Suppression of *fabB* Mutation by *fabF1* Is Mediated by Transcription Read-through in *Shewanella oneidensis*

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ABSTRACT

As type II fatty acid synthesis is essential for the growth of *Escherichia coli*, its many components are regarded as potential targets for novel antibacterial drugs. Among them, β -ketoacyl-acyl carrier protein (ACP) synthase (KAS) FabB is the exclusive factor for elongation of the *cis*-3-decenoyl-ACP (*cis*-3-C₁₀-ACP). In our previous study, we presented evidence to suggest that this may not be the case in *Shewanella oneidensis*, an emerging model gammaproteobacterium renowned for its respiratory versatility. Here, we identified FabF1, another KAS, as a functional replacement for FabB in *S. oneidensis*. In *fabB*⁺ or *desA*⁺ (encoding a desaturase) cells, which are capable of making unsaturated fatty acids (UFA), FabF1 is barely produced. However, UFA auxotroph mutants devoid of both *fabB* and *desA* genes can be spontaneously converted to suppressor strains, which no longer require exogenous UFAs for growth. Suppression is caused by a TGT TTT deletion in the region upstream of the *fabF1* gene, resulting in enhanced FabF1 production. We further demonstrated that the deletion leads to transcription read-through of the terminator for *acpP*, an acyl carrier protein gene immediately upstream of *fabF1*. There are multiple tandem repeats in the region covering the terminator, and the TGT TTT deletion, as well as others, compromises the terminator efficacy. In addition, FabF2 also shows an ability to complement the FabB loss, albeit substantially less effectively than FabF1.

IMPORTANCE

It has been firmly established that FabB for UFA synthesis via type II fatty acid synthesis in FabA-containing bacteria such as *E. coli* is essential. However, *S. oneidensis* appears to be an exception. In this bacterium, FabF1, when sufficiently expressed, is able to fully complement the FabB loss. Importantly, such a capability can be obtained by spontaneous mutations, which lead to transcription read-through. Therefore, our data, by identifying the functional overlap between FabB and FabFs, provide new insights into the current understanding of KAS and help reveal novel ways to block UFA synthesis for therapeutic purposes.

The *de novo* fatty acid synthesis (FAS) pathway, namely, type II, is the predominant, if not exclusive, route for endogenous production of fatty acids (1). The FAS pathway, the current knowledge of which derives mainly from the model organism *Escherichia coli*, is highly conserved in bacteria. Central to the pathway are reactions catalyzed by β -ketoacyl-acyl carrier protein (ACP) synthases (KAS), including FabH, FabB, and FabF. FabH is responsible for the condensation of an acyl coenzyme A (acyl-CoA) unit and a malonyl-acyl carrier protein (malonyl-ACP) unit, but cannot work with acetyl-ACP, the substrate of FabB and FabF; as a consequence, FabH could not function as a replacement for FabB or FabF (1). While FabB and FabF are exchangeable in elongation of saturated intermediates, each catalyzes a reaction with the unsaturated branch that the other cannot or at least much less effectively (2) (Fig. 1). The key reaction catalyzed by FabB is elongation of the *cis*-3-decenoyl-ACP (*cis*-3-C_{10:1}-ACP), which explains the essentiality of the enzyme to unsaturated fatty acid (UFA) biosynthesis (3). In contrast, FabF is not fully required although it is exclusively responsible for elongation of C_{16:1}-ACP (4, 5). Intriguingly, excess FabF but not FabB induces growth inhibition and viability loss by blocking fatty acid chain elongation (6).

Shewanella species, widely distributed in environments, are renowned for their respiratory versatility, which underlies their great potential for bioremediation and microbial fuel cells (7, 8). In recent years, *Shewanella oneidensis*, the extensively studied representative of the genus, has been increasingly becoming a research model for broad and diverse aspects of bacterial physiology because of its many unique traits, which are not observed in par-

adigms with *E. coli* and other Gram-negative model organisms. An example of this is fatty acid biosynthesis. *S. oneidensis* FabA, the same as its *E. coli* counterpart (9), is a bifunctional enzyme performing the dehydration of the β -hydroxyacyl-ACP (β -C_{10:0}-ACP) to *trans*-2-enoyl-ACP (*trans*-2-C_{10:1}-ACP) and the isomerization of *trans*-2-decenoyl-ACP (*trans*-2-C_{10:1}-ACP) to *cis*-2-decenoyl-ACP (Fig. 1). However, unlike the *E. coli fabA* mutant, which is a UFA auxotroph, *S. oneidensis* devoid of FabA is almost normal compared to the wild type (10). This is because *S. oneidensis* utilizes DesA to directly desaturate the membrane lipids, and, more importantly, the depletion of FabA induces DesA expression such that sufficient UFAs are produced (10). In contrast, the effects of FabB loss are much more dramatic with respect to growth. This unexpected observation is likely due to accumulation of C₁₄ fatty acids and the overall reduction in UFA production because the

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FIG 1 Type II FAS pathway after C_{10} steps in bacteria. Enzymes in black are derived from current understanding based on studies of *E. coli*, whereas those in other colors refer to *S. oneidensis* enzymes. Enzymes supported by experimental data and by genome annotation and *in silico* data are shown in red and in blue, respectively.

loss of FabB (in contrast to that of FabA) does not stimulate DesA production (11). In addition to playing an important role in the elongation of C_{14} -ACP in *S. oneidensis*, FabB when overabundant is inhibitory to growth by generating C_{18} and even longer fatty acids (11). Based on these differences, it is clear that *S. oneidensis* FabB and its *E. coli* counterpart differ from each other in that the former is much more effective in catalyzing elongation of C_{14} to C_{16} and C_{16} to C_{18} species. A consequence is that *E. coli* FabB produced at various levels fails to fully correct the growth defect of the *S. oneidensis* *fabB* mutant.

