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## The Fatty Acid Synthesis Protein Enoyl-ACP Reductase II (FabK) is a Target for Narrow-Spectrum Antibacterials for *Clostridium difficile* Infection

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### Abstract

*Clostridium difficile* infection (CDI) is an antibiotic-induced microbiota shift disease of the large bowel. While there is a need for narrow-spectrum CDI antibiotics, it is unclear which cellular proteins are appropriate drug targets to specifically inhibit *C. difficile*. We evaluated the enoyl-acyl carrier protein reductase II (FabK) that catalyzes the final step of bacterial fatty acid biosynthesis. Bioinformatics showed *C. difficile* uses FabK as its sole enoyl-ACP reductase, unlike several major microbiota species. The essentiality of *fabK* for *C. difficile* growth was confirmed by failure to delete this gene using Clostron mutagenesis and by growth inhibition upon gene silencing with CRISPR-interference antisense to *fabK* transcription or by blocking protein translation. Inhibition of *C. difficile*'s FASII pathway could not be circumvented by supply of exogenous fatty acids, either during *fabK*'s gene silencing or upon inhibition of the enzyme with a phenylimidazole-derived inhibitor **1**. The inability for fatty acids to bypass FASII inhibition is likely due to the function of transcriptional repressor FapR. Inhibition of FabK also inhibited spore formation, reflecting the enzyme's role in *de novo* fatty acid biosynthesis for the formation of spore membrane lipids. Compound **1** did not inhibit growth of key microbiota species. These findings suggest that *C. difficile* FabK is a druggable target for discovering narrow-spectrum anti-*C. difficile* drugs to treat CDI but avoid collateral damage to the gut microbiota.

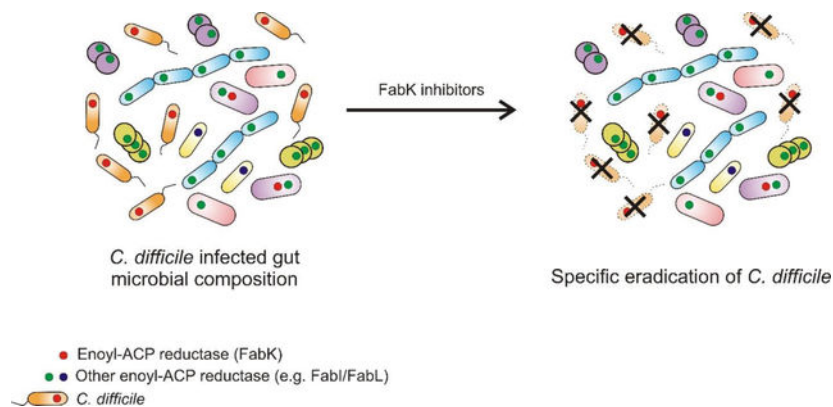
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#### SUPPORTING INFORMATION

Fatty acid biosynthesis in *Bifidobacterium* species; Sequences and general strategy of constructing anti-sense expression vectors; General chemistry procedure and synthesis of compound **1**; <sup>1</sup>H and <sup>13</sup>C NMR, HRMS and HPLC chromatogram of compound **1**; Table S1: Enoyl-ACP isoenzymes among gut microflora; Table S2: Strains and plasmids used for molecular studies; Table S3: Primers used in this study; Figure S1: Impact of antisense *fabK* RNA on *C. difficile* CD630 gene expression; References.

## Graphical Abstract



## Keywords

*Clostridium difficile*; narrow-spectrum drug target; enoyl-ACP reductase; CRISPR-interference antisense; phenylimidazole-derived inhibitor

*Clostridium difficile* is a Gram-positive, spore forming anaerobe that is the leading cause of antibiotic associated diarrhea in developed countries. In the United States, in 2011, there were an estimated ~29,300 deaths from ~453,000 cases of *C. difficile* infections (CDIs),<sup>1</sup> partly due to the emergence and spread of epidemic strains.<sup>2</sup> Broad-spectrum antibiotics are the main risk factor for CDI, as they eradicate protective gastrointestinal microbiota species enabling *C. difficile* to colonize the large bowel.<sup>3</sup> Following colonization, *C. difficile* produces toxins A and B that cause intestinal inflammation and diarrhea.<sup>3</sup> Paradoxically, for >30 years the main treatments for CDI have been the antibiotics metronidazole and vancomycin, which are not narrow-spectrum and further disrupt the microbiota during therapy.<sup>4</sup> This is thought to be a main contributing factor for why >20% of patients experience recurrent CDI after therapy.<sup>5</sup> The recently approved antibiotic fidaxomicin is narrow-spectrum, but higher costs have limited its clinical use and resistance was reported during clinical trials.<sup>6-7</sup> Besides antibiotics, other treatments in clinical use or under development include: fecal microbiota transplantation (FMT), inhibition of toxin activity by small molecules and antibodies (e.g. Bezlotoxumab) or inhibitors of germination and sporulation.<sup>8</sup> The discontinued development of surotomycin after mixed Phase 3 study findings<sup>9-10</sup> emphasize the need to maintain the CDI drug discovery pipeline with antibiotics, which are the standard-of-care for CDI. However, the discovery of narrow-spectrum antibiotics for CDI is largely serendipitous, because protein targets that are specific to *C. difficile* are either not experimentally confirmed or appear non-essential in genetic studies.<sup>11-12</sup> Thus, there is a need to identify and validate narrow-spectrum targets to discover *C. difficile* selective antibacterials that could avoid killing critical gut microbiota species. For this purpose, we investigated the drug target enoyl-acyl carrier protein (enoyl-ACP) reductase FabK that is involved in *de novo* synthesis of fatty acids.

Bacterial type II fatty acid synthesis (FASII) is conducted by a series of individual enzymes, which is distinct from the mammalian FASI pathway that is carried out by a large multi-

functional protein.<sup>13</sup> This enzymatic difference has made the FASII pathway a valid target for discovering selective antibacterials. However, targeting FASII enzymes for antibiotic discovery in Gram-positive pathogens was previously questioned,<sup>14</sup> as *Streptococcus agalactiae* was shown to use host lipids to bypass inhibition of its FASII pathway. More recent research clearly shows that not all bacteria readily use fatty acids to circumvent FASII inhibition, as this appears to be dictated by the type of genetic regulatory mechanism or whether unique lipids are required that cannot be obtained from the host.<sup>13, 15–17</sup> For example, in *Staphylococcus aureus* and *Bacillus subtilis*, expression of FASII genes is controlled by the transcriptional repressor FapR, which is released from DNA binding by malonyl-CoA, the rate limiting substrate for fatty acid synthesis.<sup>13, 17</sup> In the presence of exogenous lipids, the levels of malonyl-CoA remain high, preventing FapR from stopping expression of FASII genes. The continued activity of the pathway exhausts the cellular pool of ACP, thereby inhibiting downstream reactions that also require free ACP to manufacture phospholipids.<sup>17</sup> This has led to one paradigm that FASII pathway is druggable in pathogens bearing FapR, as its inhibition is not readily circumvented by fatty acids.<sup>13</sup>

