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RESEARCH ARTICLE

A Novel Gene Contributing to the Initiation of Fatty Acid Biosynthesis in *Escherichia coli*

Rajeshree Sanyal,[a,b] Vani Singh,[a,b] Rajendran Harinarayanan[a]

[a]Laboratory of Bacterial Genetics, Centre for DNA Fingerprinting and Diagnostics, Hyderabad, India
[b]Graduate Studies, Manipal Academy of Higher Education, Manipal, India

ABSTRACT Type II fatty acid biosynthesis in bacteria can be broadly classified into the initiation and elongation phases. The biochemical functions defining each step in the two phases have been studied *in vitro*. Among the β-ketoacyl-acyl carrier protein (ACP) synthases, FabH catalyzes the initiation reaction, while FabB and FabF, which primarily catalyze the elongation reaction, can also drive initiation as side reactions. A role for FabB and FabF in the initiation of fatty acid biosynthesis would be supported by the viability of the Δ*fabH* mutant. In this study, we show that the Δ*fabH* and Δ*yiiD* mutations were synthetically lethal and that Δ*fabH* Δ*relA* Δ*spoT* and Δ*fabH* Δ*dksA* synthetic lethality was rescued by the heterologous expression of *yiiD*. In the Δ*fabH* mutant, the expression of *yiiD* was positively regulated by (p)ppGpp. The growth defect, reduced cell size, and altered fatty acid profile of the Δ*fabH* mutant and the growth defect of the Δ*fabH* Δ*fabF fabB15*(Ts) mutant in oleate- and palmitate-supplemented medium at 42°C were rescued by the expression of *yiiD* from a multicopy plasmid. Together, these results indicate that the *yiiD*-encoded function supported initiation of fatty acid biosynthesis in the absence of FabH. We have renamed *yiiD* as *fabY*.

IMPORTANCE Fatty acid biosynthesis is an essential process conserved across life forms. β-Ketoacyl-ACP synthases are essential for fatty acid biosynthesis. FabH is a β-ketoacyl-ACP synthase that contributes to the initiation of fatty acid biosynthesis in *Escherichia coli*. In this study, we present genetic and biochemical evidence that the *yiiD* (renamed *fabY*)-encoded function contributes to the biosynthesis of fatty acid in the absence of FabH activity and that under these conditions, the expression of FabY was regulated by the stringent response factors (p)ppGpp and DksA. Combined inactivation of FabH and FabY resulted in growth arrest, possibly due to the loss of fatty acid biosynthesis. A molecule(s) that inhibits the two activities can be an effective microbicide.

KEYWORDS (p)ppGpp, *dksA*, *fabH*, fatty acid synthesis, *yiiD*

The mechanisms of fatty acid biosynthesis are conserved in prokaryotes and eukaryotes. The fatty acid biosynthetic pathway in the model bacterium *Escherichia coli* has been well studied (1, 2). For the synthesis of a fatty acid molecule, a round of initiation is followed by multiple rounds of elongation. β-Ketoacyl-acyl carrier protein (ACP) synthase I (FabB), β-ketoacyl-ACP synthase II (FabF), and β-ketoacyl-ACP synthase III (FabH) are required for the biosynthesis of fatty acids in *E. coli*. Data from many studies showed that the elongation cycle is maintained by the FabB and FabF enzymes that condense malonyl-ACP to the acyl-ACP generated during each elongation cycle so as to extend the acyl chain by two carbon units (1). The relative contribution of the β-ketoacyl-ACP synthases to the initiation of fatty acid synthesis in *E. coli* is less clear. The final step in the initiation of fatty acid synthesis is the generation of acetoacetyl-ACP by the condensation of acetyl coenzyme A (acetyl-CoA) and malonyl-ACP by FabH

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Address correspondence to Rajendran Harinarayanan, hari@cdfd.org.in.

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(3). However, purified FabB or FabF was capable of initiating fatty acid synthesis using malonyl-ACP in the absence of acetyl-ACP or acetyl-CoA. This synthesis occurred through a side reaction wherein malonyl-ACP is decarboxylated to produce acetyl-ACP and then condensed with malonyl-ACP to generate acetoacetyl-ACP (4, 5). The three β-ketoacyl-ACP synthases also catalyzed the acetyl-CoA:ACP transacylase reaction (3, 4, 6). The physiological significance of these reactions is not clear.

Inhibition of fatty acid biosynthesis signals the accumulation of the stringent response molecule (p)ppGpp (7). The accumulation of (p)ppGpp under these conditions was dependent on the interaction of ACP with the TGS (ThrRS, GTPase, and SpoT) region in the C-terminal domain of SpoT (8). SpoT is a dual-function protein capable of both synthesis and degradation of (p)ppGpp (9, 10); the other protein capable of (p)ppGpp synthesis in *E. coli* is RelA. It has been proposed that the ACP-SpoT interaction could be influenced by the ratio of unacylated ACP to acylated ACP, which could shift the balance of the two SpoT activities in favor of synthesis.

While (p)ppGpp can bind to a large number of proteins and affect multiple processes, one important target of (p)ppGpp is the RNA polymerase (RNAP), on which two binding sites have been reported (11–17). One site is at the interface of the omega and beta′ subunits, while the other is located at the interface of DksA and the beta′ subunit rim helices. DksA is a transcription factor that modulates RNA polymerase activity through the secondary channel (18). DksA potentiates both the negative and positive regulation of transcription by (p)ppGpp (19, 20). The interaction of ppGpp and DksA with RNAP can account for the altered expression of a large number of genes in response to changes in the cellular (p)ppGpp pool (18, 19, 21–23).

Genes involved in fatty acid biosynthesis are regulated by (p)ppGpp and DksA. The transcription of the *fabHDG* operon and *fadR* is directly inhibited by (p)ppGpp and DksA (24). By inhibiting the transcription of *fadR*, (p)ppGpp can also indirectly regulate fatty acid metabolism (24, 25). It has been proposed that ppGpp directly inhibits PlsB, the glycerol-3-phosphate acyltransferase of the phospholipid synthesis pathway, to modulate lipid synthesis at the level of enzyme activity (26). Expression of the *accABCD* operon was reported to be growth rate dependent, suggesting negative regulation by (p)ppGpp (27). The genes of this operon encode proteins required for the acetyl-CoA carboxylase reaction, which was reported to be inhibited by ppGpp *in vitro* (28).