There are two homologues of *E. coli* FabF, FabF1 and FabF2, encoded in the *S. oneidensis* genome (see Fig. S1 in the supplemental material), and their physiological roles have been preliminarily investigated (11). Depletion of FabF2 alone does not elicit any notable phenotype, but the additional removal of DesA results in a slight negative impact on growth. In contrast, FabF1 appears totally dispensable, seemingly due to its extremely low production (11). Moreover, a $\Delta desA \Delta fabF1 \Delta fabF2$ strain is phenotypically similar to the strain lacking both *desA* and *fabF2*. Given that FabB is the only possible alternative KAS for the role of FabFs, these observations suggest that FabB alone is nearly sufficient to carry out reactions for generation of the fatty acids that are required for survival and growth in *S. oneidensis*. That is, FabB functionally overlaps all roles played by FabF proteins but not vice versa.

Intriguingly, the *S. oneidensis* *fabB* null mutant is still able to proceed to $C_{14:1}$, implying the presence of other proteins capable of catalyzing the elongation of *cis*-3-decenoyl-ACP (11). To date, a few cases indicating that FabF enzymes can play this role have been reported, but they are exclusively in bacteria lacking a homologue

of *E. coli* FabA, including *Lactococcus lactis*, *Enterococcus faecalis*, and *Clostridium acetobutylicum* (12–14). In this sense, *S. oneidensis* presents a novel model for FabA-containing bacteria. In this study, we investigated spontaneous suppressors from a strain lacking both *desA* and *fabB* genes. We showed that the suppression is caused by a TGT TTT deletion in the sequence upstream of *fabF1*. The association of FabF1 with the suppression was then confirmed by forced expression. Although FabF1, produced in sufficient quantities, is able to complement the loss of FabB, they differ from each other in their detrimental effects when overproduced. We further showed that FabF1 suppression is mediated by transcription read-through of the Rho-independent terminator for *acpP*, an acyl carrier protein gene immediately upstream of *fabF1*. The TGT TTT deletion, within the terminator region composed of multiple tandem repeats, greatly enhances expression of *fabF1* by destroying the U tract of the terminator.

METHODS AND MATERIALS

Bacterial strains, plasmids, and culture conditions. Bacterial strains and plasmids used in this study are listed in Table 1, and sequences of the primers used are given in Table S1 in the supplemental material. All chemicals were acquired from Sigma (Shanghai, China) unless specifically noted. For genetic manipulation, *E. coli* and *S. oneidensis* strains were grown under aerobic conditions in lysogeny broth (LB) medium at 37 and 30°C, respectively. When needed, the growth medium was supplemented with chemicals at the following concentrations: 2,6-diaminopimelic acid (DAP), 0.3 mM; ampicillin, 50 μ g/ml; kanamycin, 50 μ g/ml; gentamicin, 15 μ g/ml; and oleate, 0.005%.

For physiological characterization, both LB and MS defined medium (15) containing 0.02% (wt/vol) vitamin-free Casamino Acids and 15 mM lactate as the electron donor were used in this study, and consistent results were obtained. Fresh medium was inoculated with overnight cultures grown from a single colony by 1:100 dilution, and growth was determined by recording the optical density of cultures at 600 nm (OD_{600}). As cells were mostly cultivated in the presence of oleate, which interferes with OD readings, growth was also monitored by photographing colonies or cell patches developed from a drop of culture on plates as described before (10, 11).

In-frame deletion and knock-in. In-frame deletion strains derived from *E. coli* MG1655 were constructed by the Red recombination deletion method (16). To knock in *gfp* at the *fabF1* locus in *S. oneidensis*, the *att*-based fusion PCR method initially designed for in-frame deletion was adopted (17). In brief, two fragments flanking the *fabF1* gene were amplified with primers containing *attB*, gene-specific sequences, and complementary sequences. These fragments and the PCR product of the *gfp* gene were joined by a second round of fusion PCR to produce a single fragment, with the *gfp* gene flanked by *fabF1* upstream and downstream sequences. The fusion fragments were introduced into pHGM01 by site-specific recombination using BP Clonase (Invitrogen) and maintained in *E. coli* WM3064. The resulting vector was transferred from *E. coli* into *S. oneidensis* $\Delta fabF1$ via conjugation. Integration of the knock-in constructs into the chromosome was selected by gentamicin resistance and confirmed by PCR. Verified transconjugants were grown in LB in the absence of NaCl and plated on LB agar supplemented with 10% sucrose. Gentamicin-sensitive and sucrose-resistant colonies were screened by PCR for the intended knock-in. The knock-in was then verified by sequencing.

Genetic complementation. Plasmid pHG102, which carries the constitutively active *S. oneidensis* *arcA* promoter, was used in genetic complementation of *fabB*, *fabF1*, and *fabF2* mutants (18, 19). After being verified by sequencing, the vectors were introduced into the relevant mutants for phenotypic assays.

Assessment of physiological impacts of FabF1 and FabF2 of varying concentrations. In order to assess the effects of FabF1 and FabF2 of varying concentrations on growth and morphology, their coding genes were

