-FASII grown in the absence or presence of serum. (B). BWT-FASII and EfΔFASII strains grown in 40% serum. (C) BWT-*cfa* and EfΔ*cfa* strains grown in RPMI+. (D) BWT-*cfa* and EfΔ*cfa* strains grown in 40% human serum. Only major fatty acids (>2% of total in at least one profile) are shown. Stars, fatty acids derived from serum. Green arrows, cyclopropanation in the BWT strains of unsaturated fatty acids; dark green, produced by *E. faecalis* (mainly 18:1Δ11); light green, incorporated from serum (mainly 18:1Δ9). Columns are colored as follows: dark green, BWT-FASII; dark blue, EfΔFASII; light green, BWT-*cfa*; light blue, EfΔ*cfa*; hatched, strains grown in RPMI+; empty, strains in RPMI+ supplemented with 40% human serum.

also Tables S2 and S3 in the supplemental material). 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 (PA; 16:0) (40%), vaccenic acid (VA; 18:1Δ11) (20%), and cyclopropanated VA form (19Δ11) (20%) (Fig. 3A). In contrast, membrane FA composition of bacteria grown in serum resembled the profiles of serum alone (Table S3 and Fig. 3A) (14, 26, 31). Thus, the proportion of endogenously produced 18:1Δ11 dropped to 1.7%, and that of serum-provided oleic acid (OA; 18:1Δ9) 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 exogeneous FA (6). Another notable change in the presence of serum was a decrease in the amount of C19Δ11, as reported previously (26), while cyclopropanated OA (C19Δ9), which is not found in serum, rose to 15%, indicating that *E. faecalis* CFA synthase cyclizes both 18:1Δ9 and 18:1Δ11. 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 EfΔFASII mutant strain grown in 40% human serum (Fig. 3B). The FA profiles of the EfΔFASII and BWT-FASII strains were

similar (Fig. 3B). Both strains similarly incorporated serum FAs and generated C19Δ9. 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 cellular FA composition.** To characterize the impact of the *cfa* deletion on *E. faecalis* membrane FA composition, we compared the FA profiles of EfΔ*cfa*, BWT-*cfa*, and WT strains grown in FA-free medium and in the presence of human serum (Fig. 3C and D; see also Tables S2 and S4 in the supplemental material). The BWT-*cfa* profiles were nearly identical to those of the WT (Tables S2 and S4). Compared to the BWT-*cfa* control strain in RPMI+, the EfΔ*cfa* mutant strain was devoid of C19Δ11 and was enriched in 18:1Δ11 (Fig. 3C). In serum-supplemented medium, the EfΔ*cfa* strain was devoid of C19Δ9 and enriched in 18:1Δ9 (Fig. 3D). These data confirm that the EfΔ*cfa* strain is defective for cyclopropanation of unsaturated fatty acids, mainly 18:1Δ11 in RPMI+ and 18:1Δ9 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, EfΔFASII, 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 (38, 39). 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 at 1, 7, and 14 days postinfection (dpi) (Fig. 4A and B). Throughout the course of the experiment, the CFU counts in blood were below the detection threshold (data not shown). The CFU counts in the liver diminished 31-fold, at 14 dpi, 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 10^4 CFU 24 h postinfection (hpi) to 66% of spleens being sterile at the end of the experiment. However, day 14 spleens weighed twice as much as day 1 spleens (Fig. 4B). Finally, kidney weights increased between day 7 to day 14 (Fig. 4B). This correlated, albeit with a time lag, to the tendency in the CFU counts to increase at day 7 in BWT-FASII-infected mice (15-fold) 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 EfΔFASII-infected mice. Bacterial counts in liver, spleen, and kidneys, as well as organ weights, were similar for BWT-FASII- and EfΔFASII-infected mice at all time points (Fig. 4A and B). 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 the stationary phase (40). We reasoned that differences, if any, between EfΔcfa and BWT-*cfa* strains would be more pronounced after CFU counts reached their highest level in the wild-type strain (here BWT)-infected mice, i.e., at day 7. 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.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. The vancomycin-resistant *E. faecalis* V583 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 [see "Strain construction," below]) strains (41–43). Two mutants were constructed, EfΔFASII and EfΔ*cfa*, corresponding to the in-frame deletion of nine genes from *fabK* to *accA* (7,863 bp) and of *cfa* (1,176 bp), respectively. The respective isogenic BWT strains BWT-FASII and BWT-*cfa* were obtained. *Escherichia coli* TG1 (Invitrogen) was used for cloning

