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RESEARCH ARTICLE

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Unsaturated fatty acid synthesis in *Enterococcus faecalis* requires a specific enoyl-ACP reductase

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Abstract

The *Enterococcus faecalis* genome contains two enoyl-ACP reductases genes, *fabK* and *fabI*, which encode proteins having very different structures. Enoyl-ACP reductase catalyzes the last step of the elongation cycle of type II fatty acid synthesis pathway. The *fabK* gene is located within the large fatty acid synthesis operon whereas *fabI* is located together with two genes *fabN* and *fabO* required for unsaturated fatty acid synthesis. Prior work showed that FabK is weakly expressed due to poor translational initiation and hence virtually all the cellular enoyl ACP reductase activity is that encoded by *fabI*. Since FabK is a fully functional enzyme, the question is why FabI is an essential enzyme. Why not increase FabK activity? We report that overproduction of FabK is lethal whereas FabI overproduction only slows the growth and is not lethal. In both cases, normal growth is restored by the addition of oleic acid, an unsaturated fatty acid, to the medium indicating that enoyl ACP reductase overproduction disrupts unsaturated fatty acid synthesis. We report that this is due to competition with FabO, a putative 3-ketoacyl-ACP synthase I via FabN, a dehydratase/isomerase providing evidence that the enoyl-ACP reductase must be matched to the unsaturated fatty acid synthetic genes. FabO has been ascribed the same activity as *E. coli* FabB and we report in vitro evidence that this is the case, whereas FabN is a dehydratase/isomerase, having the activity of *E. coli* FabA. However, FabN is much larger than FabA, it is a hexamer rather than a dimer like FabA.

KEYWORDS
dehydrase/isomerase, enoyl-ACP reductase, FabI, FabK, unsaturated fatty acid synthesis

1 | INTRODUCTION

Bacterial fatty acid biosynthesis is carried out by a series of enzymatic steps, each catalyzed by a discrete enzyme encoded by a given gene, which together is called type II fatty acid synthase (Yao & Rock, 2017). Although the saturated fatty acids (SFA) and unsaturated fatty acids (UFA) profiles produced by *Enterococcus faecalis*, *Streptococcus pneumoniae*, and *Escherichia coli* are similar, there are significant differences in the enzymology of the three systems. The enoyl-ACP reductases of these bacteria differ. *S. pneumoniae* has only a flavoprotein, FabK enoyl-ACP reductase (Marrakchi et al., 2003), whereas *E. faecalis* has two enoyl-ACP reductases, an NADH-dependent FabI and a flavoprotein FabK (Zhu et al., 2013). *E. coli* has only FabI (Bergler et al., 1996).

The unsaturated fatty acid synthesis also differs among the three bacteria. In *E. coli*, FabA, the essential dehydratase/isomerase, and FabB an elongation enzyme are responsible for UFA synthesis (Feng &

Cronan, 2009). *E. faecalis* has two genes encoding homologous of FabF and FabZ and one of the FabF homologs (FabO) was assigned the same function as *E. coli* FabB and one of the FabZ homologs (FabN) was assigned the same function as *E. coli* FabA (Wang & Cronan, 2004). In *E. coli* FabB and FabF control the ratios of UFA:SFA and the C16 and C18 carbon UFAS in *E. coli* (Feng & Cronan, 2009). The key reaction in UFA synthesis is FabB which is specifically required to elongate the *cis*-3-decenoyl-ACP produced by FabA (Feng & Cronan, 2009). *S. pneumoniae* lacks FabA and FabB enzymes but has FabM which catalyzes isomerization but lacks dehydratase activity (Marrakchi et al., 2002). How *E. faecalis* regulates the synthesis of SFA and UFA remains unclear, although an *E. faecalis ΔfabK* strain has an increased ratio of UFA to SFA (Zhu et al., 2013).

We report that overexpression of either FabI or FabK results in increased SFA synthesis at the cost of UFA synthesis with FabK having a much stronger SFA overproduction phenotype than FabI. Indeed, overproduction of FabK is lethal in the absence of UFA supplementation and almost completely blocks UFA synthesis whereas overexpression of FabI has the much milder effect of slow growth. The effects of enoyl ACP reductase overproduction seemed likely to be due to competition with the enzymes of *E. faecalis* UFA synthesis, FabN, and FabO. We have demonstrated that FabN is required for UFA synthesis and have defined their biochemical activities. FabO is not essential because FabF, encoded in the main fatty acid synthesis operon, also has the ability to elongate the key FabN product.

2 | RESULTS

2.1 | The differing properties of two enoyl ACP reductases FabI and FabK in vivo

Enoyl ACP reductase catalyzes the last step of the elongation cycle of the type II fatty acid synthesis pathway (Massengo-Tiassé & Cronan, 2009) (Figure 1). The genome of *E. faecalis* expresses two enoyl ACP reductases, the NADH-dependent FabI and the flavoprotein FabK. The Δ*fabI* strain is a UFA auxotroph but retains weak fatty acid synthesis due to FabK (Δ*fabI* Δ*fabK* strains completely lack fatty acid synthesis ability; see below). *E. faecalis* FabK is a fully functional enoyl ACP reductase (Zhu et al., 2013) in vitro but is poorly expressed in vivo due to defective translational initiation (Bi et al., 2014). This weak expression is insufficient for growth and therefore FabI is required. However, FabK does moderate the fatty acid composition of *E. faecalis* (Zhu et al., 2013). Deletion of *fabK* results in increased unsaturated fatty acids (UFAs) in the membrane phospholipids suggesting that FabK competes with FabO and FabF via FabN (see below).

We first expressed *fabK* using P32, a strong constitutive promoter, on a high-copy plasmid (Figure 1). When FabK was overexpressed in the wild-type *E. faecalis* strain, it was lethal. Lethality was rescued by the addition of oleic acid indicating that FabK interfered with UFA synthesis. Overproduction of FabI gave a much less severe phenotype in that it only slowed growth (Figure 1). Oleic acid supplementation restored normal growth (Figure 1). The overexpression strains accumulated suppressors in the M17 medium and sequencing showed that *fabI*, *fabK*, and the P32 promoter in plasmids purified from suppressors lacked any mutation. Hence, the appearance of suppressors is not caused by a mutation in the genes of interest but perhaps the transcription level of the P32 promoter may be altered indirectly (e.g., by RNA polymerase mutations). Labeling with [1-^{14}C]acetate showed that overexpression of FabK or FabI resulted in increased SFA synthesis and overexpression of FabK virtually eliminated UFA synthesis (Figure 2). Note that *E. faecalis* requires only UFAs for growth, saturated fatty acids are not required by strains blocked in fatty acid synthesis (Dong & Cronan, 2022).

2.2 | Comparison of FabK and FabI expression using a regulated promoter

To obtain a more nuanced comparison between FabK and FabI we used a regulated promoter expressing the proteins in both low and high copy number plasmids. Linares and workers (Linares et al., 2014) have provided a valuable regulated promoter for *E. faecalis* gene expression: the regulatory system for agmatine degradation. Exogenous agmatine controls the expression of agmatine degradation genes (Linares et al., 2014). We used low copy (pTRK L2) or high copy (pBM02) plasmids carrying the *aguR* gene (which encodes a LuxR family transcriptional regulator of agmatine degradation) together with the promoter for the agmatine degradation genes to give induction of different levels of *fabI* or *fabK* expression by varying agmatine concentrations.