Recent studies have provided evidence that the rate of phospholipid synthesis, by setting the cell envelope capacity, was a determinant of cell size in bacteria and yeast (29, 30). (p)ppGpp was implicated as the primary determinant required for coordinating the cytoplasmic volume change that accompanies nutrient availability and fatty acid biosynthesis.

fabH, coding for β-ketoacyl-ACP synthase III, was initially reported as an essential gene (31) but was subsequently reported to be nonessential and to confer synthetic lethality in the Δ*relA* Δ*spoT* (ppGpp^0) strain (29, 32). The nonessentiality of *fabH* would suggest that the side reactions reported for the *fabB*- and *fabF*-encoded proteins may contribute to the initiation of fatty acid biosynthesis in the absence of FabH.

In this study, we present evidence that the *fabB*- and *fabF*-encoded functions do not contribute to the initiation of fatty acid biosynthesis in the *fabH* mutant and that the initiation of fatty acid biosynthesis was supported by the *yiiD*-encoded function. Heterologous expression of *yiiD* rescued the growth defect, reduced cell size, and altered the fatty acid profile of the *fabH* mutant. This, taken together with the presence of acetyltransferase and thioesterase domains, is consistent with a direct role for *yiiD* in fatty acid biosynthesis; therefore, *yiiD* was renamed *fabY*. Our genetic evidence suggested that the expression of *fabY* was positively regulated at the level of transcription by the stringent response factors (p)ppGpp and DksA. Such a regulation would be the opposite that reported for *fabH*, which is negatively regulated by (p)ppGpp and DksA (24). The physiological significance of using two gene functions for the initiation of fatty acid biosynthesis, one (*fabH*) negatively regulated and the other (*fabY*) positively regulated by (p)ppGpp/DksA, is discussed.

RESULTS

(p)ppGpp and DksA but not SpoT function is essential for growth of the Δ*fabH* strain. FabH is a β-ketoacyl-ACP synthase with a primary role in the initiation of fatty acid biosynthesis (3, 6). The *fabH* deletion that was ordinarily viable was reported to be lethal in the *relA1* Δ*spoT* and Δ*relA* Δ*spoT* genetic backgrounds based on a linkage disruption test (29); the latter strain cannot synthesize (p)ppGpp and is referred to as ppGpp^0. Unlike the ppGpp^0 strain, the *relA1* Δ*spoT* strain grows on minimal medium using glucose as the sole carbon source, which suggests that the latter strain is capable of (p)ppGpp synthesis (9). We therefore reexamined if the FabH function was essential in the *relA1* Δ*spoT* and ppGpp^0 genetic backgrounds.

We studied this using the plasmid segregation assay that is based on the rationale that an essential gene function provided from an unstable plasmid would stabilize the plasmid (see Materials and Methods for details). Plasmid pRCspoT (31), in which the *spoT* and *lacZ* genes are expressed from the *lac* promoter, was constructed using the unstable single-copy plasmid pRC7 (33). pRCspoT, but not the vector pRC7, rescued the growth defect of the ppGpp^0 strain in minimal glucose medium containing the inducer isopropyl-β-D-1-thiogalactopyranoside (IPTG), indicating that pRCspoT supported (p)ppGpp synthesis (see Fig. S1 in the supplemental material). When pRCspoT is introduced into Δ*lac* strains and allowed to grow without selection, plasmid-bearing cells form blue colonies, while those that lose the plasmid during cell division form white colonies in plates containing IPTG and the indicator 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal).

Mutant alleles of *relA* and *spoT* were introduced into the isogenic Δ*lac*/pRCspoT and Δ*lac* Δ*fabH*/pRCspoT strains, and segregation of pRCspoT was monitored using the "blue-white assay" described in Materials and Methods. In each of the strains tested, except the ppGpp^0 Δ*fabH*/pRCspoT strain, blue and white colonies were obtained (Fig. 1A), and colonies of the strains bearing the Δ*fabH* allele were smaller than those of isogenic *fabH*$^+$ strains (compare panels i to v with panels vi to x). The inability to recover white colonies in the ppGpp^0 Δ*fabH*/pRCspoT strain indicated that the ppGpp^0 (Δ*relA* Δ*spoT*) and Δ*fabH* alleles were synthetically lethal, and this was consistent with the findings of Yao et al. (29). Notably, inconsistent with their results, the *relA1* Δ*spoT* Δ*fabH* strain was found to be viable (Fig. 1A, panel ix). These results showed that (p)ppGpp but not the SpoT function was essential for the growth of the *fabH* mutant.

The synthetic lethality of the Δ*relA* Δ*spoT* Δ*fabH* strain but not the *relA1* Δ*spoT* Δ*fabH* strain suggested that the basal (p)ppGpp pool in the latter strain supported the growth of the Δ*fabH* mutant. To test if an increase in the basal (p)ppGpp pool rescued the growth defect of the *fabH* mutant, we used slow-growth conditions that are known to increase the basal (p)ppGpp pool (34). Unlike in LB medium, where the growth rate of the *fabH* mutant was significantly lower than that of the wild-type (WT) strain, their growth rates were similar in minimal glycerol medium (Fig. S2A and B).

SpoT contributes to basal (p)ppGpp pool under slow-growth conditions (34), and the *relA1* allele has very little (p)ppGpp synthetase activity (35). Therefore, in the *relA1* Δ*spoT* strain, the increase in basal (p)ppGpp accompanying slow growth can be expected to be smaller than that in the wild-type strain, and consequently, the rescue of the *fabH* growth defect is expected to be diminished. Accordingly, the growth rate of the *relA1* Δ*spoT* Δ*fabH* mutant was lower than that of the *relA1* Δ*spoT* strain in minimal glycerol medium (Fig. S2B). This supports the idea that an increase in the basal (p)ppGpp pool is required to alleviate the growth defect of the fabH mutant.