Various naturally occurring or synthetic molecules inhibit different enzymes in the FASII pathway, resulting in efficacy in murine infection models and in clinical trials in humans.<sup>13, 18–19</sup> The enoyl-ACP reductase FabI, a NADPH dependent enzyme, has been the principal target for FASII inhibitors. It catalyzes reduction of *trans*-2-enoyl-acyl carrier protein (*trans*-2-enoyl-ACP) to acyl-ACP, which is the final step in fatty acid synthesis. However, FabI is not unique in all bacterial species as there are at least three other enoyl-ACP reductases i.e. FabK, FabL and FabV (Table 1 and Table S1). For example, *S. pyogenes* and *S. pneumoniae* carry the isoenzyme FabK as their sole enoyl reductase,<sup>20</sup> while *Enterococcus faecalis* contains FabI along with FabK.<sup>21</sup> Unlike many critical gut microbiota species, *C. difficile* expresses a single isozyme of FabK, which is controlled by FapR. Herein, we show that *C. difficile* FabK is an attractive target to discover narrow-spectrum antibiotics, whose cellular action may not be compromised by exogenous fatty acids.

## RESULTS

### **FabK is the sole enoyl-ACP isoenzyme in *C. difficile*.**

Bioinformatic analysis was performed on genomes deposited in KEGG database/Genbank for different gut microorganisms. It revealed that *C. difficile* carried FabK as its sole enoyl-ACP reductase, in contrast to various important gut microbiota (Table 1 and Table S1). FabK was also the sole enoyl-ACP reductase in the related genus of *Peptostreptococcus* i.e. *C. difficile* is now classified in the *Peptostreptococcaceae* family.<sup>22</sup> In contrast, the Gram-negative *Bacteroides* that makes up a large percent of gut flora<sup>23</sup> contained the isoenzyme FabI, either with or without FabK. Similarly, *Lactobacillus* and *Enterobacteriaceae* species possessed FabI or both FabI and FabK isoenzymes. *Enterococcus faecalis* contained both FabI and FabK, while only FabI was found in *E. faecium*. Both the enoyl-ACP reductases in *E. faecalis*, as in *B. subtilis*, are able to compensate for each other.<sup>21, 24</sup> Interestingly, *Bifidobacterium* species do not appear to carry any of the known enoyl-ACP reductase isoenzymes; rather, they possess a large holoenzyme complex whose protein sequence was similar to yeast FAS complex (See Supporting Information). Taken together, the

heterologous carriage of enoyl-ACP enzymes among important gut flora, suggests that *C. difficile* FabK could be a narrow-spectrum target.

### Expression of *fabK* is essential for *C. difficile* survival.

We next confirmed that *fabK* is essential for *C. difficile* growth, using gene disruption techniques. Strains and plasmids used are listed in Supporting Information (Table S2). Firstly, we could not isolate colonies with a disrupted *fabK*, after three separate attempts of inactivating *fabK* by insertional mutagenesis with Clostron retargeted to *fabK*.<sup>25</sup> Therefore, growth inhibition was tested by two gene silencing strategies. Firstly, antisense nucleic acid fragments to *fabK* were cloned into the vector pMSPT under the control of the anhydrotetracycline (ATc) inducible Ptet promoter. The vector pMSPT was constructed from pRPF185<sup>26</sup> by including a flanking paired termini nucleotides (see Supporting Information), which is predicted to enhance gene silencing by stabilizing the antisense RNA (asRNA), as reported for *Escherichia coli*.<sup>27</sup> Induction of the asRNA to *fabK*'s mRNA inhibited growth of *C. difficile* CD630 (Figure 1A), in a dose-dependent manner at ATc concentrations 0.032  $\mu\text{g}/\text{mL}$ . In contrast, cells containing the control empty vector survived. Analysis by RT-qPCR revealed that induction of asRNA, by ATc at 0.016 and 0.128  $\mu\text{g}/\text{mL}$ , reduced the formation of cDNA from *fabK*'s mRNA by  $4.0 \pm 2.1$  and  $27.75 \pm 11.7$ -fold, respectively (Figure 1B). However, transcript levels were unaffected for the up-stream (*plsX*, phosphate acyltransferase) and down-stream (*fabD*, malonyl CoA-ACP transacylase) genes in the operon; and for monocistronic *fabZ* (3-hydroxyacyl ACP dehydratase) (Figure S1). This suggests that asRNA designed to bind to *fabK*'s 5' untranslated region inhibited translation initiation, by blocking the RBS, in a manner similar to natural antisense.<sup>28</sup>

To further confirm *fabK*'s essentiality we constructed a CRISPR interference vector, to silence gene transcription.<sup>29</sup> The vector consisted of a constitutively expressed catalytically dead Cas9, from *S. pyogenes*, which lack endonuclease activity.<sup>29</sup> The antisense cassette contained two antisense chimeras, each consisting of a target specific ~20-nucleotide base-pairing region to *fabK*, the Cas9-binding handle and the *S. pyogenes* terminator.<sup>29</sup> Both guided RNAs (sgRNAs) targeted two different regions within *fabK* and were expressed from *C. difficile*'s xylose inducible pXyl promoter.<sup>30</sup> Induction of sgRNAs to *fabK* inhibited growth of *C. difficile* CD630 (Figure 1C), in a dose dependent manner at xylose concentrations of 2–8% (w/v; g/L). Growth of CD630 containing the empty vector survived. From RT-qPCR analysis, exposure of cells to 1% (w/v; g/L) of xylose for 1 hour reduced transcription by  $8.96 \pm 4.4$ -fold (Figure 1D). While the mRNA levels for the upstream *plsX* were unchanged, the levels for down-stream *fabD* were reduced 6-fold (Figure S1). Transcription of *fabZ* was also unchanged (Figure S1). Overall, these results confirm the essentiality of *C. difficile fabK* for growth. It also demonstrates for the first time, the utility of dCas9 gene silencing for genetic manipulation of *C. difficile*.