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description ^a	Reference or source
<i>E. coli</i> strains		
DH5 α	Host for cloning	Lab stock
WM3064	$\Delta dapA$; donor strain for conjugation	W. Metcalf, UIUC ^b
BW30270	= MG1655 <i>rph</i> ⁺	CGSC 7925
HGECFabB	$\Delta fabB$ derived from BW30270	This study
<i>S. oneidensis</i> strains		
MR-1	Wild type	ATCC 700550
HG0266	$\Delta ccmF$; derived from MR-1	17
HG1856	$\Delta fabA$; derived from MR-1	10
HG2774	$\Delta fabF1$; derived from MR-1	11
HG3072	$\Delta fabB$; derived from MR-1	11
HG0197-1856	$\Delta desA \Delta fabA$; derived from MR-1	10
HG0197-3072	$\Delta desA \Delta fabB$; derived from MR-1	11
$\Delta desA \Delta fabB^S$ strain	$\Delta desA \Delta fabB$; suppressor strain ^c	This study
HG2774-GFP	<i>gfp</i> knock-in strain derived from HG2774	This study
Plasmids		
pHGM01	Ap ^r Gm ^r Cm ^r ; <i>att</i> -based suicide vector	17
pHG101	Km ^r ; promoterless vector	19
pHG102	Km ^r ; pHG101 containing the <i>S. oneidensis arcA</i> promoter	19
pHGEI01	Km ^r ; integrative <i>lacZ</i> reporter vector	25
pBBR-Cre	Sp ^r ; helper plasmid for antibiotic cassette removal	26
pHGE-Ptac	Km ^r ; broad-host-range IPTG-inducible expression vector	20
pHGE-PtacTorAGFP	pHGE-Ptac containing <i>gfp</i>	20
pHG102- <i>fabF1</i>	Expresses <i>fabF1</i> under the <i>arcA</i> promoter	This study
pPfabF1- <i>lacZ</i>	In pHGEI01, for measuring the <i>fabF1</i> promoter ^d activity	This study
pPhemA- <i>lacZ</i>	In pHGEI01, for measuring the <i>hemA</i> promoter activity	45
pPhemG2- <i>lacZ</i>	In pHGEI01, for measuring the <i>hemG2</i> promoter activity	45
pPhemC- <i>lacZ</i>	In pHGEI01, for measuring the <i>hemC</i> promoter activity	45
pPccmA- <i>lacZ</i>	In pHGEI01, for measuring the <i>ccmA</i> promoter activity	45
pPccmF- <i>lacZ</i>	In pHGEI01, for measuring the <i>ccmF</i> promoter activity	45
pPacpP- <i>lacZ</i>	In pHGEI01, for measuring the <i>acpP</i> promoter activity	This study
pHG101-F1	pHG101 containing F1 fragment	This study
pHG101-F2	pHG101 containing F2 fragment	This study
pHG101-F3	pHG101 containing F3 fragment	This study
pHG101-F4	pHG101 containing F4 fragment	This study
pHG101-F5	pHG101 containing F5 fragment	This study
pHG101-F6	pHG101 containing F6 fragment	This study
pHGE-Ptac-FabA	pHGE-Ptac containing <i>S. oneidensis fabA</i>	11
pHGE-Ptac-FabB	pHGE-Ptac containing <i>S. oneidensis fabB</i>	11
pHGE-Ptac-EcFabB	pHGE-Ptac containing <i>E. coli fabB</i>	11
pHGE-Ptac-FabF1	pHGE-Ptac containing <i>S. oneidensis fabF1</i>	This study
pHGE-Ptac-FabF2	pHGE-Ptac containing <i>S. oneidensis fabF2</i>	This study

^a Ap^r, ampicillin resistant; Gm^r, gentamicin resistant; Cm^r, chloramphenicol resistant; Km^r, kanamycin resistant; Sp^r, spectinomycin resistant.

^b UIUC, University of Illinois at Urbana-Champaign.

^c Suppressor strain that carries all mutations identified in this study.

^d *fabF1* promoter refers to *fabF1* upstream sequences amplified from the wild-type and suppressor strains.

placed under the control of the isopropyl- β -D-thiogalactopyranoside (IPTG)-inducible P_{tac} promoter within pHGE-Ptac (20). While the P_{tac} promoter within the vector in *S. oneidensis* is slightly leaky, displaying an activity of ~ 50 Miller units in the absence of IPTG, its strength increases proportionally with IPTG levels ranging from 0.001 to 1 mM, showing an activity of about 8,000 Miller units with 1 mM IPTG (21, 22).

Chemical assays. Fatty acid compositional analysis was performed essentially as previously described (10). To determine heme *c* levels, cells of the mid-exponential phase were harvested and then were lysed with lysis buffer (0.25 M Tris/HCl [pH 7.5], 0.5% Triton X-100). The protein concentration was determined with a bicinchoninic acid assay kit with bovine serum albumin (BSA) as a standard according to the manufacturer's

instructions (Pierce Chemical). The amount of heme *c* was measured following the procedure described elsewhere (23). The absolute value of heme *c* was normalized to the protein quantity.

Expression assays. Multiple methods were used to evaluate expressions of genes of interest. For reverse transcription-quantitative PCR (qRT-PCR), cells of the mid-log phase were harvested by centrifugation, and total RNA was isolated using an RNeasy minikit (Qiagen) according to the manufacturer's instructions. The analysis was carried out with an ABI 7300 96-well qRT-PCR system (Applied Biosystems) as described previously (24). The expression of each gene was determined from three replicates in a single real-time qRT-PCR experiment. The cycle threshold (*C_T*) values for each gene of interest were averaged and normalized against the *C_T* value of the *arcA* gene, whose abundance was relatively constant

FIG 2 Spontaneous suppressors from the $\Delta desA \Delta fabB$ strain. (A) Cell patches grown from a droplet of mid-log-phase culture (OD_{600} of ~ 0.2) for each indicated strain on LB plates. The morphology, especially thickness, of cell patches for each strain is illustrated by the diagrams shown at the bottom. Oleate was added to a final concentration of 0.005%. Genetic complementation performed with previously constructed vectors (*pfabA* and *pfabB*) was included. Expression levels were controlled by IPTG at concentrations selected according to previous data (11). Presented are representative results from three independent experiments. (B) Growth of the indicated strains in liquid LB. $\Delta desA \Delta fabB^s$ represents a suppressor strain of the $\Delta desA \Delta fabB$ mutant. Experiments were performed at least three times independently, and error bars represent standard deviations. WT, wild type.

during the exponential phase. Relative abundance (RA) of each gene is presented.