DksA potentiates (p)ppGpp-mediated transcriptional regulation (36), and the conserved aspartic acid residues at the tip of the coiled-coil domain are required for transcriptional regulation by DksA (18, 37). A plasmid segregation assay using unstable plasmid pRC$_{sp}$-dksA showed that the DksA function was required for the growth of the *fabH* mutant (Fig. 1B). Overexpression of DksA using pKJ537 (38), referred to here as pdksA, rescued the synthetic growth defect of the *fabH dksA* mutant, but expression of the altered protein, DksA D71N D74N (using pdksA^{NN}), or the vector control (pΔdksA) did not rescue the synthetic growth defect (Fig. 1C). This indicates that DksA-mediated

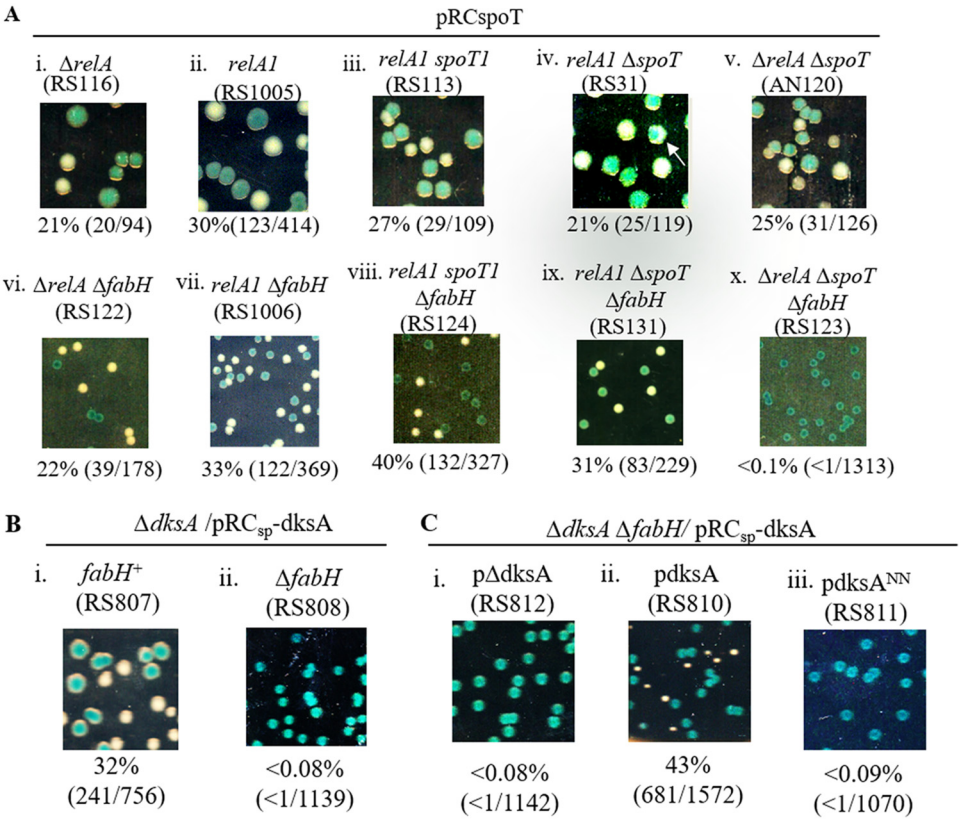

FIG 1 (p)ppGpp and DksA but not SpoT function is required for viability of the *fabH* mutant. Retention or loss of unstable plasmid pRCspoT (A) or pRC_{sp}-dksA (B and C) was scored by the blue-white segregation assay described in Materials and Methods using LB agar plates containing X-gal and IPTG. The relevant genotype of the strain and the strain number are indicated above each image showing the section of a plate. The percentage of white colonies and the total number of colonies (blue and white) used to calculate the ratio are indicated below each image. To select for plasmid pΔdksA, pdksA, or $pdksA^{NN}$, the plates in panel C were additionally supplemented with ampicillin. The arrow in panel Aiv indicates a sectored colony.

regulation through the secondary channel is required for the viability of the *fabH* mutant.

Transcriptional regulation by (p)ppGpp is necessary for growth in the absence of FabH function. Two (p)ppGpp binding sites have been identified in *E. coli* RNA polymerase (11–17) and are referred to as site 1 and site 2 (11). (p)ppGpp-mediated regulation through site 1 is dependent on the ω subunit of RNA polymerase, and that through site 2 is DksA dependent. (p)ppGpp binding at site 2 requires DksA and confers synergistic regulation of transcription along with DksA (11, 39). Using the (p)ppGpp binding-site mutants constructed by the Gourse laboratory (11), we asked if the growth of the *fabH* mutant was dependent on regulation through site 1, site 2, or both. The plasmid segregation assay showed that the inactivation of both (p)ppGpp binding sites but not site 1 or site 2 individually abolished the growth of the *fabH* mutant (Fig. 2A). This indicated that the regulation of transcription (but not the other functions) by (p)ppGpp was necessary for the growth of the *fabH* mutant.

In vitro, the N-terminal residues of ω and residues from the β' subunit together constitute functional site 1 (13). We asked if this was also the case *in vivo* by using a *fabH* mutant lacking site 2 (where the site 1 function was necessary for growth). Introduction of the β' mutations (R362A R417A K615A) or the ω mutation (Δ2-5) individually into this strain conferred growth inhibition (Fig. 2B), indicating that each component, that is, β' residues and as well as ω residues, is required for functional (p)ppGpp binding at site 1 *in vivo*.

Increases in (p)ppGpp and DksA expression suppress *dksA fabH* and $ppGpp^0$ *fabH* synthetic lethality, respectively. The *spoT202* or *spoT203* mutations that in-