### Exogenous supply of fatty acids cannot bypass *fabK*'s essentiality in *C. difficile*.

A deciding factor for FabK as potential drug target is whether exogenous fatty acids can or cannot circumvent its inhibition. To test this, we first established the concentrations at which various fatty acids were not inhibitory to *C. difficile* growth. The test panel included fatty acids common in diets and the intestinal tract, including short chain fatty acids acetic and

butyric acids.<sup>31</sup> As indicated in Table 2, fatty acids of varying saturation and chain lengths (from C4 to C20) had varying effects on growth at 32  $\mu\text{M}$  to >512  $\mu\text{M}$ . Growth was inhibited by dodecanoic acid, linolenic and arachidonic acid at 32  $\mu\text{M}$ ; linoleic acid and myristic acid at 64  $\mu\text{M}$ ; and oleic acid at 128  $\mu\text{M}$ . *C. difficile* grew in all other fatty acids at concentrations up to 512  $\mu\text{M}$ . Fatty acids were combined at sub-inhibitory concentrations to create a fatty acid mix. Growth inhibition by antisenses to *fabK* was then tested in the presence of sub-inhibitory concentrations of individual or mixed fatty acids. However, exogenous fatty acids could not rescue cell viability, during induction of asRNA to *fabK* (Figure 2).

### C. difficile FabK is druggable target.

We investigated the druggability of *C. difficile* FabK, in the presence and absence of fatty acids, using previously reported FabK inhibitors (i.e. phenylimidazole derivatives). The phenylimidazole related compounds are the best validated inhibitors of FabK and were being studied by Meiji Seika Kaisha Ltd for streptococcal infections.<sup>32–34</sup> Compounds from the phenylimidazole series were reported to: inhibit *S. pneumoniae* FabK, and not *E. coli* FabI; and to bind FabK's active site as shown by co-crystal structures with *Sp*FabK (PDB code 2Z6J) and mapping of resistance mutants.<sup>32–34</sup> As the protein sequences of *Sp*FabK (UniProt: Q9FBC5) and *Cd*FabK (UniProt: C9YKB7) are homologous (54% identity and 70% similarity), we first showed that a lead phenylimidazole analog, herein designated as compound **1** (Figure 3A), retained *in silico* binding interactions in a homology model of *Cd*FabK.<sup>33</sup> We therefore synthesized **1** and further evaluated its cellular action. Against *S. pyogenes* ATCC 19615, **1** had activities of <0.0625  $\mu\text{M}$  in broth, but it was less active against *C. difficile* CD630 (MICs >64  $\mu\text{M}$ ). Since *C. difficile* expresses efflux pumps,<sup>35</sup> **1** was retested in the presence of the pump inhibitor verapamil (100  $\mu\text{g}/\text{mL}$ ), which increased the activity of **1** to 16  $\mu\text{M}$  (Table 3). Addition of palmitic acid (100  $\mu\text{M}$ ) or the fatty acid mixture did not alter the activity of **1** against CD630 (MIC= 16  $\mu\text{M}$ ). Compound **1** showed activity against *C. difficile* R20291, a representative of the clinically dominant ribotype 027, even in the absence of verapamil; this suggests heterogeneity in efflux pump activity among *C. difficile*. The MICs of **1** against R20291 were 8 and 2  $\mu\text{M}$  in the absence and presence of palmitic acid or fatty acid mix, respectively. In contrast, 100  $\mu\text{M}$  of palmitic acid or 50  $\mu\text{M}$  of oleic acid ablated the activity of **1** against *S. pyogenes* (MIC= 1–4  $\mu\text{M}$  and 4–8  $\mu\text{M}$  or 16–64 and 64–128 fold decreases in activity with palmitic acid or oleic acid, respectively). To further examine the inability of fatty acids to bypass FASII inhibition in *C. difficile*, we tested cerulenin, an inhibitor of FabF (3-oxoacyl-ACP synthase). Cerulenin's MIC (16  $\mu\text{M}$ ) was not altered by palmitic acid, oleic acid or the fatty acid mix, unlike its activity against *S. pyogenes* (MIC= 16  $\mu\text{M}$ , which increased to 64  $\mu\text{M}$  upon addition of palmitic acid or oleic acid). Interestingly, **1** did not show antimicrobial activity against various species, including *Bacteroides* and *Lactobacillus* species, that carry different homologs of enoyl-ACP reductase isoenzymes (Table-3).

Cellular inhibition of *C. difficile* FabK by compound **1** was investigated by target overexpression. When *C. difficile* *fabK* was placed under the control of the Ptet promoter, its induction in *C. difficile* CD630 increased the MIC's of **1** in a dose dependent manner (Figure 3C). In the absence of ATc inducer, the MIC of **1** was 8 – 16  $\mu\text{M}$ , but increased to 64 – >128  $\mu\text{M}$  following addition of 0.064  $\mu\text{g}/\text{mL}$  ATc. In contrast, against the control strain

carrying the empty vector, the MIC of **1** was not significantly altered (i.e. MIC of 8 – 16  $\mu\text{M}$  and 4 – 8  $\mu\text{M}$ , with and without ATc, respectively). As further controls, overexpression of *fabK* did not affect the activities of metronidazole (MIC = 0.4  $\mu\text{M}$ ), vancomycin (MIC = 0.4  $\mu\text{M}$ ) and fidaxomicin (MIC <0.2  $\mu\text{M}$ ). This further confirms that target overexpression is an applicable strategy for testing the cellular activity of FASII inhibitors; which is also consistent with target overexpression being a mechanism for clinical resistance to the FabI inhibitor, triclosan.<sup>36</sup>

We confirmed that **1** inhibited the enoyl reductase activity of *CdFabK*, and did so in a dose dependent manner, with an  $\text{IC}_{50}$  of  $3.31 \pm 0.046 \mu\text{M}$  (Figure 3D). *CdFabK* was not inhibited by the FabI inhibitor triclosan ( $\text{IC}_{50} > 100 \mu\text{M}$ ). Under the same assay conditions, **1** was a significantly more potent against FabK from *S. pneumoniae* ( $\text{IC}_{50} = 67 \pm 0.042 \text{ nM}$ ; ~49.4 fold better  $\text{IC}_{50}$  compared to *CdFabK*), but was ~28-fold higher than previously reported observation.<sup>34</sup> Although, enzyme concentrations used in our assay (~50 nM for the monomer enzyme) was similar to that previously reported (2  $\mu\text{g}/\text{mL}$  or ~60 nM for the enzyme monomer),<sup>34</sup> the reason for this discrepancy is not immediately clear. The resulting Hill coefficients from the  $\text{IC}_{50}$  logistic curve fits (see Methods) for **1** against *CdFabK* and *SpFabK* were 0.901 (0.761 – 1.064, 95% CI) and 1.137 (0.923 – 1.409, 95% CI), respectively. These values are not significantly greater or lesser than unity (Hill slope of 1.000), suggesting that **1** displayed normal inhibitory behavior without binding cooperativity, aggregation, micellar formation or insolubility. It also suggests that while FabK is a functional dimer, its active sites function independently.<sup>33, 37</sup>