The low copy pTRK L2 plasmid carrying either *fabI* or *fabK* restored the growth of a Δ*fabI* Δ*fabK* strain upon induction with agmatine (Figure 3). The high copy pBM02 plasmid carrying either *fabI* or *fabK* gene allowed the growth of the Δ*fabI* Δ*fabK* strain with or without agmatine induction. However, compared to the *fabI* gene, the high copy plasmid carrying *fabK* was toxic for *E. faecalis* growth in the presence of agmatine (Figure 3). Labeling with [1-^{14}C]acetate was used to assay the fatty acid synthetic abilities of the complemented strains (Figure 4). The low copy plasmid carrying either *fabI* or *fabK* gene restored fatty acid synthesis upon agmatine induction whereas the high copy plasmid restored fatty acid synthesis with or without agmatine induction (Figure 5). Compared with *fabI*, the ratio of UFA to SFA was much higher when the Δ*fabI* Δ*fabK* strain was complemented with *fabK*. However, in the wild-type strain of *E. faecalis* overexpressing FabK was expressed from the low copy plasmid. [1-^{14}C]acetate labeling showed that FabK overexpression greatly reduced the ratio of UFA to SFA. We also constructed a *ΔfabI ΔfabT* strain to ask if increased expression of the fatty acid synthesis operon genes including FabK would allow growth in the absence of FabI. FabT is the repressor of the operon and loss of FabT increases the expression of the operon 2.5-3-fold (Zou et al., 2022). The increased FabK expression resulting from the absence of FabT resulted in very slow growth in the absence of FabI (data not shown).

2.3 | Mechanism of enoyl-ACP reductase overexpression toxicity

A plausible hypothesis to explain these data is that FabI and FabK toxicity is due to inhibition of UFA synthesis by competition for

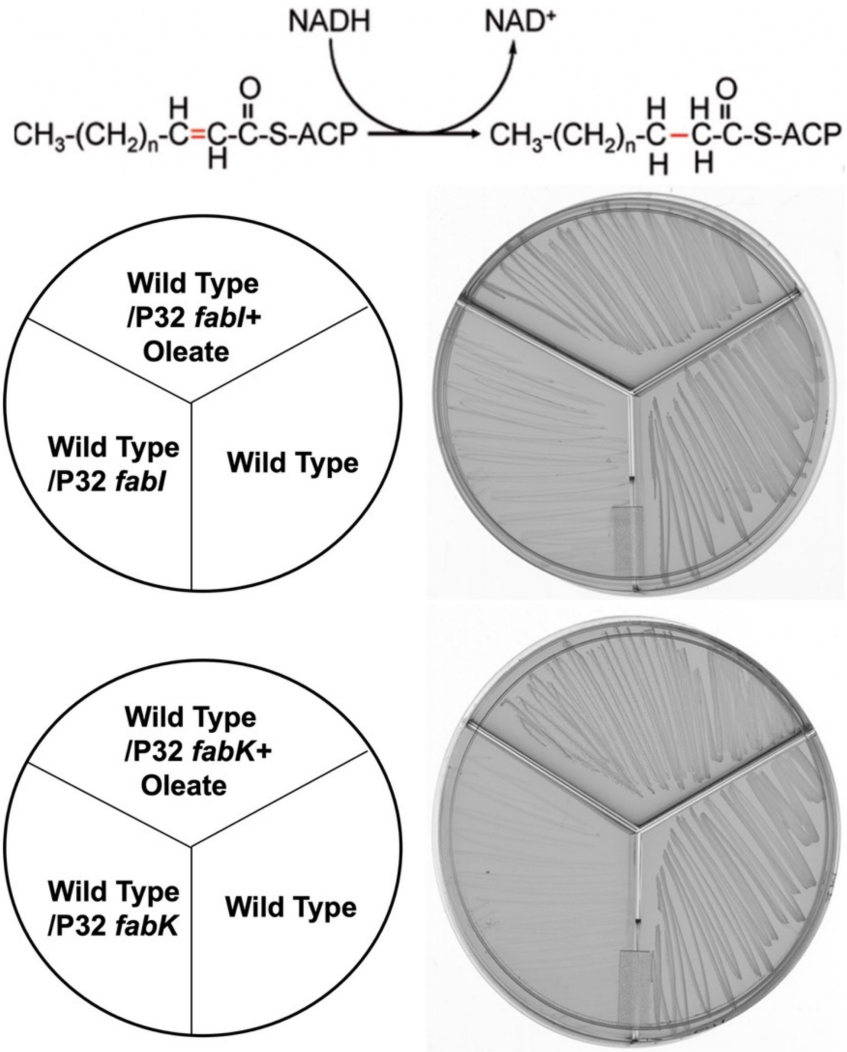

FIGURE 1 The enoyl-ACP reductase reaction and the effects of overproduction of FabI or FabK on growth and its reversal by oleate supplementation. The top plate is FabI overproduction (note the faint growth) whereas the bottom plate is FabK overproduction. The enoyl-ACP reductase reaction shown is that of FabI. The FabK reaction uses a bound flavin (FMN) for the reduction which is recycled using NADH.

substrates required for UFA synthesis The hypothesis is based on the properties of the *E. coli* UFA synthesis enzymes, FabA and FabB. The reactions of the FabN dehydratase/isomerase would proceed through the mechanism established for *E. coli* FabA (Feng & Cronan, 2009; Guerra & Browse, 1990). FabN would bind the 3-hydroxydecanoyl-ACP intermediate of saturated fatty acid synthesis, dehydrate it to *trans*-2-decenoyl-ACP and isomerize the double bond to give *cis*-3-decenoyl-ACP (Figure 5). As demonstrated for FabB, FabO and FabF would elongate the *cis*-3-decenoyl-ACP to 3-keto-*cis*-5-dodecenoyl-ACP, an irreversible reaction due to decarboxylation of the malonyl-ACP consumed in elongation.

A caveat to this scenario is that the FabO mechanism has not been directly determined nor has it been coupled to FabN. The FabN and FabO functions in UFA synthesis are based on complementation in *E. coli* mutant strains both in vivo and in cell extracts (Wang & Cronan, 2004). However, complementation only shows that a suitable product has been made and says nothing about the mechanism. Indeed, we have functionally complemented *E. coli* UFA auxotrophic strains by expressing enzymes that are mechanistically distinct from FabA and FabB (Bi et al., 2013, 2016). Therefore, we have purified the FabN and FabO proteins and studied their mechanisms in order to test the above scenario. Note that a *fabN* mutant of a different strain of *E. faecalis* was isolated for immunological investigations (Diederich et al., 2016) although no fatty acid data were reported. FabN was shown to have dehydratase/isomerase activity in vitro (Lu et al., 2005).