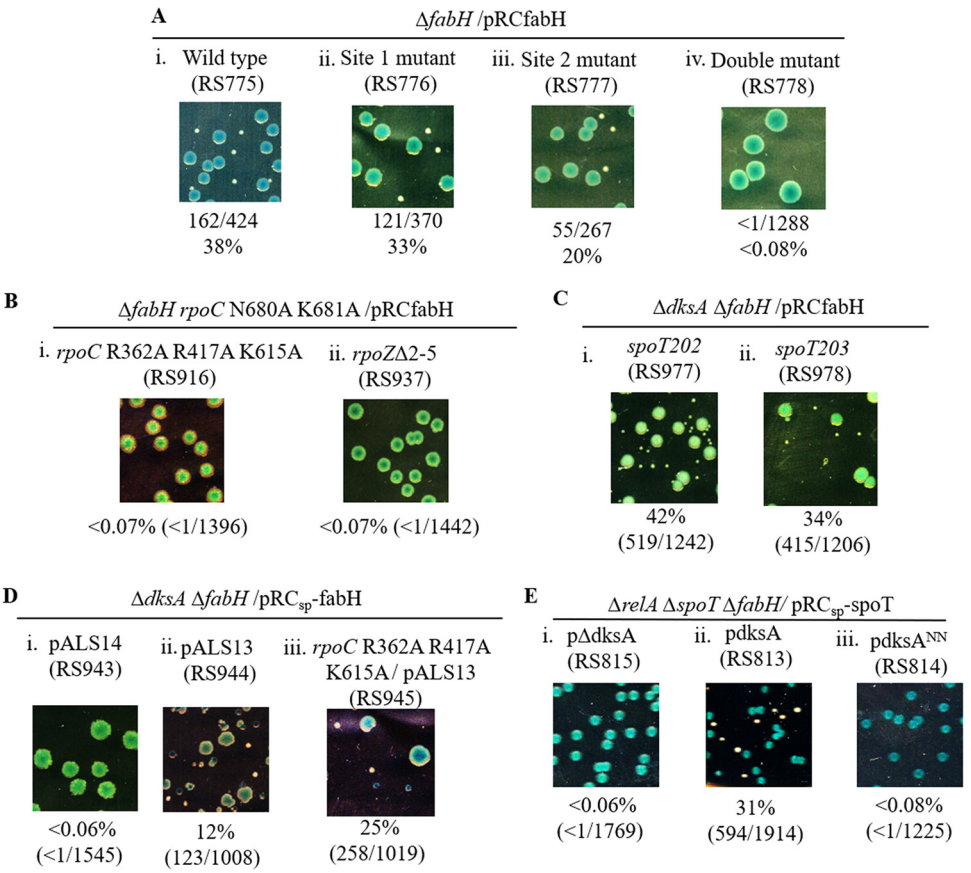

FIG 2 Role of (p)ppGpp/DksA in growth of the *fabH* mutant. Retention or loss of the unstable plasmid pRCfabH, pRC$_{sp}$-fabH, or pRC$_{sp}$-spoT was scored by the blue-white segregation assay using LB agar plates containing X-gal (A to D) or X-gal and IPTG (E). IPTG was present when studying pRCspoT segregation but not for pRCfabH because *lacZ* expression in the latter plasmid was from the *fabH* promoter. The relevant genotype of the strain and plasmid loss percentage are indicated for each panel as described in the legend to Fig. 1.

crease the basal (p)ppGpp pool (40) suppressed *fabH dksA* synthetic lethality (Fig. 2C). Similarly, the introduction of pALS13, a plasmid encoding a truncated RelA polypeptide that increases the basal (p)ppGpp pool (41), suppressed *fabH dksA* synthetic lethality (Fig. 2D, panels i and ii). Since (p)ppGpp regulation through site 2 is DksA dependent, suppression of *fabH dksA* synthetic lethality by the increase in the basal (p)ppGpp level was expected to be mediated through site 1. To test this, site 1 was inactivated by the introduction of the *rpoC* mutations (R362A R417A K615A) into the *fabH dksA*/pALS13/ pRC$_{sp}$-fabH strain. Surprisingly, suppression of *dksA fabH* synthetic lethality by pALS13 continued to be observed (Fig. 2D, panel iii). Although the sizes of the white colonies in Fig. 2C and D are small, they are viable since they continued to show growth upon streaking. The reason for the heterogeneity in the size of the blue colonies associated with the presence of pALS13 is not clear. Overexpression of DksA but not DksA^{NN} (DksA protein with altered amino acid residues [D71N D74N]) suppressed ppGpp^0 *fabH* synthetic lethality (Fig. 2E, panels ii and iii). These results indicate that DksA is not required for growth rescue by elevated basal (p)ppGpp levels and that (p)ppGpp is not required for growth rescue during DksA overexpression. Furthermore, rescue following DksA overexpression seems to be mediated through the secondary channel of RNAP.

***yiiD* is a multicopy suppressor of *fabH*-associated synthetic lethal phenotypes.** The ppGpp^0 Δ*fabH*/pRCspoT strain grew poorly in the absence of IPTG, that is, during decreased SpoT expression. Using a genomic library constructed in plasmid pACYC184, the genes(s) that rescued the growth defect of the ppGpp^0 Δ*fabH*/pRCspoT strain was identified. Rescue of the growth defect of the ppGpp^0 Δ*fabH*/pRCspoT strain by four

such clones is shown in Fig. S3A. Sequencing of the plasmid-chromosome junctions showed that one clone carried *dksA* and adjacent genes and that another carried N-terminally truncated SpoT, complete *trmH*, and truncated *recG* (Fig. S3B). The recovery of the clone carrying DksA was expected, because, as described above, overexpression of DksA supported the growth of the ppGpp^0 Δ*fabH* strain. The truncated *spoT* gene supported the growth of the ppGpp^0 strain in minimal glucose medium (data not shown), suggesting that there was (p)ppGpp synthesis in the presence of the plasmid; these multicopy suppressors were not studied further.

Two clones carried the *dtd* gene and different lengths of flanking regions having genes of unknown function (Fig. S3B). A plasmid segregation assay showed that each clone could support the growth of the ppGpp^0 Δ*fabH* strain (Fig. S3C). We focused on the *yiiD* and *dtd* open reading frames (ORFs) that were present in both plasmids. Their role in the suppression of the growth defect was tested using the pCAyiiD and pCAdtd clones from the ASKA collection along with pCAfabH, which served as a positive control. pCAfabH and pCAyiiD but not the vector pCA24N suppressed the growth defect of the ppGpp^0 *fabH*/pRCspoT strain when SpoT expression was lowered by the removal of IPTG (Fig. 3A); growth rescue by *yiiD* was comparable to that seen with *fabH*, and the leaky expression of these proteins in the absence of the inducer IPTG was sufficient for suppression. pCAdtd did not rescue the growth defect of the ppGpp^0 *fabH*/pRCspoT strain (data not shown). To further verify that *yiiD* expression was capable of supporting the growth of the ppGpp^0 *fabH* strain, the plasmid segregation assay was performed using pBADyiiD, where the expression of *yiiD* was arabinose regulated. The ppGpp^0 Δ*fabH*/pRC$_{sp}$-spoT strain was transformed with pBADyiiD and the vector pBAD24, and the ability of the transformants to grow without pRC$_{sp}$-spoT was studied using the plasmid segregation assay. pBADyiiD but not the vector pBAD24 supported pRC$_{sp}$-spoT-independent growth in the presence of arabinose (Fig. 3B). Similarly, the presence of pCAyiiD but not the vector pCA24N supported pRCfabH-independent growth of the Δ*dksA*::Kan Δ*fabH*/pRCfabH strain (Fig. 3C). We conclude that the expression of *yiiD* from a multicopy plasmid suppressed the ppGpp^0 *fabH* and Δ*dksA*::Kan Δ*fabH* synthetic lethal phenotypes.