### Inhibition of FASII reduces spore production.

Sporulation by *C. difficile* is important for endogenous CDI recurrence and dissemination of this pathogen. Studies in *B. subtilis* show that *de novo* synthesis of fatty acids and membrane lipids is highly active during sporulation, to form the inner and outer membranes of spores.<sup>38–39</sup> To test if depletion of FASII mRNA affects spore production, we analyzed the rate of sporulation in the presence or absence of asRNA to *fabK*'s mRNA or with empty vector (pMSPT). Induction of asRNA with 0.016 and 0.128  $\mu\text{g}/\text{mL}$  of ATc significantly reduced spore formation by ~2- $\log_{10}$ , whereas spore formation was unaffected in cells containing the empty vector control (Figure 4A). The spore index (number of spores/total number of viable cells) for asRNA to *fabK* was comparable to cells expressing an asRNA to *spoIIID* (Figure 4A), encoding an essential regulator of mother-cell specific gene expression that ensures correct spore formation. We next tested cerulenin at (1 $\times$  and 4 $\times$  MIC), showing it reduced spore numbers by ~2 logs (Figure 4B), comparable to the positive control acridine orange (113  $\mu\text{M}$ ). In contrast, vancomycin (0.2 and 1  $\mu\text{M}$ ) was inactive, as it does not primarily inhibit sporulation.<sup>40–41</sup>

## DISCUSSION

While there is an important need for narrow-spectrum antibacterials to treat CDI, it remains uncertain how to design inhibitors specifically targeting *C. difficile*, to reduce collateral damage to important gut microbes. We approached identifying a narrow-spectrum drug target for *C. difficile* by assessing known macromolecular synthetic pathways that contain

proven antibacterial drug targets. In this regard, FabK was identified as the sole enoyl-ACP reductase in *C. difficile* strains. Enoyl-ACP reductase displays extensive sequence and structural diversity among bacteria, with there being four different enoyl-ACP isoenzymes, FabI, FabL, FabV and FabK.<sup>42</sup> Out of the four isoenzymes FabI, FabL and FabV are members of the short chain dehydrogenase reductase (SDR) superfamily, and are related by structure and mechanism; whereas FabK is a TIM barrel flavin containing protein with no structural similarity to the members of SDR superfamily.<sup>42</sup> While this precludes the discovery of broad-spectrum inhibitors of enoyl-ACP reductases, it is suitable for identifying narrow-spectrum antibacterials. Indeed, phenylimidazole derivatives, such as compound **1**, were reported to specifically inhibit FabK with no inhibition of FabI.<sup>34</sup> This suggests that structural differences of FabK to SDR enzymes could be exploited to derive selective inhibitors for *C. difficile*. Challenging this concept is that gut flora such as *Bacteroides* species carry an SDR enzyme, alone or in combination with FabK. Previously, it was reported that in species carrying two enoyl-ACP reductases, inactivation of one isoenzyme is not sufficient to shunt the FASII pathway.<sup>24, 43–44</sup> Although, compound **1** showed anti-*C. difficile* activity and did not inhibit representative gut species, studies will be required to determine if this is due to: an inability to inhibit both isoenzymes in gut species; or whether poor penetration across the outer membrane prevented cellular activity against Gram-negative *Bacteroides*. Indeed, poor penetration into Gram-negatives by lipophilic compounds, such as compound **1**, may also contribute to species selectivity for future FabK inhibitors. Nonetheless, the lower activity of **1** against *Cd*FabK, compared to *Sp*FabK, suggests that co-crystallization and biophysical analysis of its molecular interactions will be needed to guide optimization of phenylimidazole analogs for *C. difficile*.

Our attempts to delete *CdfabK* using Clostron were unsuccessful, while the same technique successfully deleted the non-essential gene for D-proline racemase,<sup>11</sup> highlighting *fabK*'s essentiality. Consequently, growth was inhibited by gene silencing of *fabK* and by compound **1**. These results support findings from *Himar1* mutagenesis of *C. difficile*, where eleven fatty acid biosynthesis genes, including *fabK*, could not be deleted to produce viable colonies.<sup>45</sup> Importantly, sporulation was reduced upon genetic inhibition of *fabK* and chemical inhibition of the FASII pathway by cerulenin. This might suggest that FASII inhibition could reduce episodes of recurrence that arise from endogenous spores that survive in the colon; it is estimated that half of the recurrent CDI cases occur from endogenous spores, while others are due to reinfection with spores from the environment.<sup>46</sup>

Another factor favoring FabK as a drug target is its FASII pathway appears to be controlled by the FapR regulator, explaining why exogenous fatty acids could not prevent inhibition by either compound **1** or antisenses to *fabK*. Conversely, *S. pyogenes* that bears the FabT repressor, bypassed inhibition with compound **1**, when supplied with exogenous fatty acids. Nonetheless, it is unlikely that *C. difficile* is immune to developing genetic resistance to FabK inhibitors, either by mutations that decrease binding to the target site or allow cells to use of exogenous fatty acids without depleting ACP. This was shown in *S. aureus*, where mutations to the initiation enzyme FabD decreased its catalytic activity, thereby increasing available free ACP for the synthesis of phospholipids from host fatty acids.<sup>47</sup> For FabK

inhibitors with future clinical potential, it will be required to determine whether high concentrations of inhibitors in the intestines may prevent the emergence of resistance.

## CONCLUSIONS

We provided the first experimental evidence that *CdFabK* is a potential drug target, showing that its cellular inhibition may not be readily bypassed by fatty acids, including those from dietary and host origins. This may be due to the function of the FapR regulator in *C. difficile*, which we speculate that like *S. aureus*, plays a role in preventing bypass of FASII inhibition by exogenous fatty acids. The low homology of *CdFabK* to other enoyl-ACP reductases may further permit the rational design of species specific inhibitors, afforded by compound **1** as a chemical starting point. However, as FabK moves forward in a structure-based drug discovery process, it will be critical to optimize for molecules that are more potent, soluble, non-absorbed from the intestinal tract and not protein bound to fully leverage inhibition of *C. difficile* vegetative cells and sporulation.<sup>41, 48</sup> We anticipate that such *C. difficile* specific FabK inhibitors will not only inactivate this pathogen, but also allow the resident microbiota to repopulate the large bowel to prevent recurrent CDI.