For the integrative *lacZ* reporter system, fragments indicated in the text or figure legends were cloned into the reporter vector pHGE101 to generate transcriptional fusions (25). The resultant vectors were then verified by sequencing and transferred into relevant strains by conjugation. To eliminate the antibiotic marker, helper plasmid pBBR-Cre was transferred into the strains carrying a correctly integrated construct (26). Mid-log phase cultures were harvested, aliquoted, and subjected to β -galactosidase activity assays as described before (25).

Expression of *fabF1* was assessed by *gfp* knock-in at the *fabF1* locus. Expression of green fluorescence protein (GFP) in the mid-log-phase cultures was visualized using a Zeiss LSM-510 confocal microscope as described previously (20). Quantification was performed with a fluorescence microplate reader (M200 Pro; Tecan) as described previously (27). In brief, mid-log-phase cultures of each test strain carrying GFP fusions were collected, washed with phosphate-buffered saline containing 0.05% Tween 20, and resuspended in the wash buffer to an OD_{600} of 0.1. Then 100- μ l cell suspensions were transferred into black 384-well plates at various time intervals, and fluorescence was measured using a fluorescence microplate reader (M200 Pro) with excitation at 485 nm and detection of emission at 515 nm. The relative signal intensities were calculated by normalizing test strains carrying GFP to that producing GFP from the *arcA* promoter.

Identification of transcriptional start sites. *S. oneidensis* cells were grown in LB with the required additives to the mid-log phase and collected by centrifugation, and RNA extraction was performed using the RNeasy minikit (Qiagen, Shanghai, China) as described before (28). RNA was quantified by using a NanoVue spectrophotometer (GE Healthcare). The transcriptional start sites of *acpP* and *fabF1* were determined using rapid amplification of cDNA ends (RACE) according to the manufacturer's instructions (Invitrogen, Shanghai, China) as recently used (29). In brief, reverse transcription was conducted on preprocessed RNA without 5'-phosphates followed by two rounds of nested PCRs. The PCR products were subjected to agarose gel separation, with purification of the 5'-RACE products, and inserted into the pMD19-T vector (TaKaRa, Dalian, China) for direct DNA sequencing. The first DNA base adjacent to the 5'-RACE adaptor was regarded as the transcription start site.

Construction of vectors for mutation rate assay. Promoterless plasmid pHG101, present in ~ 5 copies in *S. oneidensis*, was used to assay the effects of tandem repeat (TR) copy numbers on mutation rates (19, 26). DNA fragments of interest were generated by PCR with primers given in Table S1 in the supplemental material. An *acpP*-free fragment was generated by fusion PCR and cloned into pHG101. The resulting vector was used as the template for subsequent PCR, including a TR-free fragment that was also generated by fusion PCR. All fragments were cloned into pHG101, and the resultant vectors were introduced into the relevant strains.

Bioinformatics and statistical analyses. RNA secondary structures were drawn with the XRNA suite of tools with manual modification (<http://rna.ucsc.edu/rnacenter/xrna/xrna.html>) as described before (30). For statistical analysis, values are presented as means $\pm$ standard deviations (SD). Student's *t* tests were performed for pairwise comparisons of groups.

RESULTS

Suppressor strains of the $\Delta fabB \Delta desA$ strain. During our investigation into the physiological role of *S. oneidensis* FabB, we found by chance that cell patches grown from a droplet of the $\Delta desA \Delta fabB$ culture on LB agar plates containing oleate were speckled with brown-red colonies (suppressor strains) when incubation was extended (Fig. 2A). These strains were no longer auxotrophic for UFAs and displayed growth comparable to that of the wild type (Fig. 2B). As the suppression occurs spontaneously and does not require oleate for growth on plates, we reasoned that we may be able to obtain them on plates free of oleate. Indeed, in the absence of oleate, a similar colony appeared from the $\Delta desA \Delta fabB$ mutant (Fig. 2A). These observations suggest that the $\Delta desA \Delta fabB$ strain was prone to mutation. Although culture droplets of the $\Delta desA \Delta fabA$ strain were significantly thinner and paler than those of the $\Delta desA \Delta fabB$ strain, such mutants were not found (Fig. 2A). Growth defects in both double mutants were corrected by expressing the missing *fabA* or *fabB* gene at proper levels as specified before because excess FabB is detrimental (11, 20), thus validating the mutations. Notably, no suppressors were obtained from the *fabB* single mutant (data not shown), implying that the loss of the additional route (DesA) for UFA biosynthesis prompts suppressing mutations.

Suppression is likely associated with FabF1. As shown above, with respect to the color of the culture droplets, both double mutants appeared lighter, especially the $\Delta desA \Delta fabA$ mutant, but the suppressor strains were similar to the wild type. Given that *S. oneidensis* develops brown-red colonies due to its high content of *c*-type cytochromes (24), these differences imply the possibility of impaired cytochrome *c* biosynthesis in both mutants. However, multiple lines of evidence presented in the supplemental material demonstrated that mutations in the suppressors are not associated with *c*-type cytochromes although the loss of both FabA and DesA seems to hamper cytochrome *c* biosynthesis during exponential growth (see Document S1 and Fig. S2 in the supplemental material).