The altered fatty acid composition of the *fabH* mutant is largely rescued by *yiiD* expression. The fatty acid composition is altered in the *fabH* mutant of *E. coli* (29) and following the overexpression of FabH (3). We asked if the rescue of the *fabH* growth defect by *yiiD* expression was associated with changes in the composition of fatty acids. The fatty acid methyl ester (FAME) composition was determined in the wild-type and *fabH* mutant strains carrying the plasmid vector pCA24N and the *fabH* mutant expressing *yiiD* from pCAyiiD. The percentages of $C_{14:0}$, $C_{16:0}$, and $C_{16:1}$ fatty acids were each decreased in the *fabH* mutant; together, they represented only 38.1% of the major fatty acid species in the *fabH* mutant, compared to 67.1% in the wild type. This was rescued to a large extent by the overexpression of *yiiD* (59.2%) (Table 1). The ratio of $C_{16:1}$ to $C_{18:1}$ decreased ~10-fold in the *fabH* mutant compared to the wild type, and this was alleviated to an ~3-fold decrease following the expression of *yiiD*. We observed a significant increase in the amount of the minor fatty acid species $C_{19:0}$ cyclo ω8c in the *fabH* mutant (5.22% of the total) compared to the wild type (0.56% of the total), and this was reversed by the expression of *yiiD* in the *fabH* mutant (0.63% of the total). These results showed that YiiD expression could compensate to a large extent for the altered fatty acid composition of the *fabH* mutant. Although the biochemical function performed by YiiD is not apparent from these results, given that the *yiiD* gene product has an N-terminal acetyltransferase domain and a C-terminal thioesterase domain (see Discussion), the above-described results suggest that YiiD activity could directly contribute to fatty acid synthesis. Therefore, we have renamed *yiiD* as *fabY* and refer to it here as *fabY*.

Overexpression of *fabY* rescues the growth and cell size defects of the *fabH* mutant. Since the primary function of FabH was in the initiation of fatty acid biosynthesis (3), it may be assumed that the slow growth of the *fabH* mutant arose from the reduced fatty acid biosynthetic capacity of the cell. Due to the reduced fatty acid

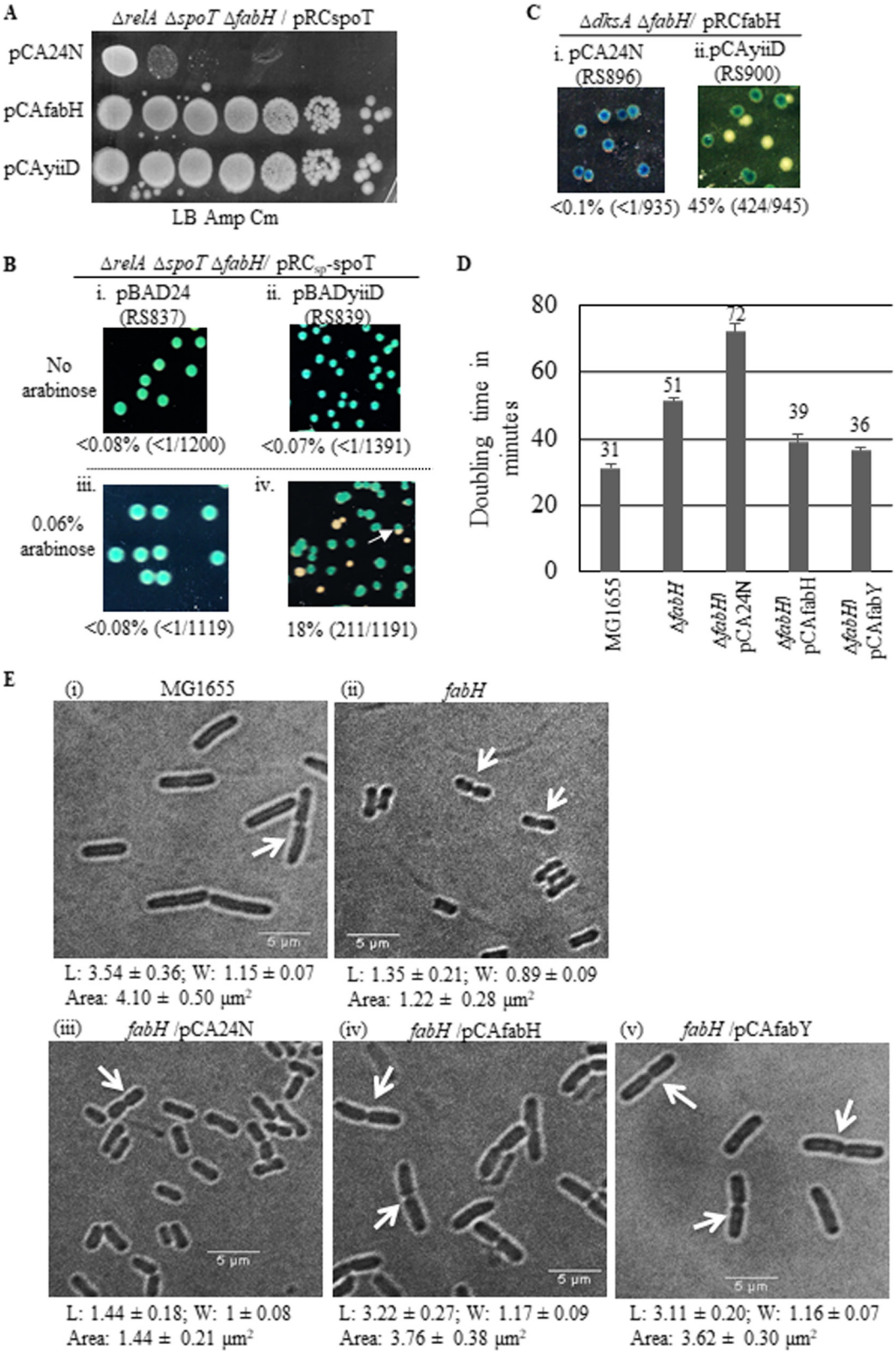

FIG 3 Expression of *yiiD* from plasmids suppresses ppGpp⁰ *fabH* and *dksA fabH* synthetic lethality and rescues the growth defect and cell size defect of the *fabH* mutant. (A) The rescue of ppGpp⁰ Δ*fabH* synthetic lethality by the ASKA plasmids pCAfabH and pCAyiiD was studied by spotting serial dilutions of the strains whose relevant genotypes are indicated. pCA24N is the vector used for the cloning of *fabH* and *yiiD*. (B) Retention or loss of unstable plasmid pRC_{sp}-spoT was scored by the blue-white plasmid segregation assay in LB agar plates containing X-gal and IPTG in the presence or absence of arabinose. The arrow indicates a sectored colony. (C) Retention or loss of unstable plasmid pRCfabH was studied in LB agar plates containing