2.4 | Properties of the purified FabN and FabO proteins

Hexahistidine-tagged versions of both proteins were expressed in *E. coli* and purified by Ni-cheleate chromatography. FabN had a subunit molecular weight of 16.2 kDa as measured by denaturing gel electrophoresis, a value similar to that of *E. coli* FabA (Figure S1). A surprise was that size exclusion chromatography gave a value of 111 kDa (Figure S2) indicating that FabN is a hexamer in solution rather than a dimeric protein like FabA. *E. coli* FabZ, a close cousin of FabN by sequence alignment (Wang & Cronan, 2004), is hexameric and gives a similar value upon size exclusion chromatography. FabZ and FabA have identical folds and dimer interfaces. Indeed, FabZ is a trimer of FabA-like dimers whereas FabA is a dimer. Unlike FabN and FabA, FabZ proteins lack

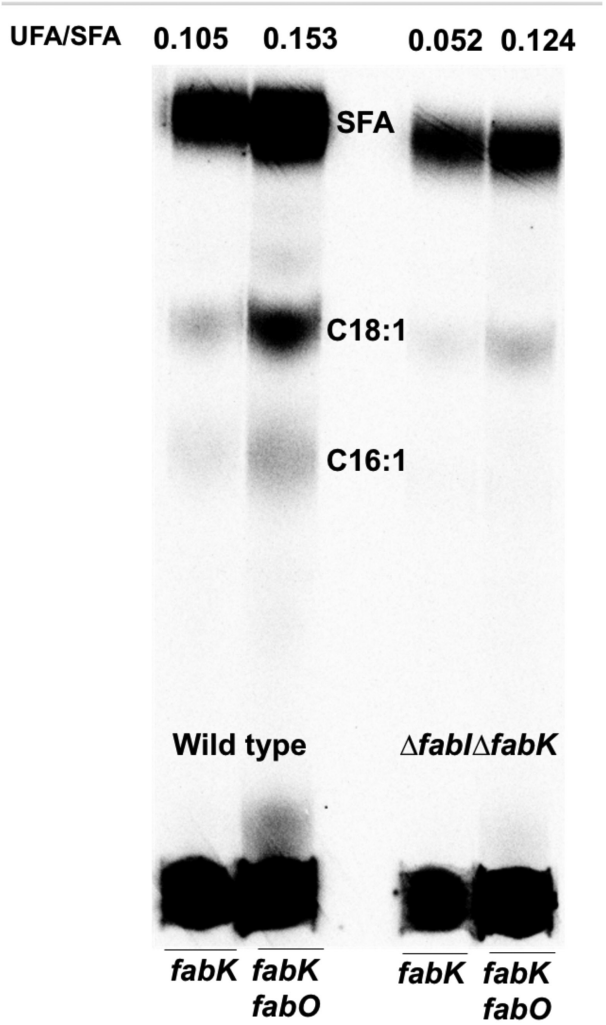

FIGURE 2 Effects of FabI or FabK overproduction on [^{14}C] acetate incorporation into phospholipid acyl chains. The lanes of the left panel are FabI overproduction, whereas the lanes of the right panel are FabK overproduction. There is an empty lane in between. The FabI and FabK labeling was done in duplicate. The figure is an autoradiograph of a Ag-TLC plate.

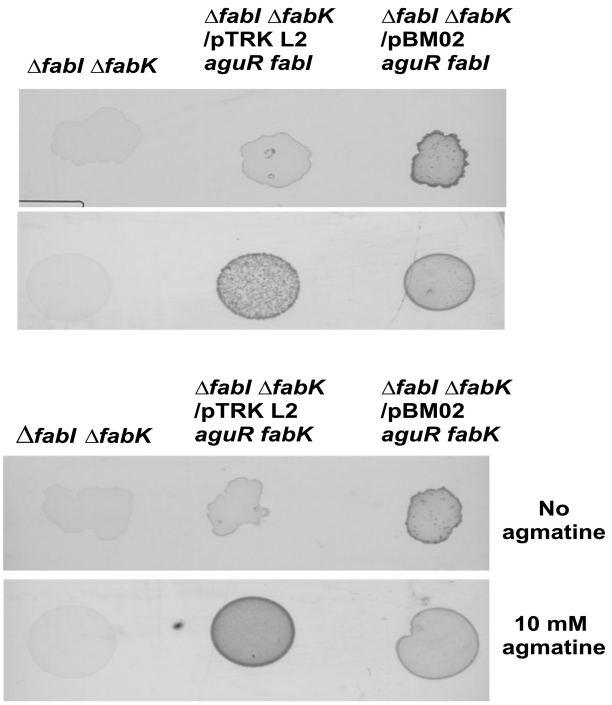

FIGURE 3 Growth of *ΔfabI ΔfabK* strains with or without *fabI* (top panel) or *fabK* (bottom panel) under agmatine control in either the low copy plasmid, pTRKL2, or the high copy plasmid, pBM02. Cultures were spotted on plates and incubated overnight. Note the faint growth of the *ΔfabI ΔfabK* strain lacking a plasmid (left spottings).

isomerase activity due to the architecture of the acyl chain binding pocket (Dodge et al., 2019). FabN is the first example of a hexameric dehydratase/isomerase. Previous workers had performed size exclusion chromatography analyses on purified FabN and FabZ but did not note the large size of FabN relative to FabA (Lu et al., 2005).

The FabO structure seems similar to that of *E. coli* FabB. FabO had a subunit molecular weight of 43.2 kDa as measured by denaturing gel electrophoresis (Figure S1) and a size exclusion chromatography molecular weight of 104 kDa indicating a dimeric protein (Figure S2). Based on prior studies in *E. coli* and the *E. faecalis* genome we expected that FabO and FabF are the only long-chain 3-ketoacyl-ACP synthase enzymes in *E. faecalis*. To test this premise, we constructed a *ΔfabO ΔfabF* double mutant strain, and [1-^{14}C]acetate labeling showed that the strain completely lacked the ability to synthesize long-chain fatty acids (Figure 6). When the FabO mutation was complemented with the wild-type gene, long chain fatty acid synthesis returned but only the C16:1 UFA was abundantly synthesized because FabF is required to elongate the C16:1-ACP to C18:1-ACP (Dong & Cronan, 2021).

2.5 | FabN has dehydrase/isomerase activity and functions with FabO

We determined the enzymatic activities of FabN by running reactions in parallel with the paradigm dehydratase/isomerase, *E. coli* FabA (Figure 7). Both enzymes dehydrated 3-hydroxydecanoyl-ACP to a mixture of *cis*-3-decenoyl-ACP and *trans*-2-decenoyl-ACP. The two enzymes gave essentially the same products (lane 3, FabA; lane 4, FabN). Although we could not separate the decanoyl isomers, two species were present, a dark band migrating just ahead of a light band. Upon addition of a 3-ketoacyl-ACP synthase, only the light band remained to suggest that the dark band was *cis*-3 decenoyl-ACP (enoyl-ACPs are not 3-ketoacyl-ACP synthase substrates). Note that previous workers reported FabN dehydratase/isomerase activity very similar to that of FabA but coupled the activities to a *Streptococcus pneumoniae* 3-ketoacyl-ACP synthase (Lu et al., 2005).