By ruling out the possibility of *c*-type cytochromes and their

FIG 3 Characteristics of the suppressor strain. (A) Upstream sequence of the *S. oneidensis* *fabF1* gene. The translation start and stop sites are in lowercase, italic, and underlined (*gtg* for *fabF1* and *atg* and *taa* for *acpP*). The coding sequence of the *acpP* gene is in lowercase, italic, and blue. The transcription start site for *acpP* is in bold green. Tandem repeats are in bold, and those with dashed lines below the letters had deletions. Within the tandem repeats, underlined sites were mutated. (B) *FabF1* is able to restore the growth defect of the Δ desA Δ fabB mutant. Expression of *fabF1* is driven by the *arcA* promoter. (C) Direct DNA sequencing of the 5'-RACE products of the *fabF1* gene. The arrow denotes the transcriptional start site.

synthesis as a suppressing factor, we reasoned that there must be other KAS enzymes that can fulfill the role of *FabB* in suppressor strains. We therefore sequenced all other KAS genes, including *fabF1*, *fabF2*, *fabH1*, *fabH2*, and *fabH3*, for mutations from 5 suppressor strains (see Fig. S1 in the supplemental material). Indeed, in all strains under examination, mutations were found exclusively to be a TGTTTT deletion in the region upstream of the *fabF1* gene (Fig. 3A). There are two TGTTTT repeats in tandem, one of which is lost. The same result was obtained from sequencing the same region of 5 new suppressor strains generated from a new round of the experiment, implying a role for the deletion in suppression. To test if the *fabF1* gene is associated with the suppression, we placed the *fabF1* gene under the control of the *arcA* promoter (P_{arcA}), which is constitutively active at levels similar to those for the *fabB* promoter (11, 18). Expression of the *fabF1* gene driven by P_{arcA} largely corrected the growth defect of the Δ desA Δ fabB strain (Fig. 3B). Given that the *fabF1* gene was barely transcribed in the wild-type strain (11), these data suggest that *FabF1*, when produced enough in *S. oneidensis*, is a determining factor for the suppression, likely functioning as a *FabB* replacement with respect to growth.

***S. oneidensis* FabF1 and FabB are functionally overlapping but not identical.** *FabF1* has been proposed to play a totally dispensable role in *S. oneidensis* physiology based on its extremely low expression (11). Association of *FabF1* with the suppression revealed by the forced expression raises a question about the proposal. In *E. coli*, *FabF* rather than *FabB* elicits detrimental effects when overproduced (6, 31). However, this may not be the case in *S. oneidensis* because its excess *FabB* results in lethality of cells (11). To test this, we manipulated *FabF1* and *FabF2* levels by the IPTG-controlled promoter P_{tac} within pHGE-Ptac and monitored their effects on the growth of the wild-type and Δ desA Δ fabB strains. In order to precisely interpret data, the levels of *FabF1*, *FabF2*, and *FabB* proteins produced in the wild type by IPTG induction were directly compared in the form of GFP fusion proteins. The *gfp* fusion constructs used previously were placed under

the control of promoter P_{tac} within pHGE-Ptac, and expression was visualized and quantified as before (11). In agreement with previous quantification, all fusion proteins were produced at comparable levels with IPTG at any given concentration (see Fig. S3 in the supplemental material).

With IPTG at up to 1 mM, neither *FabF1* nor *FabF2* had any effect on the growth deficiency of the Δ desA Δ fabB strain (Fig. 4A), consistent with the notion that these two proteins are functionally relevant with *FabB* but not *FabA*. Expression of *fabF1* in the presence of 0.2 mM IPTG eliminated the growth difference between the wild-type and Δ desA Δ fabB strains (Fig. 4), reinforcing the possibility that production of enough *FabF1* can fully complement the *FabB* loss. However, IPTG at other test levels failed to achieve the same result. A general trend was that the further away from 0.2 mM, the more significant the growth difference, supporting the fact that *FabF1* functions in a dose-dependent manner. Notably, without IPTG, slight growth of the Δ desA Δ fabB strain was observed (Fig. 4A), which is due to the leakiness of the promoter (21, 22). The detrimental effect of excess *FabF1* was confirmed in the wild-type strain (Fig. 4A). Apparently, this effect was not comparable to that resulting from excessive *FabB* (11), which prevents growth in the presence of IPTG at 0.2 mM or above (Fig. 4).

In the case of *FabF2*, with IPTG at all test concentrations, effects were evident but much less significant than those resulting from *FabF1* expression (Fig. 4). While this observation indicates a slight functional overlap between *FabF2* and *FabB*, it confirms that *FabF2* could not fully complement the *FabB* loss. Moreover, there seemed to be little adverse effect, or none at all, when excess *FabF2* was present, contrasting both *FabB* and *FabF1*. Taken together, these findings indicate clearly that *S. oneidensis* *FabF1* is largely a functional replacement for *FabB* but differs from the latter in that it would not have a severe detrimental impact on physiology.

Transcription read-through underlies the suppression. To address the conflicting observations between the suppressing ef-

FIG 4 Effects of FabF1 and FabF2 in various abundances on growth. Expression of *fabF1* and *fabF2* at various levels was achieved by using the IPTG-inducible P_{tac} promoter within pHGE- P_{tac} , which is slightly leaky in *S. oneidensis*. The vector with the *fabF1* and *fabF2* construction was introduced into the indicated deletion strains by conjugation. (A) Effects on growth on agar plates. Experiments were conducted in the absence and presence of IPTG from 0.05 to 1 mM on LB plates. Presented are representative results from three independent experiments. (B) Effects on growth in liquid media. The wild type and the $\Delta EcfabB$ mutant carrying the empty vector were used as controls. Experiments were performed at least three times independently, and error bars (<10% of presented data) were omitted for clarity.

fect of forced expression of *fabF1* and the extremely low expression of the *fabF1* gene by its upstream sequence (up to 400 bp relative to the translational start codon), transcript levels of the *fabF1* gene in the wild-type and suppressor strains grown to the

mid-log phase were measured using qRT-PCR. The difference was substantial, with TGT TTT deletion suppressor strains transcribing the gene ~ 30 times more (Fig. 5A). We then knocked in a copy of the *gfp* gene at the *fabF1* locus, and the fluorescence was visu-