## MATERIALS AND METHODS

### Strains, growth conditions and chemicals.

*C. difficile* was grown in Brain Heart Infusion (BHI) agar or broth, supplemented with 15  $\mu\text{g/mL}$  thiamphenicol when appropriate. Cultures were grown at 37°C in an anaerobic chamber (Don Whitley A35 anaerobic chamber). Strains and plasmids used in this study are in Tables S2. *E. coli* was grown in Luria-Bertani (LB) medium, supplemented with required antibiotics when appropriate (ampicillin 100  $\mu\text{g/mL}$ , kanamycin 50  $\mu\text{g/mL}$  or chloramphenicol 35  $\mu\text{g/mL}$ ). *S. pyogenes* was grown in BHI medium supplemented with 10 g/L tryptone and 5 g/L yeast extract (BHITY) at 37 °C in presence of 5% CO<sub>2</sub>. *S. aureus* was grown in Muller Hinton (MH) broth. Fatty acids were purchased from: Sigma (decanoic acid, dodecanoic acid, butyrate, octanoic acid, oleic acid and stearic acid), Merck Millipore (arachidonic acid), Acros organics (acetic acid, linoleic acid, myristic acid and palmitic acid) and Chem Impex Inc (linolenic acid).

### Construction of anti-sense and target overexpression vectors.

The vector pMSPT was used to express ATc inducible asRNAs to the mRNA of *fabK* or *spo0A*. Further details on the design of pMSPT is in the Supporting Information. Vector pMSPT was derived by cloning a paired termini region, synthesized by GenScript in pUC57, into *BamHI/SacI* sites of pRPF185. Antisense fragments were designed and synthesized to span 50-bases pairs upstream and downstream of the start codon, to mimic naturally occurring bacterial asRNAs that block translation by binding to the ribosome binding site. The antisense fragments were cloned into the *SphI/XhoI* sites of pMSPT. We constructed the first Crispr-interference vector for *C. difficile* and provide further details in the Supporting Information. Essentially, we first modified vector PMTL84151<sup>49</sup> by introducing *C. difficile* pXyl promoter<sup>30</sup> for xylose inducible gene expression, followed by cloning *dcas9* from *S.*



*pyogenes* under a xylose inducible promoter. The sgRNA to *fabK* was synthesized by GenScript and cloned into *SalI* restriction site of the constructed vector pXW<sub>xyI</sub>-dcas9.

Overexpression of *fabK* was achieved by cloning the gene into pRPF185. The plasmids and primers used in this study are in Table S2 and Table S3. The plasmid pRPF185 was digested with BamHI/SacI and a similarly digested PCR amplicon of *fabK* was ligated into the vector. The derived construct, termed pRPF185-*fabK*, contained the gene with its own ribosomal binding site, expressed from the P<sub>tet</sub> promoter.

### Insertional inactivation of *fabK*.

To genetically delete *fabK* in the CD630 derivative strain JIR8094, we applied Group IIA ClosTron mutagenesis. Essentially, the intron retargeting cassette was designed by Perutka algorithm at [www.clostron.com](http://www.clostron.com) and a 344 bp target region synthesized and cloned into pMTL007C-E2 by DNA2.0 Inc. The resulting plasmid pMTL007C-E2 *fabK* was conjugated into *C. difficile* JIR8094 and mutagenesis attempted as described.<sup>25</sup>

### Susceptibility tests and effects on growth.

Growth inhibition following induction of asRNA to *fabK* mRNA was performed by agar dilution method as described previously.<sup>50</sup> Essentially, serial dilutions of anhydrotetracycline (ATc) were added to molten BHI agar and spotted with *C. difficile* to a final inoculum of ~10<sup>5</sup> CFU/spot, using a Bench top pipettor (Sorenson BioScience Inc). Plates were incubated for 24 h, before recording the lowest concentration of ATc that inhibited growth. Susceptibility to test compounds was determined by microbroth dilution in 96-well microtiter plates under anaerobic growth condition as described.<sup>37</sup> For *S. pyogenes* and *S. aureus* aerobic growth conditions were maintained. The MIC was determined as the lowest compound concentration inhibiting visible growth at 24 h.

### Quantitative Reverse-Transcription PCR Analysis.

RT-qPCR experiments were done as described previously<sup>40</sup> with slight modifications. Briefly, *C. difficile* CD630 was grown in BHI broth to OD<sub>600</sub> ~0.2 (T<sub>0</sub>). Next, ATc was added to final concentrations of 0.016 or 128 µg/mL and cultures incubated for 1 h before adding two volumes of RNA protect (Qiagen) solution and harvesting by centrifugation. Total RNA was extracted from cell pellets using: 700 µL of Qiazol lysis reagent (Qiagen) followed by lysing cells in a Fastprep® cell disrupter for 5 min and then isolating RNA with RNeasy mini kit (Qiagen). From one µg of RNA, complementary DNA (cDNA) was synthesized using qScript cDNA supermix (Quanta biosciences). qRT-PCR was done using qScript SYBR Green RT-qPCR master mix (Quanta biosciences) in Applied Biosystems ViiA7 RT-PCR system. Results were calculated using the 2<sup>-Ct</sup> method<sup>51</sup>, with transcript levels normalized to 16S rRNA. The primers used are listed in Table S3.

### Synthesis of FabK inhibitor, phenylimidazole-derivative.

Compound **1** was synthesized by following reported procedures<sup>32, 34</sup> with modifications (Scheme S1, see Supporting Information).<sup>52-53</sup> The structure was characterized by <sup>1</sup>H and <sup>13</sup>C NMR, and HRMS. Purity was determined to be >99% by reverse phase C18-HPLC. General chemistry procedures, synthetic scheme including reagents and conditions, full

characterization data of compound **1** and its associated  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra, HRMS, and HPLC chromatogram, are provided in Supporting Information.

### Computational modelling of CdFabK.z

A composite homology model of the CdFabK enzyme was created using the published structures of the *S. pneumoniae* (pdb ID 2z6j) and *Porphyromonas gingivalis* (pdb ID 4IQL) FabK enzymes as templates.<sup>33, 37</sup> *P. gingivalis* loop positions were used for missing loop regions in the *S. pneumoniae* template. The Prime software package available in the Schrödinger molecular modeling suite (Schrödinger Release 2018-1: Prime, Schrödinger, LLC, New York, NY, USA) was used to build and refine the model.<sup>54</sup> The CdFabK sequence from strain 630 was used for the model sequence. Secondary structures were predicted, and sequences were aligned automatically using ClustalW (high sequence identity) with manual adjustment as necessary. The energy-based option was used for model building, wherein residues not matching the templates, including structural discontinuities, are constructed and refined using iterative energy calculations. The resulting CdFabK model is believed to be high-quality due to the high degree of homology to the template structures. Following generation of the CdFabK homology model, compound **1** was docked into the active site using the Glide docking program with XP (extra precision) settings.<sup>55-57</sup> The unbiased docking recapitulated the experimental binding pose of the phenylimidazole analog from the SpFabK structure.