In our work, the activity of FabO was assayed by running reactions in parallel with the paradigm 3-ketoacyl-ACP synthase I, *E. coli* FabB (Figure 7). The reactions were run in an *E. coli* fatty acid synthesis system reconstituted from purified proteins but lacking an enoyl-ACP reductase with the additions and omissions as given in the figure. In addition to the

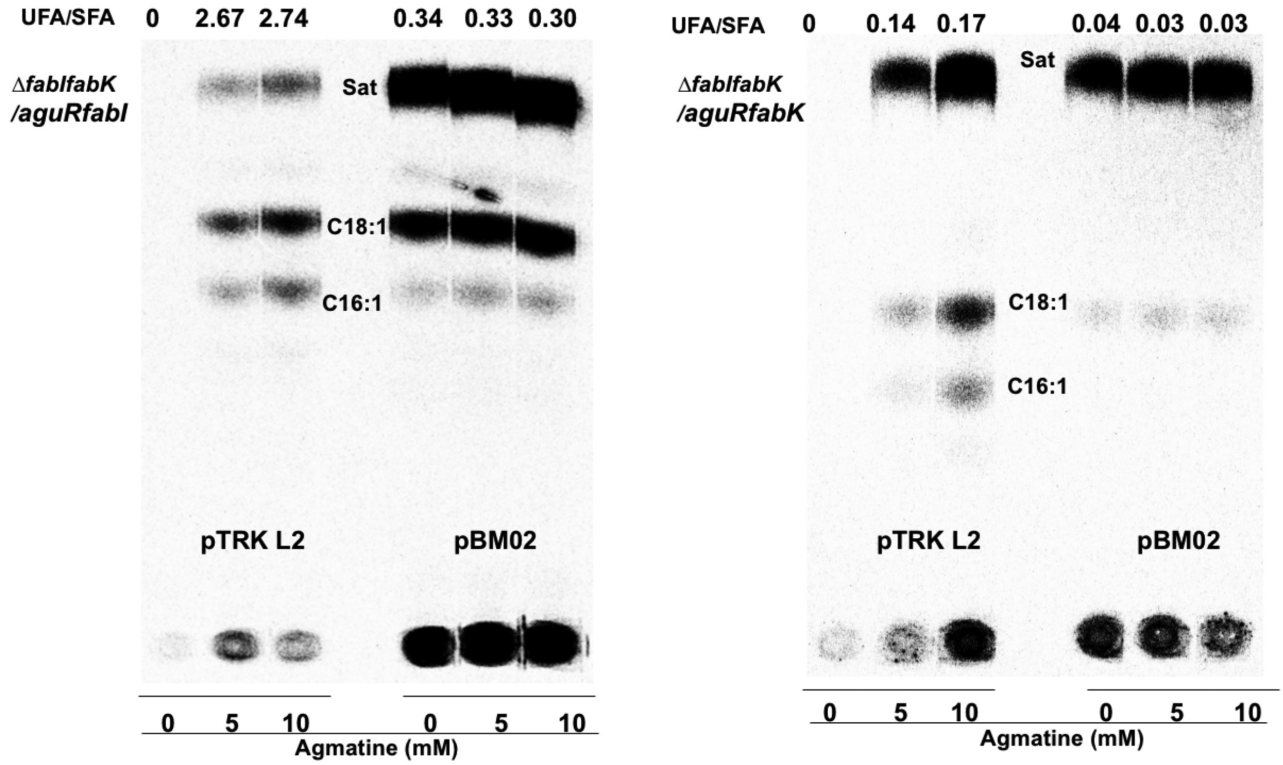

FIGURE 4 Effects of agmatine regulated expression of FabI (left panel) or FabK (right panel) in the *ΔfabI ΔfabK* strain. The low copy plasmid pTRK L2 and high copy plasmid pBM02 were used as shown. The figure is an autoradiograph of two Ag-TLC plates.

E. coli proteins, *Vibrio harveyi* acyl-ACP synthetase (AasS) was used to convert 3-hydroxydecanoic acid to 3-hydroxydecanoyl-ACP. FabG was present to reduce the unstable 3-ketoacyl-ACPs produced by FabB and FabO to stable 3-hydroxyacyl-ACPs. FabB was run with either FabA or FabN to produce the elongation substrate and similarly, FabO was run with either FabA or FabN. The two 3-ketoacyl-ACP synthases gave essentially identical products with a given dehydratase/isomerase. However, although the two dehydratase/isomerases gave the same banding patterns, the intensities varied probably due to reactions occurring after 3-ketoacyl-ACP reduction and the inability of synthesis to proceed further. The band migrating just below the 3-OH C12:1(Δ5c) band is probably the dehydration product catalyzed by the dehydratase/isomerase. FabA catalyzed dehydration has a broad substrate specificity in vitro (Heath & Rock, 1996) although in vivo the isomerase reaction is specific to the C10 species. Having determined the in vitro activities of FabN and FabO, we deleted the encoding genes and determined their phenotypes.

2.6 | Deletion strains of *fabN* and *fabO*

Nonpolar deletions of both genes were constructed. The *ΔfabN* strain was an oleate auxotroph as previously reported in immunological investigations (Diederich et al., 2016) (Figure S3), and is completely defective in UFA synthesis (Figure S4). To our surprise, the *ΔfabO* strain grew without oleate supplementation. Moreover, a *ΔfabN ΔfabO* strain was constructed and was found to grow without oleate when complemented with FabN plasmids (Figure S3).

These data argued that the other long-chain 3-ketoacyl-ACP synthase, FabF, encoded in the large fatty acid synthesis operon, also had the ability to elongate *cis*-3-decenoyl-ACP. This is not unprecedented, FabF homologs of two other members of the Lactobacillales have this ability (Lu et al., 2005; Morgan-Kiss & Cronan, 2008). To test the involvement of FabF, we constructed a *ΔfabO ΔfabF* strain which proved an oleate auxotroph. This strain was complemented for growth without oleate by a *fabO* plasmid providing evidence that FabO elongates *cis*-3-decenoyl-ACP in vivo (Figure 6). Labeling with [^{14}C]acetate showed that the *ΔfabO ΔfabF* completely lacked UFA synthesis (Figure 6), although SFA synthesis proceeded. Complementation with *fabO* restored UFA synthesis.

Purified FabF was directly shown to elongate *cis*-3-decenoyl-ACP (Figure S5). This is the first case of two proteins in the same bacterium produced under the same transcriptional regulation having *cis*-3-decenoyl-ACP elongation activity.

2.7 | The in vivo properties of *E. faecalis* FabF and FabO

Deletion of *fabO* or *fabF* significantly decreased the ratio of UFA to SFA (Figure 6). The FA compositions were determined by GS-MS (Table 1). The growth phenotypes of these mutant strains and their complemented strains were also determined (Figure S5). Hence FabO and FabF differ in that FabF is required for normal synthesis of the longer UFA, *cis*-vaccenic acid (C18Δ11) (Dong & Cronan, 2021). whereas FabO is more proficient than FabF in elongation of *cis*-3-decenoyl-ACP.

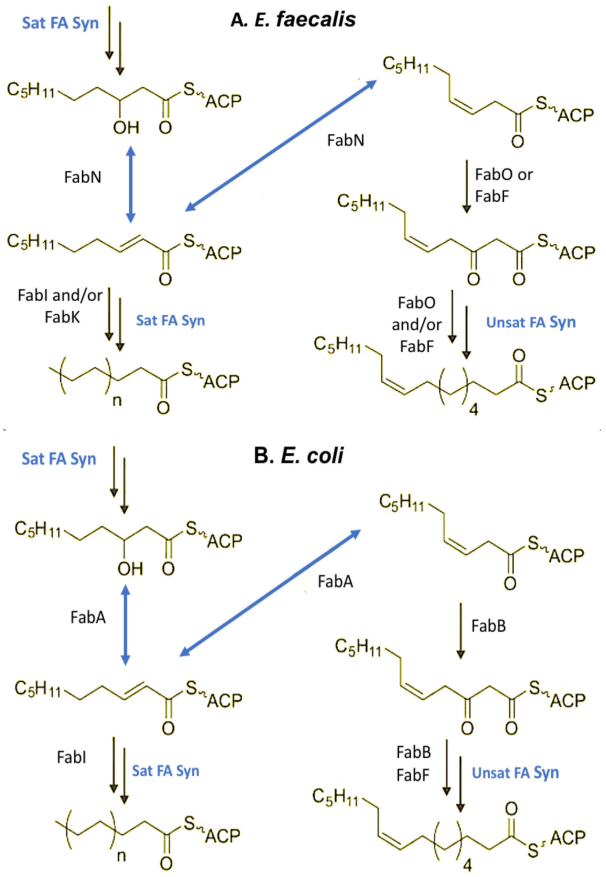

FIGURE 5 The branch points of fatty acid synthesis in *E. facaelis* and *E. coli*. (a) The proposed *E. facaelis* pathway and (b) the *E. coli* pathway. The molecule in the upper right-hand corner of each panel is *cis*-3-decenoyl-ACP.