(Continued on next page)

TABLE 1 Profile of major fatty acids in the wild type, the *fabH* mutant, and the *fabH* mutant expressing *yiiD*

	% fatty acid content for:					
	MG1655/pCA24N		Δ*fabH*/pCA24N strain		Δ*fabH*/pCAyiiD strain	
Fatty acid species	Avg	SD	Avg	SD	Avg	SD
12:00	4.14	0.01	3.53	0.13	4.42	0.01
14:00	8.68	0.08	3.49	0.11	6.31	0.04
14:0 3-OH	9.31	0.27	8.80	0.32	9.39	0.19
16:1 ω7c	16.88	0.52	7.14	1.60	16.09	0.09
16:00	37.41	0.27	25.71	0.09	35.85	0.53
17:0 cyclo	8.76	0.65	7.05	1.25	3.81	0.13
18:1 ω7c	7.50	0.26	32.99	1.30	20.76	0.77
19:0 cyclo ω8c	0.56	0.11	5.22	2.17	0.63	0.01
18:00	0.62	0.03	1.41	0.16	1.20	0.04
Total	93.86		95.34		98.46	

biosynthesis, the *fabH* mutant has a 70% reduced cell volume and 50% reduced area compared to the wild-type strain (29). We asked if the overexpression of *fabY* rescued the growth and cell size defects of the *fabH* mutant. The doubling time of the *fabH* mutant was significantly longer than that of the wild-type strain (Fig. 3D). We studied the rescue of the *fabH* growth defect using the plasmid pCAyiiD (pCAfabY), where *fabY* was expressed from an IPTG-inducible promoter. The addition of 1 mM IPTG inhibited the growth of the *fabH*/pCAfabH and *fabH*/pCAfabY strains (data not shown); we therefore studied growth without IPTG. Introduction of the plasmid vector pCA24N slowed down the growth of the *fabH* mutant (Fig. 3D); the reason for this is not clear. On the other hand, the presence of pCAfabY or pCAfabH increased the growth rate of the *fabH* mutant. For the measurement of cell size, the log-phase cultures used for doubling time measurements were used for microscopy. As expected, the cell size of the *fabH* mutant was smaller than that of the wild type (Fig. 3E). Plasmids pCAfabH and pCAfabY but not the vector pCA24N increased the cell size of the *fabH* mutant, and the size of the cells was similar to that of the wild-type strain (Fig. 3E). These results indicate that restoration of fatty acid synthesis was sufficient to rescue the growth and cell size defects of the *fabH* mutant and lend support to the model that fatty acid biosynthesis plays a central role in regulating the size of *E. coli* cells (29, 30).

Positive regulation of *yiiD* transcription by (p)ppGpp and DksA in the absence of FabH function. The data obtained with the site 1^- and/or site 2^- RNA polymerase alleles supported the idea that the loss of (p)ppGpp-mediated transcriptional regulation was responsible for ppGpp^0 Δ*fabH* lethality (Fig. 2A). Since expression of *fabY* from multicopy plasmids suppressed the synthetic lethal phenotype, one possibility was that reduced *fabY* expression was the cause of synthetic lethality in the ppGpp^0 Δ*fabH* and Δ*dksA* Δ*fabH* strains.

To study the transcriptional regulation of *fabY*, a *fabY-lac-kan* fusion was generated at the chromosomal *fabY* locus by FLP-mediated recombination using the plasmid pKG137 and the Δ*yiiD*::FRT (FLP recombination target) allele derived from the Keio collection (32, 42). The Δ*yiiD* mutant did not exhibit any growth defect (data not shown). As shown in Fig. S4, *fabY* is the last gene in an operon comprising the *yihW*, *yihX*, *yihY*, and *dtd* genes. β-Galactosidase expression from the *fabY-lac* fusion was

FIG 3 Legend (Continued)
X-gal. The percentages of white colonies were estimated as described in the legend to Fig. 1. (D) The doubling time of cells was determined for wild-type and Δ*fabH* strains in LB medium and for Δ*fabH*/pCA24N (RS453), Δ*fabH*/pCAfabH (RS186) (white colony), and Δ*fabH*/pCAyiiD (RS187) (white colony) strains in LB medium containing Cm. The mean values from three independent experiements are plotted, and error bars represent the standard errors of the means. (E) Representative fields with cells of the indicated strains. Arrows indicate "8"-shaped cells that are close to cell division and used for length, width, and area measurements. The mean length (L), width (W), and area obtained from 50 "8"-shaped cells for each strain are indicated (see "Estimation of cell size by microscopy" in Materials and Methods for details).