### Protein expression and purification.

The *fabK* genes from *C. difficile* CD630 or *S. pneumoniae* R6 were cloned within the NdeI/BamHI sites of pET15b. The *CdfabK* construct was codon optimized, synthesized, and cloned by GenScript (Piscataway, NJ), whereas *SpfabK* was obtained by PCR using primers in Table-S3. Proteins were overexpressed in *E. coli* BL21-Gold (DE3) cultivated at 37°C in Terrific Broth with 100 µg/mL ampicillin by transferring 1% (v/v) overnight culture into fresh medium. Cells were grown until OD<sub>600</sub> of ~ 0.6 and protein expression induced with 0.1 mM IPTG. To enhance the expression, 0.5 mM flavin mononucleotide (FMN) was added along with IPTG. After 18 h at 18°C, cells were harvested by centrifugation at 18000 × g for 15 min and the pellet resuspended in buffer A (20 mM Tris buffer [pH 7.4] with 300 mM NH<sub>4</sub>Cl). Cell suspensions were supplemented with 1 mM DTT, 5% glycerol and 10 mM imidazole, 0.5 mg/mL lysozyme, 10 µg/mL DNaseI, 5 mM MgCl<sub>2</sub>, 0.5% Triton-X 100, 25 mM sucrose (for CdFabK stabilization), and EDTA-free Protease inhibitor. In case of CdFabK, the Tris in buffer A was replaced with 50 mM HEPES buffer (pH 8.0) with 10 µg/mL benzamidine. Cells were lysed by pulsed sonication with 50% amplitude for 8 min, lysates centrifuged at 39000 × g for 15 min and the supernatant was passed through 0.22 µm filters. Proteins were purified by affinity chromatography on HisTrap HP column (GE Life Sciences) and eluted with 500 mM imidazole. Eluted proteins were further purified by gel filtration on Superdex 200 PG (GE Life Sciences) using respective buffer A with 18% glycerol and 3 mM DTT. After purification, concentrated proteins were stored at -80°C in 35% glycerol.

### IC<sub>50</sub> determination.

Enzyme reactions were carried out in an assay buffer consisting of 100 mM HEPES pH 8.0, 500 mM NH<sub>4</sub>Cl, 10% glycerol, and 0.125 mg/mL  $\gamma$ -Globulins; 10% DMSO with 150  $\mu$ M crotonyl-CoA (substrate) and 150  $\mu$ M NADH (cofactor). Compounds were incubated with 50 nM of enzyme in three-fold dilutions ranging from 100 to 0.005  $\mu$ M and 100 to 0.0017  $\mu$ M for *CdFabK* and *SpFabK*, respectively. After 10 min, the reaction was started by adding substrate and cofactor. Reaction kinetics were monitored for 10 min by measuring NADH fluorescence at 340/460 nm. Experiments were carried out in duplicates for each enzyme. IC<sub>50</sub> values were calculated using the four-parameter logistic (Hill) curve analysis in GraphPad Prism 7; using the equation  $y = \text{bottom} + (\text{top} - \text{bottom}) / (1 + 10^{-(\text{LogIC}_{50} - x) * \text{HillSlope}})$ , where x is the logarithm of concentration and y is the response.

### Sporulation assay.

This assay was performed as described previously<sup>41</sup> to assess the effect of inhibiting FASII on sporulation. Briefly, *C. difficile* CD630 harboring respective plasmids were grown overnight on BHI agar plate with 0.1% (w/v) sodium taurocholate and 15  $\mu$ g/mL of thiamphenicol. Colonies were inoculated in fresh BHI broth and grown to OD<sub>600</sub>  $\approx$  0.3, before adding ATc at concentrations (0.016 and 0.128  $\mu$ g/mL) that did not inhibit growth in broth. Total viable count and heat-resistant spores were analyzed over 5 days.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## ACKNOWLEDGMENTS