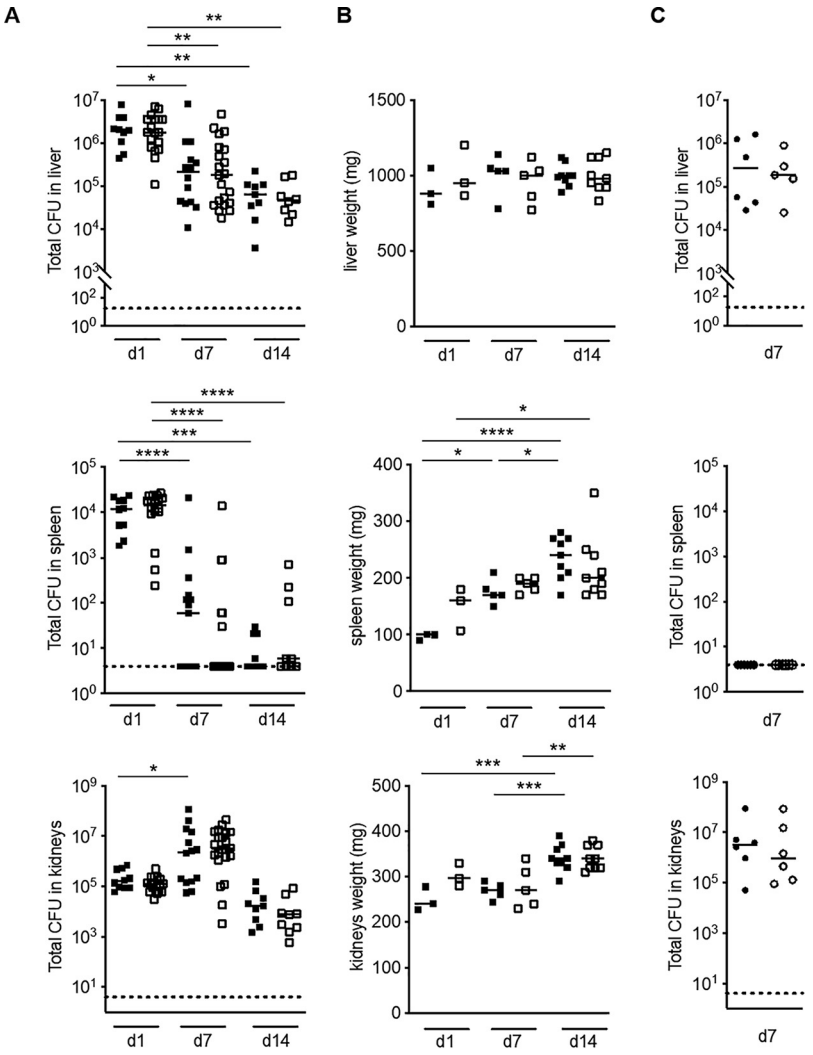

FIG 4 EfΔFASII and BWT-FASII infection in a mouse septicemic model. (A) CFU shown by scatter graph plots at 1, 7, and 14 days postinfection (dpi) of bacteria recovered from BWT-FASII- and EfΔFASII-infected mice. Nine to 20 mice were intravenously infected with 6×10^8 to 8×10^8 CFU *E. faecalis* BWT-FASII (closed squares) or EfΔFASII (open squares) strains. (B) Organ weights shown by scatter graph plots at 1, 7, and 14 dpi of 3 to 9 mice infected by the EfΔFASII or BWT-FASII strains. Symbols are as in panel A. (C) CFU shown by scatter graph plots at 7 dpi of viable bacteria recovered from EfΔ*cfa*- and BWT-*cfa*-infected mice. Six mice were intravenously infected with 6×10^8 to 8×10^8 CFU *E. faecalis* BWT-*cfa* (closed circles) or EfΔ*cfa* (open circles) strains. Upper panels, liver; middle panels, spleen; lower panels, kidneys. Each symbol represents the number of CFU per organ of one infected mouse or the weight of one organ. Median ranges are represented. Statistical analysis was as follows: 2-way analysis of variance (ANOVA) and a Bonferroni posttest (A and B) Mann-Whitney test (C). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$; ****, $P < 0.0001$.

experiments. *E. faecalis* was cultured in Todd-Hewitt (TH) broth or agar (Difco Laboratories, Detroit, MI), supplemented with 0.1% Tween 80 (Sigma) when appropriate, or in RPMI+ medium (RPMI 1640 supplemented with 1× amino acids; Gibco, Life Technologies), 1× vitamins (Sigma-Aldrich), 1% glucose, and 0.1 mM HEPES (Gibco, Life Technologies). *E. coli* was cultured in trypticase soy (TS) medium (Difco Laboratories,). When needed, antibiotics were used at the following concentrations: for *E. coli*, ticarcillin, 100 μg/ml, and erythromycin, 160 μg/ml; and for *E. faecalis*, erythromycin, 50 μg/ml. Cerulenin (Sigma) was added at 8 μg/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 Ampli*Taq* Gold polymerase as described by the manufacturer (Applied Biosystems). The molecular weight standard used in agarose gels is the 1 kb Plus DNA ladder (Life Technologies). Amplification products were purified using a QIAquick PCR purification kit (Qiagen). PCR

products and plasmids used were sequenced with an ABI 310 automated DNA sequencer, using the Prism dye terminator cycle sequencing kit (Applied Biosystems). 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 in the supplemental material.