FIG 5 Transcription read-through underlies the suppression. (A) Expression of *fabF1* and *acpP* revealed by qRT-PCR in various strains. The abundance of mRNAs for *fabF1* and *acpP* in indicated strains at the mid-log phase was assayed by qRT-PCR. Expression levels are represented by signal intensity ratios of *fabF1* and *acpP* to *arcA*. (B) Assessment of on-site expression levels of *fabF1* by *gfp* knock-in. A copy of the *gfp* gene was used to replace the chromosomal *fabF1* as described in Materials and Methods, and its intensities were visualized and quantified. A copy of the *gfp* gene under the control of the *arcA* promoter was introduced into the wild type as a control. (C) Activity of indicated DNA fragments as promoters. *PacpP* represents the *acpP* promoter only. Other DNA fragments under test were composed of the *acpP* promoter and intergenic sequence between *acpP* and *fabF1*. (D) Rho-independent terminator in the wild-type and TGT TTT suppressor strains. Tandem reports and point mutations were marked out in the wild-type sequence. In panels A, B, and C, experiments were performed at least three times independently, and error bars represent standard deviations.

FIG 6 Tandem repeats likely underlie suppressing mutations. Fragments illustrated here were produced by PCR, cloned into promoterless vector pHG101, and introduced into the $\Delta desA \Delta fabB$ strain. Colonies colored brown-red that developed on cell patches grown from a droplet of mid-log-phase cultures (~ 0.2 of OD_{600}) for each indicated strain on LB plates were counted. Fragment F6 is the promoter sequence of ~ 300 bp for the *arcA* gene, regarded as DNA unrelated to the region. Experiments were performed at least three times independently.

alized and quantified with a microscope and a microplate reader, respectively (Fig. 5B). In agreement with the qRT-PCR data, suppressor strains displayed drastically increased fluorescence intensities compared to those for the wild type. Nevertheless, the wild type exhibited notable fluorescence, contrasting with the finding that no signal was observed from the *gfp* gene driven by the *fabF1* upstream sequence (11). This difference implies that *fabF1* may not be transcribed from the region immediately upstream of its coding sequence.

To explain the discrepancy between data from the promoter activity assay (*lacZ* reporter) and qRT-PCR, we first made attempts to determine the transcriptional start site for the *fabF1* gene on mRNAs prepared from the wild-type and TGTTTT deletion suppressor strains using 5'-RACE. The analysis failed to identify any site for the *fabF1* gene from either sample but revealed one for the *acpP* gene (-39 relative to the translational initiating code), the gene before the *fabF1* gene (Fig. 3A and C). Failure to identify a promoter immediately upstream of the *fabF1* coding sequence implies a lack of a promoter for the *fabF1* gene, although the possibility that the expression of the *fabF1* gene driven by its own promoter is simply below the detection limit of the method could not be ruled out. By using the *lacZ* reporter, the *acpP* promoter was found to be unusually robust, $\sim 5,000$ Miller units (Fig. 5C). Based on these data, we predicted that the increased expression of the *fabF1* gene in the suppressor strain is probably due to the read-through transcription from P_{acpP} , a result of the TGTTTT deletion. This prediction was supported by an *in silico* analysis: there is a Rho-independent transcription terminator in the region covering the TGTTTT deletion (Fig. 5D). This terminator is characterized by a GC-rich hairpin followed by a U tract (32). Apparently, the deletion destroyed the U tract.

To test whether read-through explains the suppression, we cloned the entire region covering the *acpP* promoter and coding sequence as well as the sequence between *acpP* and *fabF1* (~ 600 bp) (Fig. 3A). However, we failed to transform the ligated sequence into *E. coli* after many tries but were able to do so when the *acpP* gene was not included (fragment F3 in Fig. 6), implying that overexpression of the *S. oneidensis acpP* gene may be lethal to *E. coli* cells. This coincides with the observation that overproduction of *E. coli* ACP strongly inhibits growth of *E. coli*, although ACP is

one of the most abundant proteins in bacteria (33, 34). Based on β -galactosidase activities, this long *acpP*-free fragment of the wild-type version was weak, but the TGTTTT deletion was highly active, confirming that the mutation critically affects transcription of the *fabF1* gene from the *acpP* promoter (Fig. 5C). These data, collectively, indicate that expression of the *fabF1* gene is primarily, if not exclusively, from the transcription driven by the *acpP* promoter and the Rho-independent transcription terminator dictates its expression levels.

Suppressor mutations occur within a fragment composed of tandem repeats. Since the suppressing mutations are exclusively the TGTTTT deletion, it is likely that the mutation occurs within a fragment that is hypermutable. An *in silico* analysis revealed that the fragment covering the Rho-independent transcription terminator is composed of multiple tandem repeats (TRs), nucleotide sequences which are prone to strand slippage replication and recombinant events (35). To test if these TRs are the reason for suppressing mutations, we introduced them into $\Delta desA \Delta fabB$ vectors carrying either TR-containing or TR-free fragments within multiple-copy-number plasmid pHG101 (Fig. 6), because it has been established that mutation rates increase exponentially with increasing numbers of repeat units (35). The copy number of pHG101, which is based on the RK2 replicon, is about 4 to 7 per cell in *E. coli* and is estimated to be in a similar range in *S. oneidensis* (26, 36). Clearly, the double mutant carrying the TR-containing fragments F1, F3, and F4 became hypermutable (Fig. 6; see also Fig. S4A in the supplemental material), whereas TR-free fragments F2, F5, and F6 did not significantly alter mutation rates compared to those of the vector-free control. Based on the ratio of suppressors from $\Delta desA \Delta fabB$ vectors carrying the TR and TR-free fragments, we estimated that the presence of TRs increased the mutation rate at least 10-fold (Fig. 6A).