The UFA/SFA ratios of the *ΔfabF* strain are 1.3 whereas that of the *ΔfabO* strain is 1.0 (Table 1). In the wild-type strain FabO and FabF act together to give the UFA/SFA ratio of 2.3 (Table 1).

The above data argue that there is a formal competition between the isomerization reaction of FabN and the two enoyl-ACP reductases. If isomerization wins, an unsaturated acyl chain is made. If an enoyl-ACP reductase wins, a saturated acyl chain is produced (Figure 5). Two aspects of the competition would be important. First, acyl chains with a *cis*-3 double bond are not enoyl-ACP reductase substrates. Second, all the FabN reactions are reversible such that *cis*-3-decenoyl-ACP could be converted to *trans*-2-decenoyl-ACP: the enoyl-ACP reductase substrate. This can be avoided by FabO- or FabF-catalyzed elongation to 3-keto-*cis*-5-dodecenoyl-ACP. Hence, overall the enoyl-ACP reductases are competing with FabO and FabF via FabN. The greater toxicity of FabK relative to FabI would be due to the greater efficiency of FabK in competing with FabN, FabO, and FabF.

2.8 | Testing the competition hypothesis

In order to test the competition hypothesis, we constructed a plasmid expressing both FabK and FabO (each gene had its own P32 promoter). This plasmid was expressed in parallel with an analogous plasmid carrying only FabK in both wild-type and *ΔfabI ΔfabK* strains (Figure 8). The effect of FabO overexpression on growth in the presence of FabK overexpression was very modest, only a few colonies were formed. However, [1-^{14}C]acetate labeling showed that FabO overexpression restored UFA synthesis to wild-type cells inhibited by FabK (Figure 9). Although the restoration was modest in that the UFA/SFA ratio was only 50% greater than that of the FabK inhibited strain, these results indicate that the competition between enoyl-ACP reduction and elongation of *cis*-3-decenoyl-ACP takes place in vivo. Given better matching of the activities of the two enzymes, it may be possible to avoid FabK overexpression toxicity.

3 | DISCUSSION

The toxicity of the *E. faecalis* enoyl-ACP reductases, FabI and FabK is due to the inhibition of UFA synthesis. The reactions of the FabN dehydratase/isomerase proceed through the mechanism established for *E. coli* FabA. FabN binds the 3-hydroxydecanoyl-ACP intermediate of saturated fatty acid synthesis, dehydrates it to *trans*-2-decenoyl-ACP, and isomerizes the double bond to give *cis*-3-decenoyl-ACP. FabO then elongates the *cis*-3-decenoyl-ACP to 3-keto-*cis*-5-dodecenoyl-ACP. The FabO and FabF reactions are irreversible due to decarboxylation of the malonyl-ACP consumed in elongation. In vitro the *trans*-2-decenoyl-ACP and *cis*-3-decenoyl-ACP intermediates of the FabA reaction are not enzyme bound and are released into the solution (Guerra & Browse, 1990). This follows from the demonstration that FabA reaches a distribution of β-OH, 24%, *trans*-2, 38%, and *cis*-3; 38% (mol %) at equilibrium regardless of which of the three C10-ACP substrates was the starting material (Guerra & Browse, 1990). Since only one substrate molecule can occupy each active site, the others will be free in the solution.

Hence, there is competition between the isomerization reaction of FabN and the two enoyl-ACP reductases. If isomerization wins, an unsaturated acyl chain is produced. If an enoyl-ACP reductase wins, a saturated acyl chain results (Figure 5). Hence, overall, the enoyl-ACP reductases are competing with FabO and FabF via FabN. When overexpressed using equivalent constructs FabK is much more effective than FabI in growth inhibition. Hence, high-level expression of the chromosomal *fabK* would compromise growth. The chromosomal *fabK* is located in the large fatty acid synthesis gene operon and is transcribed with the other genes (the *acpP* gene is immediately upstream of *fabK*) and thus is under the same FabT-dependent transcriptional regulation as *fabK* and *fabI*. However, FabK is poorly expressed, there is only a trace of enoyl-ACP reductase activity in a *ΔfabI* strain which is insufficient to support growth (Bi et al., 2014; Zhu et al., 2013). Previously, we isolated suppressors that allowed a *ΔfabI* strain to grow without UFA supplementation. All suppressors mapped in the ribosome binding region of the *fabK* mRNA and increased complementarity to the 16S RNA (Bi et al., 2014). This argues that the weak expression of FabK is due to poor translational initiation. Note that all suppressors isolated were fairly weak perhaps because strong suppression would be lethal.