Strain construction. The primers used for the construction of the in-frame EfΔFASII and EfΔ*cfa* mutant strains are listed in Table S1. Primers used are based on the published genome sequence of *E. faecalis* V583 (GenBank accession number NC_004668.1) (41). The ΔFASII deletion was constructed by using splicing by overlap extension PCR as described previously (44). 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 the In-Fusion PCR cloning kit (Clontech), cloning the two PCR-amplified fragments into pG1, previously linearized in a one-step reaction, following the manufacturer's recommendations. The resulting intermediate plasmids, pG1ΔFASII and pG1Δ*cfa*, were checked by sequencing the inserts. Electroporation of VE14089 and allelic exchange were performed as described previously with minor modifications (44, 45). For each construction, starting from a single clone in which the intermediate plasmid was integrated by homologous recombination, we selected clones in which the second homologous recombination event had taken place. One class of clones was deleted for the considered gene, and the other reverted to the wild-type genotype. An isolated reverted clone was termed back-to-the-wild type (BWT). The BWT strains are isogenic to their deleted counterparts, i.e., they should possess the same secondary mutations if any came up during the construction steps (43). That they displayed the WT phenotype was checked for all *in vitro* phenotypes. For the EfΔFASII strain construction, 0.1% Tween 80 (Sigma), a rich source of FA containing 70% oleic acid (OA; 18:1Δ9), was added during 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 phosphate-buffered saline (PBS) and subcultured (1:100) in RPMI+ and in RPMI+ supplemented with 40% human serum. In these experiments, the bacterial cultures carried out in RPMI+ were further diluted in RPMI+ before assessing growth. Growth was determined by measuring absorbance at 600 nm (OD_{600}). Experiments were repeated three times.

RNA extraction and RT-PCR. RNA was extracted from bacteria by the phenol-chloroform technique as described previously (46). RNA (2 to 5 μg) was treated with DNase I (Promega) according to the 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 the manufacturers' instructions. PCRs were carried out on cDNA, as well as on genomic DNA (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) to the mid-log phase (OD_{600} = 0.3) in RPMI+ with or without the addition of 40% human serum. 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 comparative threshold cycle ($2^{-\Delta\Delta CT}$) method, using *rpoB* as the reference gene (47).

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+ and diluted 100-fold in fresh RPMI+ cultures with or without 40% human serum. Cells were harvested after 14 h of growth (stationary phase) under 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 column (30 m × 30.25 mm × 30.25 mm; Agilent, France), for all analyses except those shown in Fig. 3D, or on a ZB-Wax capillary column (30 m × 0.25 mm × 0.25 mm; Phenomenex, France), for that shown in Fig. 3D, as described previously (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 the 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 Paris Descartes University animal care and use committee and by the Ministère de l'Education Nationale, l'Enseignement Supérieur et la Recherche (2015032714098562_v1, APAFIS no. 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, 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 medium supplemented with 0.1% Tween 80, were diluted 1:100 into 50 ml of TH-Tween 80 medium and grown until an OD_{600} of 0.6. The bacterial suspensions were washed twice in 0.9% NaCl and resuspended in 0.9% NaCl to obtain a final concentration of 6×10^8 to 8×10^8 CFU/ml in 500 μl for intravenous injections. Mice were injected in the tail vein using a 28-gauge 0.5-in. needle.

Dissemination assay and organ analysis. Randomized groups of 9 to 21 mice or of 6 mice were infected with the BWT-FASII and EfΔFASII or BWT-*cfa* and EfΔ*cfa* strains, respectively, and mice were sacrificed by cervical dislocation at days 1, 7, and 14 postinfection or at day 7, postinfection; 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% Tween 80 (TH-Tween agar). CFU were enumerated after 24 h of incubation at 37°C.

Statistical analysis. Statistical analyses were performed using a 2-way analysis of variance (ANOVA) and a Bonferroni posttest or a Mann-Whitney test (Prism 8). A P value of ≤ 0.05 was considered statistically significant.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, XLSX file, 0.02 MB.

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