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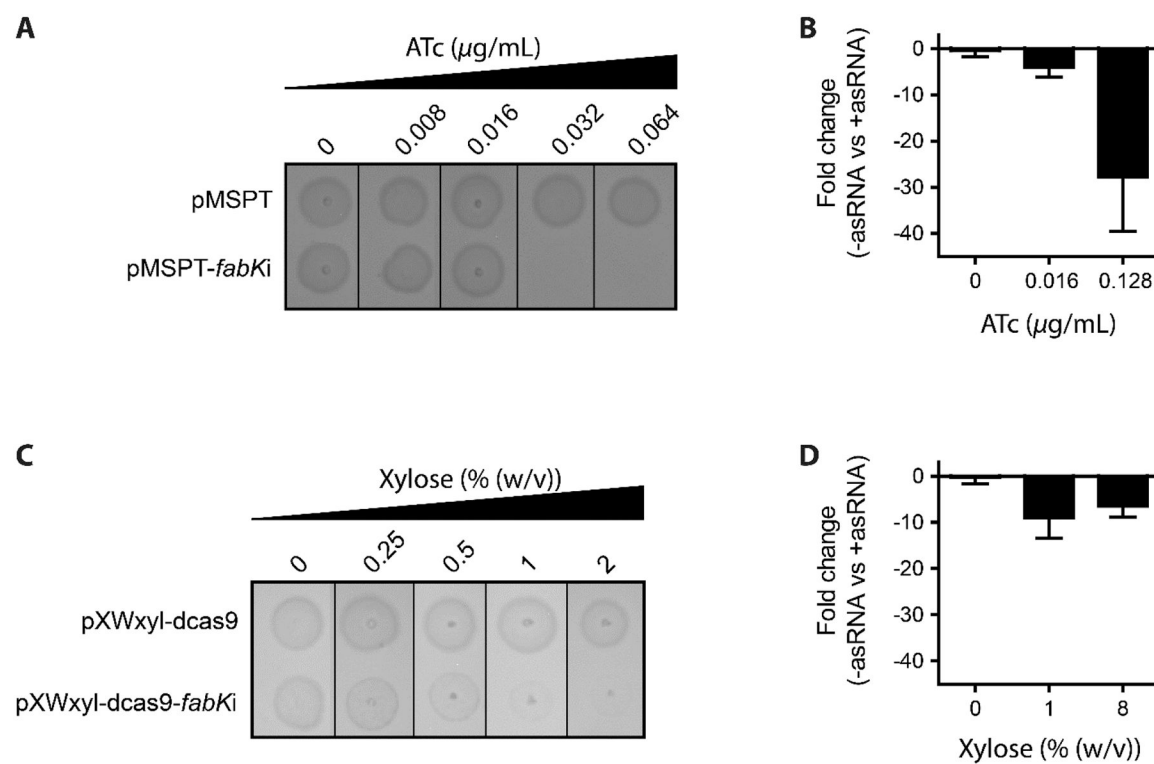
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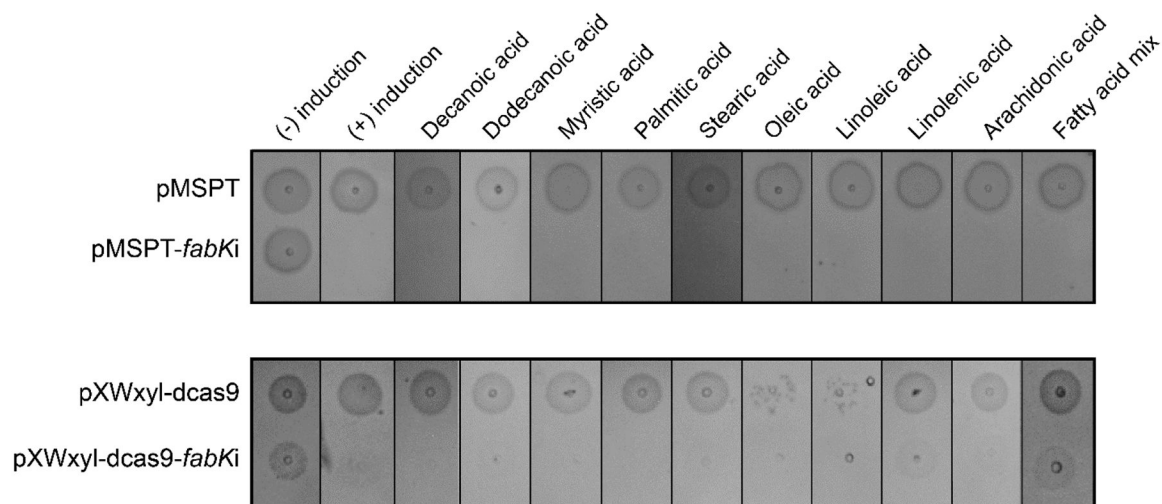
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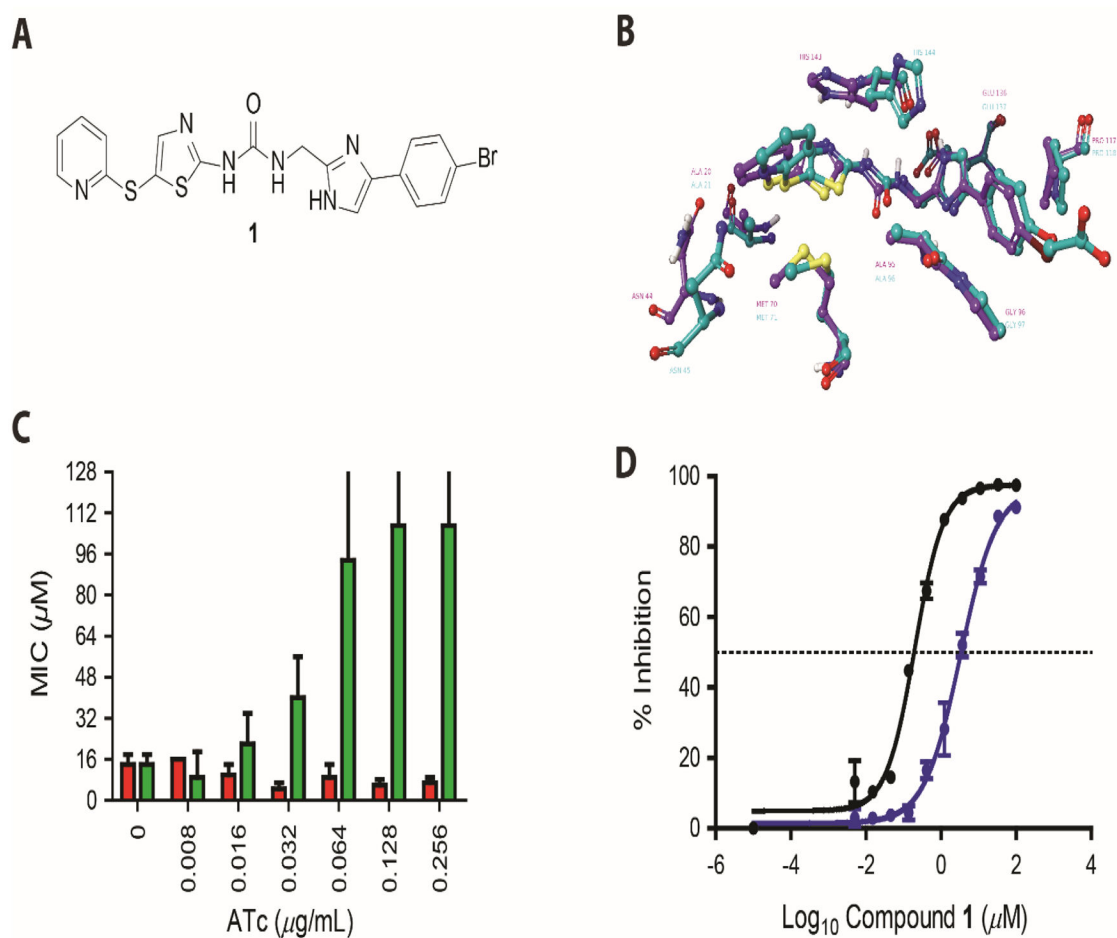
**Figure-1: Impact of antisense *fabK* RNA on *C. difficile* CD630 growth and gene expression. (Panel A & C)**

*C. difficile* CD630 strains carrying empty plasmid or plasmids encoding antisense *fabK* RNA were analyzed on BHI agar. From overnight cultures 3  $\mu\text{l}$  was spotted on BHI agar plates containing ATc (A) and xylose (C). (Panel B & D) The mRNA levels for *fabK* was analyzed by RT-qPCR. Cultures were grown to  $\text{OD}_{600} \approx 0.3$  and antisense RNA expression induced with indicated concentrations of ATc (panel B) or xylose (panel D). Cells were harvested after 1 h of induction and the fold change calculated for differences of mRNA between strains containing the empty vector versus those with antisense cassettes.