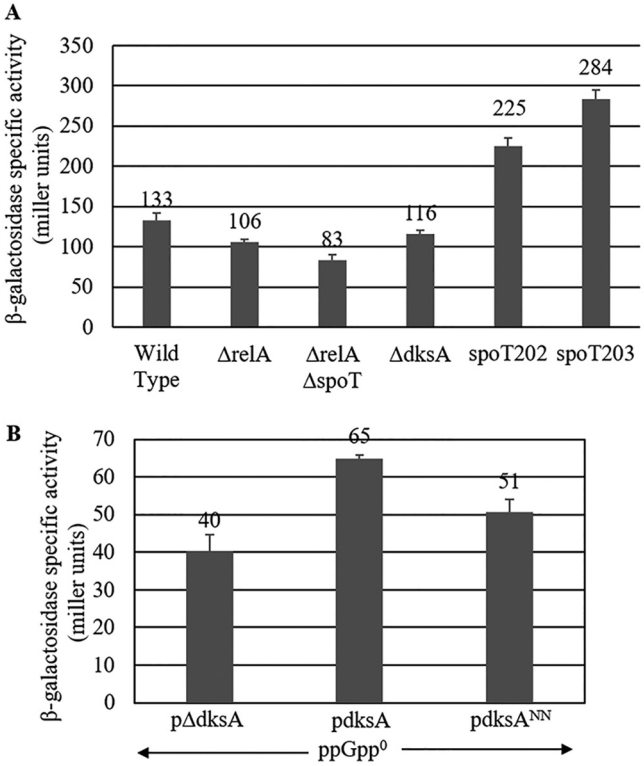

FIG 4 Transcriptional regulation of *yiiD-lac* expression by (p)ppGpp/DksA. Strains lacking the native *lac* operon and carrying the *fabH-lac* fusion were grown in LB medium, and the β-galactosidase activity was measured by collecting cells at mid-log phase. The relevant genotype of each strain is indicated. Values are the mean values from 3 to 5 independent experiments, and the error bars indicate standard errors of the means. (A) Wild-type (RS198), Δ*relA* (RS223), Δ*relA* Δ*spoT* (RS224) (white colony), Δ*dksA* (RS959), *spoT202* (RS243), and *spoT203* (RS245) strains; (B) ppGpp^0 strain carrying the plasmid pdksA (RS964), pdksA^{NN} (RS965), or pΔdksA (RS966).

studied in an otherwise wild-type strain, the *relA* mutant, ppGpp^0, and strains with elevated basal (p)ppGpp levels. Compared to the wild type, the *relA* and ppGpp^0 strains showed a slight decrease in β-galactosidase activity, while the strains with elevated (p)ppGpp levels showed ~2-fold-increased activity (Fig. 4A). The β-galactosidase activity in the *dksA* mutant was not significantly different from that of the wild-type strain. Since the overexpression of DksA (but not DksA^{NN}) suppressed ppGpp^0 Δ*fabH* synthetic lethality, we asked if this affected *fabY* expression. In the ppGpp^0 strain, a slight increase in activity was observed following the overexpression of DksA and, to a lesser extent, DksA^{NN} (compared to the vector) (Fig. 4B). These results are consistent with a weak positive regulation of *fabY* expression by (p)ppGpp, because, starting with the ppGpp^0 strain, increasing the basal (p)ppGpp pool correlates with an increase in *fabY-lac* expression (ppGpp^0 < *relA* < WT < *spoT202* < *spoT203*).

We wanted to study *fabY* expression in the Δ*fabH* mutant. However, transductants could not be recovered despite several attempts to introduce the *fabY-lac* fusion into the *fabH* mutant by phage P1 transduction using the linked kanamycin (Kan) marker. Using the plasmid segregation assay, we tested if the Δ*fabH* and Δ*fabY* mutations were synthetically lethal. The unstable plasmid pRCfabH was introduced into the Δ*fabY* mutant, and subsequently, the Δ*fabH*::Kan allele was introduced by phage P1 transduction to construct the Δ*fabY*::FRT Δ*fabH*::Kan/pRCfabH strain. Plasmid loss frequencies were compared between the Δ*fabY*::FRT Δ*fabH*::Kan/pRCfabH and the Δ*fabY*::FRT/pRCfabH strains using the plasmid segregation assay. Loss of pRCfabH was observed in the Δ*fabY* strain (white colonies) but not in the Δ*fabY* Δ*fabH* background (Fig. 5A), indicating that the *fabY*-encoded function was essential for the growth of the *fabH*

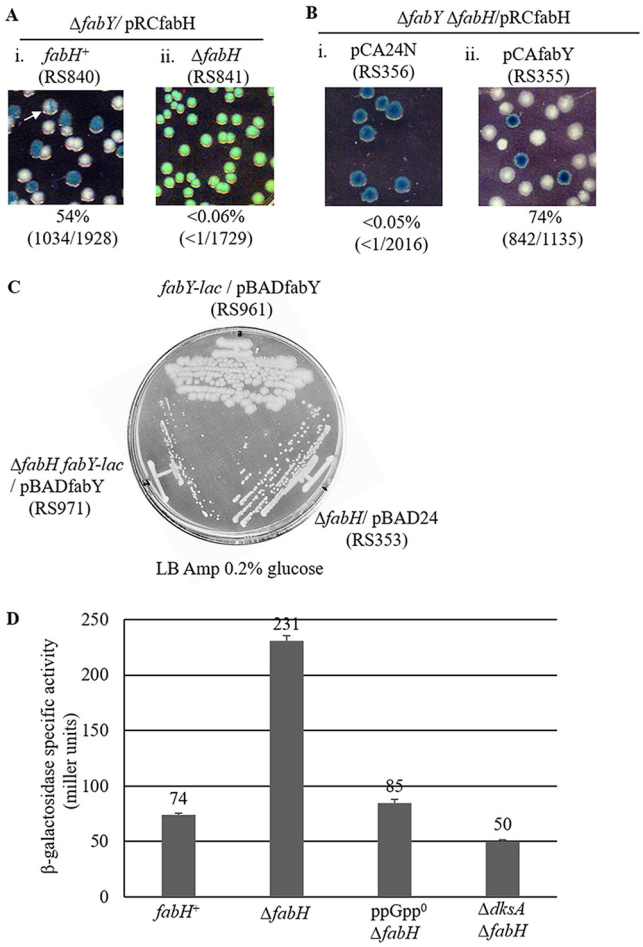

FIG 5 Synthetic lethality of Δ*fabH* and Δ*fabY* mutations and positive regulation of *fabY* transcription by (p)ppGpp and DksA in the absence of FabH function. (A and B) Retention or loss of unstable plasmid pRCfabH was scored using the blue-white plasmid segregation assay in LB agar plates containing X-gal. The percentages of white colonies were estimated as described in the legend to Fig. 1. The relevant genotype and strain number are indicated above each image. The arrow indicates a sectored colony. (C) Strains were streaked on LB agar plates containing 0.2% glucose and Amp and photographed after 24 h of incubation at 37°C. (D) *yiiD-lac* expression in strains carrying the Δ*fabH* lesion was measured. All strains carried the pBADfabY plasmid. In the strains with the Δ*fabH* mutation, β-galactosidase activity was measured in LB medium using an arabinose concentration that suppressed the lethality and supported growth similar to that of the *fabH* mutant. *fabH*$^+$/pBADfabY (RS961) and Δ*fabH*/pBADfabY (RS971) strains were cultured in LB medium with 0.2% glucose (no arabinose), the Δ*relA* Δ*spoT* Δ*fabH*/pBADfabY (RS981) strain was cultured in LB medium containing 0.06% arabinose, and the Δ*dksA* Δ*fabH*/pBADfabY (RS982) strain was cultured in LB medium containing 0.02% arabinose. The β-galactosidase activities are the mean values from four independent experiments, and the bars indicate the standard errors of the means.

mutant. Consistent with this idea, the plasmid segregation assay showed that the loss of pRCfabH could be tolerated in the Δ*fabY* Δ*fabH*/pCAfabY background (Fig. 5B).