To determine mutations in these new suppressors, 30 mutations chosen randomly from the $\Delta desA \Delta fabB$ vector with the TR fragment were subjected to sequencing. Among them, 23 carried the TGTTTT deletion, confirming that this mutation occurs at the highest frequency. The remaining included 1 GGC deletion, 2 GCC deletions, and 4 point mutations (2 G $\rightarrow$ A, the first G within GGCGGC; 1 G $\rightarrow$ A, the third G within GGCGGC; and 1 C $\rightarrow$ A, the second C within GCCGCC) (Fig. 3A and 5D). The new mutations were then tested for their ability to affect *fabF1* expression. As expected, the GCC deletion and C $\rightarrow$ A point mutations also greatly enhanced the read-through, albeit less effectively than the TGTTTT deletion (Fig. 5C). This is reasonable because these mutations only affect the stability of the GC-rich hairpin (Fig. 5D). These results, on the one hand, support the fact that the TR sequence underlies suppressing mutations and, on the other hand, reinforce the fact that the *fabF1* transcription read-through is responsible for the suppression.

***S. oneidensis* FabB and FabF are distinct in complementing *E. coli fabB* mutants.** Previously, we have shown that both *E. coli* FabB (*EcFabB*) and FabF (*EcFabF*) were able to complement the *S. oneidensis fabB* mutant, but when overproduced neither had a detrimental impact as severe as that caused by *S. oneidensis* FabB (11). As *S. oneidensis* FabF1 is the first example in Gram-negative FabA-containing bacteria that can complement the FabB loss, it is worth testing whether this protein can also function as a functional replacement for *EcFabB*. To this end, we constructed an *EcfabB* mutant, which relies on oleate for growth (see Fig. S4B in the supplemental material). Vectors expressing *EcfabB* and *S. one-*

FIG 7 Effects of *S. oneidensis* FabB and FabF1 on an *E. coli* $\Delta EcfabB$ strain. (A) Effects on growth. $\Delta EcfabB$ cells carrying plasmids encoding *S. oneidensis* FabB and FabF1 were grown in the presence of IPTG from 0.05 to 1 mM. The wild type (relative to the *fabB* mutation) and the $\Delta EcfabB$ mutant carrying the empty vector were used as controls. Experiments were performed at least three times independently, and error bars (<10% of presented data) were omitted for clarity. (B) Effects on membrane fatty acid composition. Cultures grown as described for panel A with two levels of IPTG to the late-log phase were collected for fatty acid composition determination. Asterisks indicate statistically significant differences (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; $n \geq 3$). Experiments were performed at least three times independently, and error bars represent standard deviations.

idensis fabB, *fabF1*, and *fabF2* under the control of P_{tac} were introduced into this $\Delta EcfabB$ strain. In the presence of IPTG at 0.1 to 0.5 mM, *EcFabB* restored growth of the mutant fully (Fig. 7A), validating the view that the phenotype of this *EcfabB* mutant is due to the intended mutation. However, when IPTG was added to 0.05 mM and 1 mM, growth restoration was substantial but not full. While imperfect complementation at the lower end is likely due to insufficient production, that at the higher end may be due to overproduction. When IPTG levels were at 0.05 mM, *S. oneidensis* FabB restored growth most effectively (Fig. 7A). In the presence of IPTG at 0.1 mM, overproduction of *S. oneidensis* FabB inhibited growth significantly, and at higher test concentrations growth was barely observed. Compared to that of FabB, the efficacy of FabF1 in growth restoration was relatively low, rendering the mutant growth poor with IPTG at 0.1 mM or lower. When FabF1 was produced more (by IPTG at 0.2 and 0.5 mM), growth was comparable to that resulting from FabB produced in the presence of 0.05 mM IPTG. Interestingly, further overproduction of FabF1 (by 1 mM IPTG) impaired growth modestly. In contrast to both FabB and FabF1, FabF2 at all test levels was insufficient to restore growth, indicating that this protein may not complement the *EcFabB* loss (data not shown). The observed effects of *S. oneidensis* FabB and FabF1 on growth of the *EcfabB* mutant resembled those observed in *S. oneidensis* (11), implying that these two proteins function in *E. coli* in a similar manner.

We then performed gas chromatography-mass spectrometry (GC-MS) analysis of the membrane fatty acid composition of the $\Delta EcfabB$ strain expressing *S. oneidensis fabB* and *fabF1* with IPTG at 0.05 and 0.2 mM, respectively (Fig. 7B). *S. oneidensis* FabB produced with 0.05 mM IPTG slightly altered the composition from that in the $\Delta EcfabB/EcfabB$ strain (with 0.2 mM IPTG) by increasing C_{14} species, presumably due to insufficient production. Im-

portantly, when heavily overexpressed (with 0.2 mM IPTG), this protein substantially enhanced production of C_{18} , with an accompanying decrease in the level of C_{16} species. In contrast, differences in compositions between the $\Delta EcfabB/EcfabB$ and $\Delta EcfabB/fabF1$ strains were minor. Based on these data, we suggest that the detrimental effect of excess FabB in *S. oneidensis* is likely due to the accumulation of long-chain fatty acids, a scenario reported in the *S. oneidensis* cells overproducing FabB.

DISCUSSION

A type II FAS pathway is undoubtedly critical for bacteria because it is the predominant, if not exclusive, route for fatty acid biosynthesis. The pathway splits into saturated fatty acid (SFA) and UFA synthesis arms at the 10-carbon stage (C_{10}) (Fig. 1). In *E. coli*, on which the current understanding of the pathway and its constituents is largely built, KAS enzymes (FabB, FabF, and FabH) that catalyze 3-ketoacyl-ACP synthase reactions have been extensively studied (1). Both FabB and FabF participate in elongation of long-chain acyl-ACP in both arms to control fatty acid composition and impact the rate of fatty acid production (3, 4, 34, 37). Unlike FabH, which has a Cys-His-Asn active site triad, FabB and FabF enzymes have common Cys-His-His triad as their active sites. As a consequence, functional complementation between them thus is expected. Indeed, both enzymes perform well at all steps in the SFA arm and most steps in the UFA arm. However, loss of FabB but not FabF causes an UFA auxotroph. This is because the reaction catalyzed by FabB exclusively is an essential step for UFA production but that catalyzed by FabF exclusively is not (4, 5).