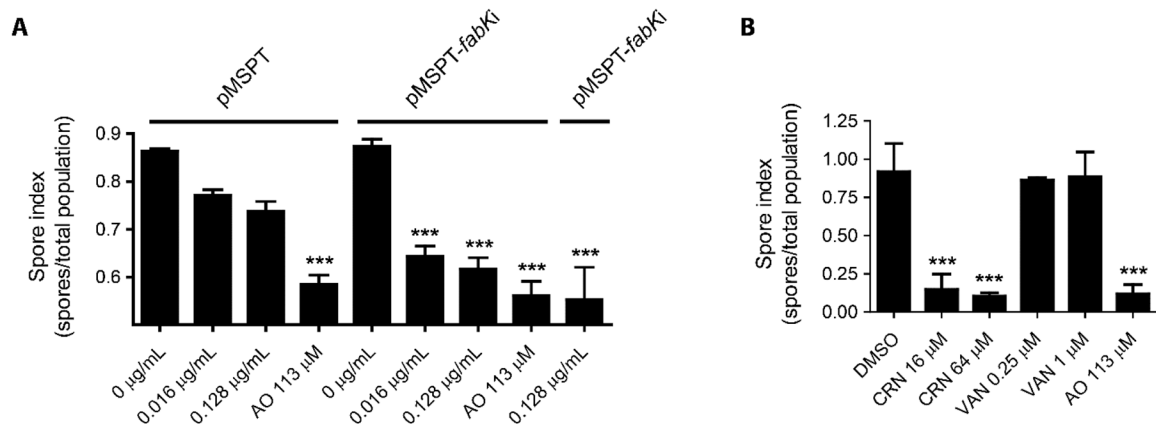


**Figure-2: Effect on cell growth in presence of antisense *fabK* RNA and exogenous fatty acids.** Overnight cultures of *C. difficile* CD630 harbouring respective plasmids were analyzed on BHI agar plates with respective concentrations of fatty acids. Antisense RNA expression was induced by 0.256  $\mu\text{g}/\text{mL}$  ATc (top panel) or 1% (w/v) xylose (bottom panel). Cell viability was tested in presence of individual fatty acids i.e. decanoic acid (256  $\mu\text{M}$ ), dodecanoic acid (8  $\mu\text{M}$ ), myristic acid (16  $\mu\text{M}$ ), palmitic acid (256  $\mu\text{M}$ ), stearic acid (256  $\mu\text{M}$ ), oleic acid (32  $\mu\text{M}$ ), linoleic acid (16  $\mu\text{M}$ ), linolenic acid (8  $\mu\text{M}$ ) and arachidonic acid (8  $\mu\text{M}$ ). The fatty acid mixture was used at sub-inhibitory concentrations of individual fatty acids; in some cases partial growth recovery was observed with the xylose inducible Crispr-interference; with addition of 1% (w/v) xylose, cells were more sensitive to oleic acid and linoleic acid.



**Figure-3: Characterization of compound 1 activity against CdFabK.**

(A) Chemical structure of phenylimidazole derivative **1**. (B) Homology modeling of **1** in active site of FabK. The comparative model of the *C. difficile* FabK (green) is shown aligned with the experimental model of *S. pneumoniae* FabK. Compound **1** is shown docked into the *C. difficile* FabK active site, closely recapitulating the experimental structure of the bound compound, which is an analog **1**. Active site residues are visible within 3 angstroms of the inhibitor, showing 100% identity between the binding sites of the two enzymes. (C) Minimum inhibitory concentrations (MICs) for **1** against *C. difficile* CD630 overexpressing FabK. MIC's for *C. difficile* CD630 harboring respective plasmids were analyzed in BHI broth with respective concentration of compounds. The MIC's against empty vector control pRPF185 (red bars) and FabK overexpressing pRPF185-*fabK* (green bars) are shown for 4 biological replicates. The data included MIC's that were >128 µM, as reflected by error bars beyond 128. (D) Sigmoidal plot showing inhibition of CdFabK (purple line) and SpFabK (black line) to NADH by **1**. The assay was performed with fixed concentration of cofactor and substrate but three-fold dilution of the **1** ranging from 100 to 0.005 and 33 to 0.0017 µM respectively for CdFabK and SpFabK. NADH fluorescence was measured at 340/460 nm and the IC<sub>50</sub> values were determined through Hill curve analysis in Graph pad prism 7.



**Figure-4: Effect of antisense *fabK* on sporulation.**

(Panel A, B & C) Sporulation was analyzed over 5 days as the total number of spores/total population (spore forming and vegetative cells). (A) Colonies obtained from BHI agar plate with 0.1% (w/v) taurocholate were inoculated into BHI medium and grown until  $\text{OD}_{600} \approx 0.3$ , at which antisense RNA expression was induced by addition of 0, 0.016 or 0.128  $\mu\text{g/mL}$  of ATc or 113  $\mu\text{M}$  acridine orange (AO). (B) Comparison of inhibition of sporulation by cerulenin (CRN; MIC=16  $\mu\text{M}$ ) and vancomycin (VAN; MIC=0.25  $\mu\text{M}$ ). DMSO and AO were negative and positive controls.

**Table-1:**

Distribution of enoyl-ACP isoenzymes among gut microflora.

Genus/Organism <sup>a</sup>	FabI	FabK	Repressor
<i>Bacteriodes</i>	+	±	FabT
<i>Bifidobacterium</i> <sup>b</sup>			Not Found
<i>C. difficile</i>		+	FapR
<i>Enterobacterium</i>	+	±	FapR
<i>E. faecalis</i>	+	+	FabT
<i>E. faecium</i>	+		FabT
<i>Faecalibacterium</i>		+	FabT
<i>Lactobacillus</i>	+	±	FabT
<i>Peptostreptococcus</i>		+	FabT
<i>Propionibacterium</i>	+		FabT
<i>Staphylococcus</i>	+		FapR
<i>Streptococcus</i> <sup>c</sup>		+	FabT

<sup>a</sup>Not shown: FabV is primarily found in *Vibrio* species, FabL in *Bacillus* species.

<sup>b</sup>Yeast-like Fatty Acid synthase

<sup>c</sup>Known to use endogenous fatty acids to bypass FASII

**Table-2:**Inhibition concentrations of *C. difficile* CD630 by different fatty acids

Fatty acid	Abbreviation	MIC ( $\mu$ M)
Acetic acid	C2:0	>512
Butyric acid	C4:0	>512
Octanoic acid	C8:0	>512
Decanoic acid	C10:0	>512
Dodecanoic acid	C12:0	32
Myristic acid	C14:0	64
Palmitic acid	C16:0	>512
Stearic acid	C18:0	>512
Oleic acid	C18:1 9	128
Linoleic acid	C18:2 9,12	64
Linolenic acid	C18:3 9,12,15	32
Arachidonic acid	C20:4 5,10,11,14	32

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**Table-3:**

Minimum inhibitory concentrations (MICs)<sup>a</sup> for compound **1** against various bacteria

Organism	Enoyl-ACP reductase	MIC ( $\mu$ M) - Palmitic acid + Palmitic acid	
<i>C. difficile</i> CD630	FabK	16	16
<i>C. difficile</i> R20291	FabK	8	2
<i>S. pyogenes</i> ATCC 19615	FabK	<0.0625	4
<i>C. perfringens</i> HM310	FabV	>64	>64
<i>B. ovatus</i> HM222	FabI & FabK	>64	>64
<i>L. reuteri</i> LTH 5448	FabI	>64	>64
<i>S. aureus</i> Newman	FabI	>64	>64

<sup>a</sup>Vancomycin's MIC against test strains ranged from 0.25–1  $\mu$ M. With exception of *S. pyogenes* MICs were done with addition of verapamil (100  $\mu$ g/mL).

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