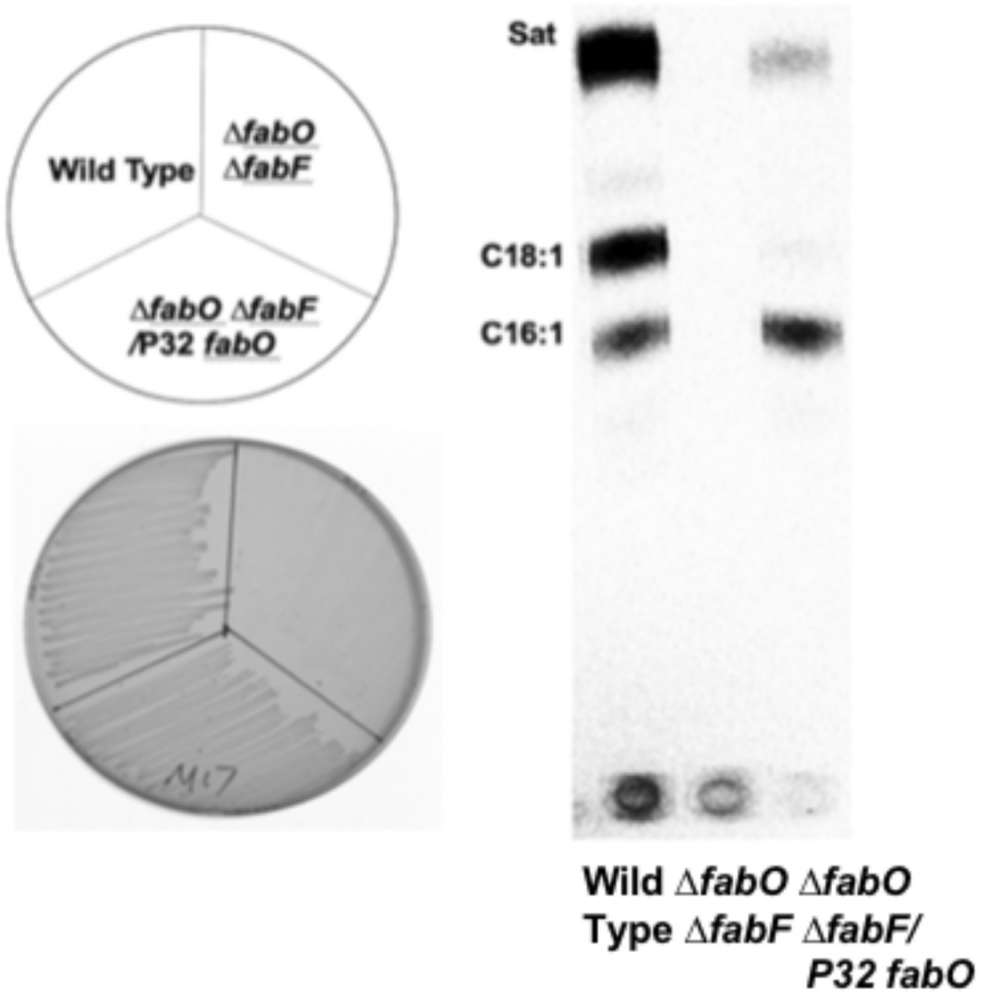

FIGURE 6 Growth and [^{14}C]acetate labeling of the wild-type strain, the *ΔfabO ΔfabF* strain and the *ΔfabO ΔfabF* strain complemented with a *fabO* plasmid. The figure is an autoradiograph of a Ag-TLC plate.

A possible scenario for the two *E. faecalis* enoyl-ACP reductases is that FabK expression had to be weakened to allow UFA synthesis to proceed. However, the enoyl-ACP reductase is required in each fatty acid synthesis cycle so *fabI* was acquired perhaps by lateral gene transfer from one of the many species of bacteria that inhabit the colon. It seems that the enoyl-ACP reductase and the enzyme that introduces the *cis* double bond must be carefully matched to ensure UFA synthesis. This is the case in *E. faecalis*, where FabI is paired with FabN and FabK expression is largely absent. Another example is the related *Streptococcus pneumoniae* which lacks FabN, FabO, and FabI. This bacterium has FabM, an isomerase that converts *trans*-2-decenoyl-ACP to *cis*-3-decenoyl-ACP (Marrakchi et al., 2002) but FabM cannot catalyze dehydration of 3-hydroxy-ACP. Attempts to complement a *fabA* mutant of *E. coli* with FabM failed due to competition from *E. coli* FabI. However, when *E. coli* FabI was inactivated and replaced with the *S. pneumoniae* FabK enoyl-ACP reductase, FabM functionally replaced FabA (Lu et al., 2005).

Why do FabK and FabI differ in their abilities to inhibit fatty acid synthesis? As shown for FabA (see above) (Guerra & Browse, 1990) since FabN makes that same mixture of products as FabA (Figure 7), it does not sequester the *trans*-2- and *cis*-3-decenoyl-ACP intermediates: they are released into the solution. Hence, the *trans*-2-decenoyl-ACP intermediate is "fair game" for enoyl-reductase catalysis by either FabK or FabI. In *E. faecalis* poor expression of FabK precludes competition with FabI, but why does not FabI block UFA synthesis? A straightforward mechanism would be partial inhibition of FabI by *cis*-3-decenoyl-ACP. This would ensure a sufficient supply of *cis*-3-decenoyl-ACP for UFA synthesis and allow the enoyl-reductase steps of long-chain fatty acids synthesis to proceed. Inhibition could be competition at the enoyl-reductase active site (competitive inhibition) or more likely allosteric inhibition. This hypothesis posits that FabK is not inhibited by *cis*-3-decenoyl-ACP. This seems plausible because the presence of the FMN cofactor renders the active site necessarily different from that of FabI. Testing this hypothesis will not be straightforward. The acyl chain, *cis*-3-decenoic acid, is not commercially available and the *Vibrio harveyi* acyl-ACP synthetase (AasS) does not accept *cis*-3-decenoic acid as a substrate (Jiang et al., 2010).

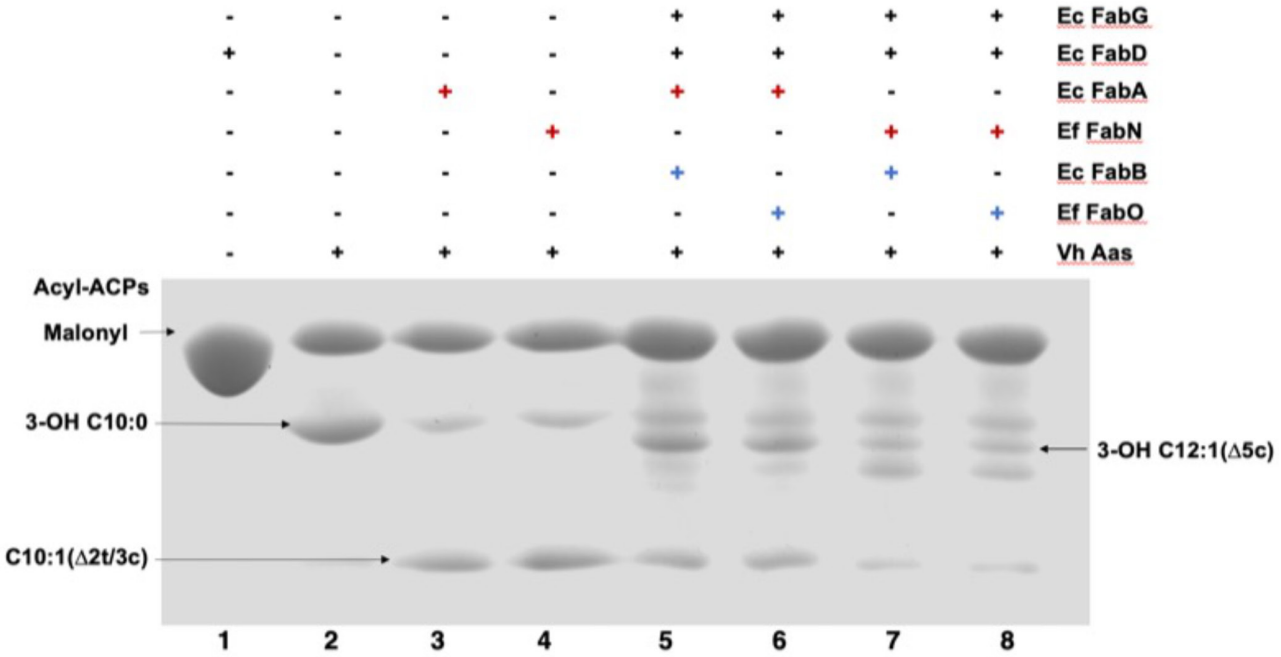

FIGURE 7 In vitro comparisons of FabN with FabA and of FabO with FabB. Lanes 1 and 2 are standards, lanes 3 and 4 compare the dehydration/isomerization activities of FabA (lane 3) and FabN (lane 4). In lanes 5 to 8, a reconstituted one-cycle UFA synthesis system composed mainly of purified *E. coli* proteins was utilized. *Vibrio harveyi* acyl-ACP synthetase was used to convert 3-hydroxydecanoic acid to its ACP thioester in situ. The red plus signs indicate the enzymes to be compared.