To date, FabF proteins that are able to fulfill the role of *E. coli* FabB are exclusively from bacteria lacking homologues for *E. coli* FabA and FabB (12, 13). This is reasonable as these FabF proteins carry out all required steps for UFA production. In this study, we

report the first evidence that in *S. oneidensis*, a FabA-containing bacterium possessing both FabB and FabF proteins, FabF1 is able to complement the FabB loss. There is a caveat: FabF1 has to be produced at significantly enhanced levels because the basal production is too low to make a difference. In addition, FabF2 is also implicated in playing the role of FabB, albeit much less effectively. The difference in the abilities of FabF1 and FabF2 to replace FabB is similarly evident in *E. coli*, indicating that FabF1 essentially functions as FabB. Based on sequence similarities, FabF1 can be confidently assigned to be a homologue of *EcFabF* (see Fig. S1 in the supplemental material). Moreover, the synteny (genetic organization) for *fabF1* and *EcfabF* are the same, *acpP-fabF*. In contrast, *fabF2* is clustered with *fabG2*, a scenario widely found in bacteria. Despite these differences, FabF1 and FabF2 have levels of sequence similarities to *EcFabB* that are comparable. Conceivably, there must be intrinsic differences explaining the abilities of FabF1 and FabF2 to complement the FabB loss, which is under investigation.

We have previously demonstrated that FabB loss renders *S. oneidensis* cells susceptible to accumulation of C₁₄ fatty acids, indicating that the strain lacking FabB is still able to proceed to C₁₄ (11). While this discovery does not rule out the participation of FabB in elongation of the *cis*-3-decenoyl-ACP (*cis*-3-C_{10:1}-ACP), it is certain that there is another protein(s) that is also able to carry out this essential step. Based on the findings reported here, it is probable that both FabF1 and FabF2 play substitution roles for FabB at the same time. Despite this, the growth defect resulting from FabB loss in the *desA* deletion background was corrected by either protein alone, apparently due to the low production of FabF1 and the low efficacy of FabF2. This coincides with the severe growth defect of an *E. coli fabH* mutant. FabH, once regarded an essential enzyme for fatty acid (FA) biosynthesis and viability (2), is removable in *E. coli* (38). As the step catalyzed by FabH is essential, either FabB and FabF proteins or a new type of KAS, such as that in *Pseudomonas aeruginosa* (39), may substitute for FabH. Given that these substituents are not comparable with FabH in functional effectiveness, the growth of the *fabH* mutant is impaired substantially (38). In *S. oneidensis*, FabB is sufficient to support normal fatty acid synthesis as the loss of both FabF1 and FabF2 causes no noticeable growth defect (11). However, FabB appears to catalyze a certain step(s) less effectively than FabF2 because in the *desA* deletion background, the removal of *fabF2* results in a slight growth defect (11). Clearly, there is only limited functional overlap between FabB and FabF2 in *S. oneidensis*.

Because of its extremely low expression, the physiological impact of FabF1 on the wild type is not detectable. However, when expressed sufficiently, FabF1 can fully complement the FabB loss, indicating that both enzymes not only catalyze the same steps but also have similar activities. Despite this, they are not identical. *S. oneidensis* FabB, similar to *E. coli* FabF rather than FabB, induces lethality when overproduced (11). In contrast, FabF1 does not have such an effect. Thus, FabF1 seems to function as a backup for FabB in *S. oneidensis*: when cells have FabB, its expression is shut off, but when FabB and *DesA* are depleted, its quantity increases and it takes over the role. Conceivably, there may be other conditions that can trigger the read-through; efforts are being taken to find them.

The enhancement in FabF1 production in suppressor strains is a result of mutations, causing transcription read-through of a typical Rho-independent transcription terminator for *acpP*, the gene

located immediately upstream of *fabF1* (40). The hallmark features of such a terminator are a short GC-rich inverted repeat sequence followed by a run of A residues on the template strand (41). After transcription, a hairpin structure is formed by the inverted repeat sequence, which triggers termination within the U tract (40). The GC-rich inverted repeat sequence and the U tract of the *acpP* transcription terminator are composed of four different tandem repeats (TRs), which are hypermutable because DNA replication slippage and recombination at the loci are prone to occur (35, 42). Although all TRs are inherently unstable, mutation rates vary greatly between TRs, which mainly depend on their sequence features, including numbers of repeated units, unit length, and repeat purity (43). The most important factor is the number of repeat units; repeat variability increases exponentially with increasing numbers of repeat units. This perfectly explains why the additional copies of the DNA fragments covering these TRs within the *fabF1* upstream region substantially elevate mutation rates. Repeat variability also increases with increasing unit length (44), a notion consistent with the finding that the TGT TTT deletion occurs at a frequency substantially higher than others.

In general, TRs are commonly positioned as *cis*-regulatory elements in the intergenic region in proximity to a promoter. A consequence of the mutation is that the activity of the promoter is affected, leading to the altered transcription of the downstream genes. Our data presented an intriguingly different case. Instead of modulating the activity of the *fabF1* promoter, the mutations, especially the TGT TTT deletion, substantially impair the capacity of the terminator for the *acpP* gene, allowing the occurrence of read-through transcription from the *acpP* promoter. Such an arrangement requires that the promoter must be robust, a caveat that the *acpP* promoter apparently satisfies.

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