TABLE 1 Fatty acid compositions of wild type, Δ*fabF*, and Δ*fabO* strains

Fatty acids % of total	Wild type	△*fabF*	△*fabO*
C14:0	0.7 ± 0.2	1.7 ± 0.7	1.0 ± 0.3
C16:0	24.9 ± 1.3	33.9 ± 5.2	39.6 ± 2.4
C16:1 (9)	5.7 ± 2.2	51.5 ± 5.2	2.8 ± 0.3
C18:0	4.6 ± 0.7	8.4 ± 1.3	8.5 ± 0.7
C18:1 (11)	60.9 ± 2.6	3.6 ± 0.7	41.0 ± 2.3
C19:0-cyclo	3.2 ± 0.1	0.9 ± 0.6	7.1 ± 0.2
UFA/SFA	2.3	1.3	1.0

Note: Cyclo denotes cyclopropane fatty acid methyl ester.

Finally, the question arises why *fabK* has not been deleted or the protein inactivated since it is nonessential (at least in the lab). This was previously discussed (Bi et al., 2014) and we refer the interested reader to that report. There are 137 nt between *acpP* and *fabK* in an otherwise tightly packed operon. Our best guess is that a small RNA binds the mRNA and somehow provides a good ribosome binding site to allow high-level FabK synthesis in the mammalian gut.

4 | MATERIALS AND METHODS

4.1 | Materials

Malonyl-CoA, NADPH, fatty acids, antibiotics, M17 medium, and agmatine sulfate salt were purchased from Sigma-Aldrich. DNA polymerases, Gibson assembly kit, restriction endonucleases and T4 ligase were from NEB. Sodium [1-^{14}C]acetate (specific activity, 58.6 mCi/mmol) was provided by Moravek. Ni-NTA resin and the DNA purification kits were from Qiagen, and the silver nitrate silica gel thin layer plates were from Analtech. All the other reagents were of the highest available quality. Oligonucleotide primers were synthesized by Integrated DNA Technologies and DNA sequencing was provided by ACGT, Inc.

4.2 | Bacterial strains, plasmids, and incubation

The bacterial strains and plasmids used in this study are listed in Table S1 and the primers used for this study are listed in Table S2. *E. coli* cells were incubated at 37°C in a Luria-Bertani medium (tryptone, 10 g/L; yeast extract, 5 g/L; NaCl, 10 g/L) whereas *E. faecalis* cells were cultured at 37°C in M17 medium (Sigma). In a few cases, we used AC medium (tryptone, 10 g/L; yeast extract, 10 g/L; glucose 1 g/L; K_2HPO_4 5 g/L) which contains low levels of fatty acids. Antibiotics were added in the following concentrations (in mg/L): kanamycin sulfate 30 for *E. coli*; erythromycin at 250 for *E. coli* and 10 for *E. faecalis*; chloramphenicol 30 for *E. coli* and 10 for *E. faecalis*; fatty acids were added at 0.1 mM unless otherwise stipulated.

4.3 | Construction of *E. faecalis* deletion strains

The *E. faecalis* deletion strain was constructed using the methods described before (Dong & Cronan, 2022; Zou et al., 2022) and the PCR

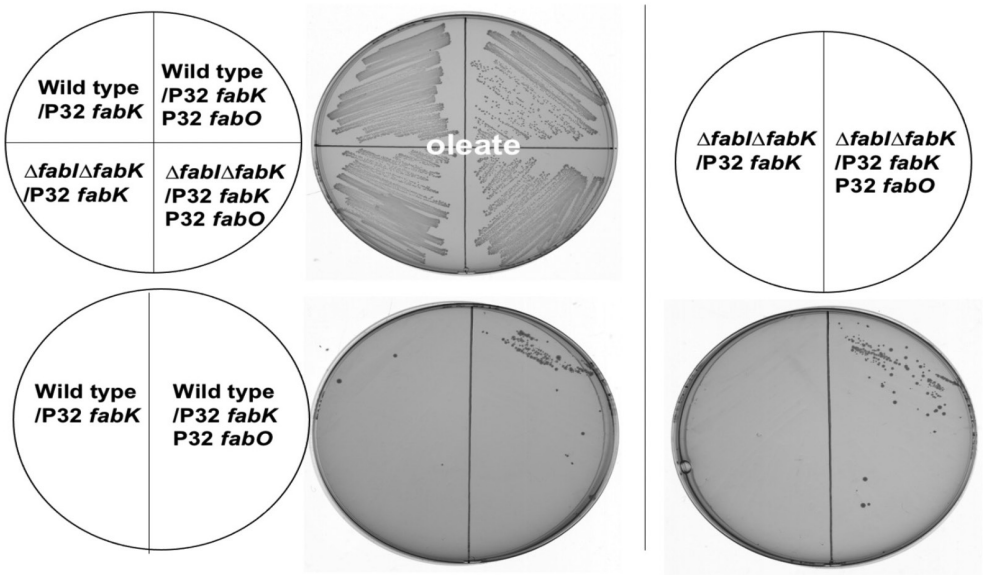

FIGURE 8 Effect of coexpression of FabO on FabK inhibition of growth. The left panel is the wild-type strain. The upper plate contained 100 μM oleate. The right panel is the *ΔfabI ΔfabK* strain.

primers in Table S2. A cassette for deletion of the genes was constructed by overlap PCR. Upstream and downstream ~500 bp DNA fragments were assembled by overlap PCR and then inserted into temperature-sensitive vector pBVGh using Gibson assembly. The plasmid was transformed into competent cells of *E. faecalis* FA2-2 by electroporation. One blue transformant colony was streaked on M17 or AC agar medium containing 10 μg/ml erythromycin and 0.1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and incubated overnight at 42°C to confirm the blue colony indicating chromosomal integration of the plasmid. One such single crossover strain was cultured in AC liquid medium with 5 mM oleic acid and 20% serum for 4 h at 30°C and then shifted to 42°C overnight. This step was repeated several times and the final culture was diluted and plated on AC agar containing X-Gal and 200 μM oleic acid and incubated for 24–48 h at 42°C. Genomic DNAs from white colonies, which represent double-crossover events, were extracted, and screened by PCR analysis to give the deletion strain.

The *fabI*, *fabK*, *fabN*, *fabF*, *and fabO* expression plasmids were constructed by inserting the *fab* genes together with a P32 promoter or agmatine-induced promoter into a shuttle plasmid vector either pBM02 or pTRK L2 through Gibson assembly. Transformation of *E. faecalis* deletion strains gave the complementation strains. The growth phenotype of the strains was detected through the M17 medium.

4.4 | Thin layer chromatography (TLC) analysis of radioactive labeled fatty acid methyl esters from membrane phospholipids

To test bacterial de novo fatty acid biosynthesis, *E. faecalis* strains were started at OD of 0.5 in an M17 medium and incubated at 37°C in the presence of 1 mCi/L sodium [1-^{14}C]acetate for about 3 h. For the auxotroph strains, wash them three times with M17 liquid medium before [1-^{14}C]acetate labeling. The cells were lysed in a methanol-chloroform (2:1) solution and the phospholipids were extracted in chloroform and then dried under nitrogen. The fatty acyl groups were methylated by 25% (w/v) sodium methoxide, extracted by hexanes, and processed for TLC analysis on Analtech silica gel containing 20% silver nitrate in toluene at −20°C. The plates (called Ag-TLC in figure legends) containing the [^{14}C] labeled fatty acid methyl esters were exposed on the phosphorimager GE Typhoon FLA700 Scanner and then analyzed by ImageQuant TL software.

Monounsaturated methyl esters separate from saturated esters because the double bonds interact with the silver nitrate in the plate (π–π interactions). Under the right conditions (toluene as solvent run at −20°C), monounsaturated methyl esters separate according to the position of the double bond. Hence, Δ9 C16 runs slower than Δ11 C18.

4.5 | Gas chromatography–mass spectrometry analysis of the fatty acids of cell membrane phospholipids

E. faecalis strains were inoculated at a logarithmic phase in the M17 medium. The cells were lysed by methanol-chloroform (2:1) solution and the phospholipids were extracted into chloroform and dried under a nitrogen stream. The fatty acyl groups were methylated by transesterification using 25% (w/v) sodium methoxide in methanol, extracted with hexanes, and sent to the Carver Metabolomics Center of the University for gas chromatography–mass spectrometry analysis. The same procedure was used in the presence of exogenous oleic acid since sodium methoxide in methanol does not methylate free fatty acids.

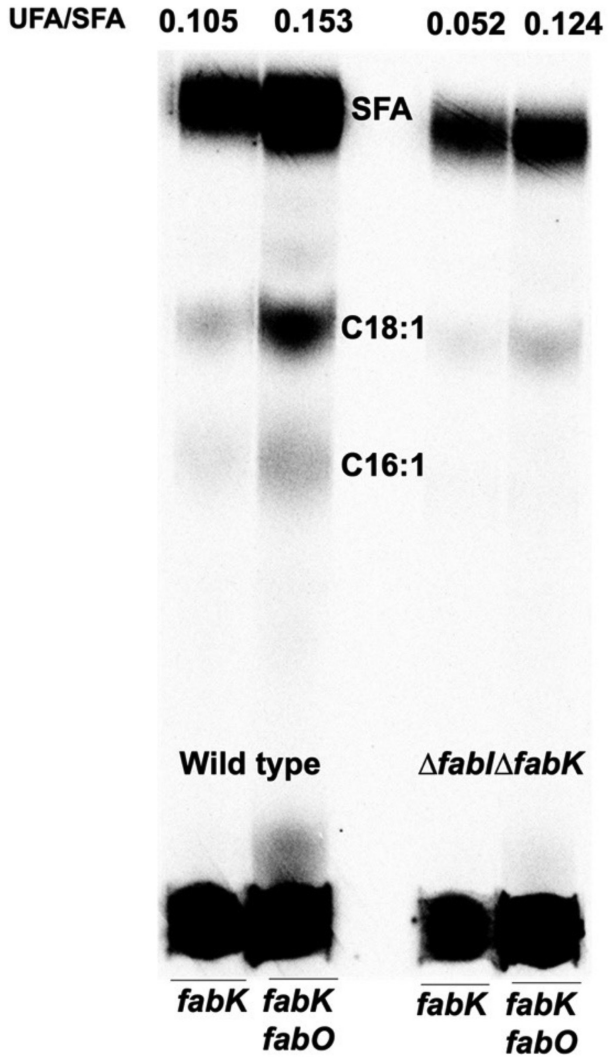

FIGURE 9 Labeling with [^{14}C]acetate of the wild-type and *ΔfabI ΔfabK* strain carrying isogenic plasmids encoding FabK or FabK plus FabO. The figure is an autoradiograph of an Ag-TLC plate.

4.6 | Expression and purification of His_6-tagged FabN, FabO, and FabF proteins

The *E. faecalis fabN*, *fabO*, and *fabF* genes were amplified from the genomic DNA of strain FA2-2 with primers (Table S2) containing designed restriction sites. The PCR fragments were digested and ligated to pET28b digested with the same enzymes to yield expression plasmids (Table 1). Vector pET28b carrying *fabO* was transformed into the BL21(DE3) and *fabN* and *fabF* were transformed into the BL21(Tuner). The transformant of *fabO* was incubated in LB medium at 37°C with 30 μg/ml kanamycin to OD_{600} of 0.6 and then was induced by 1 mM IPTG for another 4 h incubation. The transformants of *fabN* and *fabF* were incubated in LB medium at 18°C with 30 μg/ml kanamycin to OD_{600} of 0.6 and then were induced by 0.2 mM IPTG for overnight incubation. The cells were harvested and lysed in lysis buffer (20 mM Tris–HCl [pH 7.5] 500 mM NaCl and 10 mM imidazole). The supernatant was loaded onto the Ni-NTA column. The column was eluted with a wash buffer (20 mM Tris–HCl [pH 7.5] 500 mM NaCl and 40 mM imidazole) and then the tagged proteins were eluted with the same buffer containing 500 mM imidazole. The eluted proteins were dialyzed against 20 mM Tris–HCl (pH 7.5) 500 mM NaCl. The purified proteins were monitored by SDS-PAGE. The *E. coli* FabD, FabG, FabA, *Vibrio harveyi* AasS, and *E. coli* holo-ACP proteins were purified as described previously (Bi et al., 2013). The solution structures of FabN, FabO, and FabF were analyzed by size exclusion chromatography on a Superdex 200 10/300 GL column (GE Healthcare) using an AKTA Purifier10 at 0.3 ml/min in phosphate running buffer (20 mM Tris–HCl (pH 7.5) 500 mM NaCl). Finally, glycerol is added to the protein after dialysis of 15% and stored at −80°C.

4.7 | Assay of *E. faecalis* FabN, FabO, and FabF proteins activities in vitro

Fatty acid synthesis was reconstituted in vitro to assay FabN, FabO, and FabF activity using the purified enzymes that catalyze the fatty acid biosynthesis essentially. 3-hydroxydecanoyl-ACP was synthesized in a preincubation reaction using *Vibrio harveyi* AasS (Jiang et al., 2010). Briefly, the reaction mixtures contained 20 μM ACP, 10 mM ATP, 10 mM $MgSO_4$, 5 mM dithiothreitol, 0.1 M Tris–HCl (pH 8.0), 100 μM 3-hydroxydecanoic acid, and AasS (0.2 μg) in a final volume of 20 μl were incubated at 37°C for 1 h. To assay *E. faecalis* FabN, the following incubation of 1 μg each of *E. coli* FabD, FabG, and FabB, 100 μM NADPH, 100 μM malonyl-CoA, and 1 μg of *E. coli* FabA or 1 μg of *E. faecalis* FabN was added. To assay *E. faecalis* FabO, the following incubation of 1 μg each of *E. coli* FabD, FabG, and FabA, 100 μM NADPH, 100 μM malonyl-CoA, and 1 μg of *E. coli* FabB or 1 μg of *E. faecalis* FabO was added. To assay *E. faecalis* FabF, the following incubation of 1 μg each of *E. coli* FabD, FabG, and FabA, 100 μM NADPH, 100 μM malonyl-CoA, and 1 μg of *E. faecalis* FabO or 1 μg of *E. faecalis* FabF was added. The resulting mixture was incubated for an additional 20 min and the reaction products were analyzed by conformationally sensitive gel electrophoresis on 17.5% polyacrylamide gels containing 2.5 M urea. The gel was stained with Coomassie Brilliant Blue R250.

AUTHOR CONTRIBUTIONS

Huijuan Dong: Conceptualization; investigation; methodology; writing – original draft. **John E. Cronan:** Conceptualization; data curation; funding acquisition; investigation; methodology; supervision; writing – review and editing.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

ETHICS STATEMENT

This research did not use human subjects or animals. The recombinant DNA work was done according to the NIH Guidelines for Research Involving Recombinant or Synthetic Nucleic Acid Molecules.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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