Since Δ*fabH* was synthetic lethal with Δ*fabY*, in order to study *fabY* expression in the *fabH* background using the *fabY-lac* fusion, *fabY* was provided using pBADfabY (referred to as pBADyiiD previously). In order to mimic the physiological state of the *fabH* mutant, *fabY-lac* expression was studied under conditions where the growth of the *fabH fabY-lac*/pBADfabY strain was similar to that of the *fabH* mutant. In the absence of arabinose and the presence of glucose, growth of the *fabH fabY-lac*/pBADfabY strain was similar to that of the *fabH*/pBAD24 strain (Fig. 5C) and suggested that the aggregate FabY function in the Δ*fabH fabY-lac*/pBADfabY strain could be similar to that in the Δ*fabH* mutant. Under these growth conditions, we found that the β-galactosidase activity in the Δ*fabH fabY-lac*/pBADfabY strain was 3-fold higher than that in the isogenic *fabH*$^+$ strain (Fig. 5D). Furthermore, the increased *fabY-lac* expression in the *fabH*

mutant was dependent on (p)ppGpp or DksA; expression was 2.7- and 4.5-fold decreased in the absence of ppGpp and *dksA*, respectively (Fig. 5D). Note that the DksA requirement for *fabY* expression can be clearly observed in the Δ*fabH* background but not in the *fabH*$^+$ background (compare Fig. 4A and 5D). These results support the idea that a reduction in FabH function could signal (p)ppGpp- and DksA-dependent induction of *fabY* expression.

Suppression of *fabH*-associated synthetic lethality by the stringent RNAP alleles or overexpression of (p)ppGpp/DksA requires FabY function. The stringent RNA polymerase alleles support stringent transcriptional regulation independent of (p)ppGpp or *dksA* (10, 43–45). We tested if some of these alleles rescued ppGpp^0 *fabH* and *dksA fabH* synthetic lethality. The plasmid segregation assay showed that three alleles tested, *rpoBL571P*, *rpoBT563P*, and *rpoBH1244Q*, suppressed *dksA fabH* and ppGpp^0 *fabH* synthetic lethality (Fig. S5). Consistent with the idea that the stringent RNAP alleles can mimic the transcriptional effects of (p)ppGpp (10, 43–45), all the alleles increased *fabY* expression in the ppGpp^0 strain (Fig. 6A). The increase was less pronounced in the case of the *rpoBH1244Q* allele (also referred to as *rpoB**35*) than with the *rpoBL571P* and *rpoBT563P* alleles. The increase in *fabY-lac* expression and the suppression of ppGpp^0 *fabH* lethality by the stringent alleles reinforce the association between the two phenotypes. In fact, the suppression of ppGpp^0 Δ*fabH* and Δ*dksA* Δ*fabH* synthetic lethality by the stringent RNAP alleles was *fabY* dependent (Fig. 6B). Δ*fabH* Δ*fabY* synthetic lethality was not suppressed by the stringent alleles (Fig. 6C). Furthermore, the rescue of Δ*dksA* Δ*fabH* synthetic lethality by the *spoT202* and *spoT203* alleles was lost upon *fabY* inactivation (Fig. 6D). *fabY* was also required for the suppression of ppGpp^0 Δ*fabH* lethality by DksA overexpression (data not shown). These results strongly suggested that the positive regulation of *fabH* transcription by (p)ppGpp and DksA was required for the growth of the *fabH* mutant.

***fabH* expression is not driven by a single well-defined promoter.** Since *fabY* is part of an operon with at least three other genes, namely, *yihX*, *yihY*, and *dtd*, these other genes may be functionally related to *fabY*. Therefore, the contribution of these genes to the growth of the *fabH* mutant was examined. A nonpolar deletion of each gene (so as to not affect the expression of *fabY*) was made in the Δ*fabH*/pRCfabH strain, and the plasmid segregation assay was used to score for synthetic growth defects. None of these mutations conferred synthetic lethality with the Δ*fabH* mutation (Table S2), indicating that only the *fabY*-encoded function but not those of the other genes in the operon was required for the growth of the *fabH* mutant.

In order to identify a promoter(s) of *fabY*, polar insertions or insertions-deletions were introduced at different locations upstream of the *fabY* gene (Fig. S4), and their effect on *fabY-lac* expression was studied (Fig. 7). The effect of each insertion on the growth of the *fabH* mutant was also monitored (Table S3) since a reduction in *fabY* expression can confer growth inhibition to the Δ*fabH* mutant. A promoter has been reported upstream of *yihX* from a genome-wide transcription start site mapping study (46). To study the role of this promoter in *fabY* expression, two deletions-insertions were introduced so as to delete a 50-bp (Δ50::Cm) or a 100-bp (Δ100::Cm) fragment upstream of the *yihX* start codon and replace it with the chloramphenicol (Cm) cassette from plasmid pKD3 (47) (Fig. S4). (p)ppGpp-mediated regulation was still evident in the strains with the deletion; that is, as observed in the strain without the deletion, *fabY* expression was decreased in the ppGpp^0 strain and increased in the presence of *spoT202* or *spoT203* mutations that increase the basal (p)ppGpp level (Fig. 7A). The plasmid segregation assay performed using the *fabH*/pRCfabH strain without the deletion and isogenic strains with the deletions-insertions showed that the removal of the reported promoter did not affect the growth of the *fabH* mutant; the white colonies showed comparable growth with and without the deletion (Fig. 7B). This indicated that the *yihW-yihX* intergenic region was not the major determinant for *fabY* expression and its regulation by (p)ppGpp.

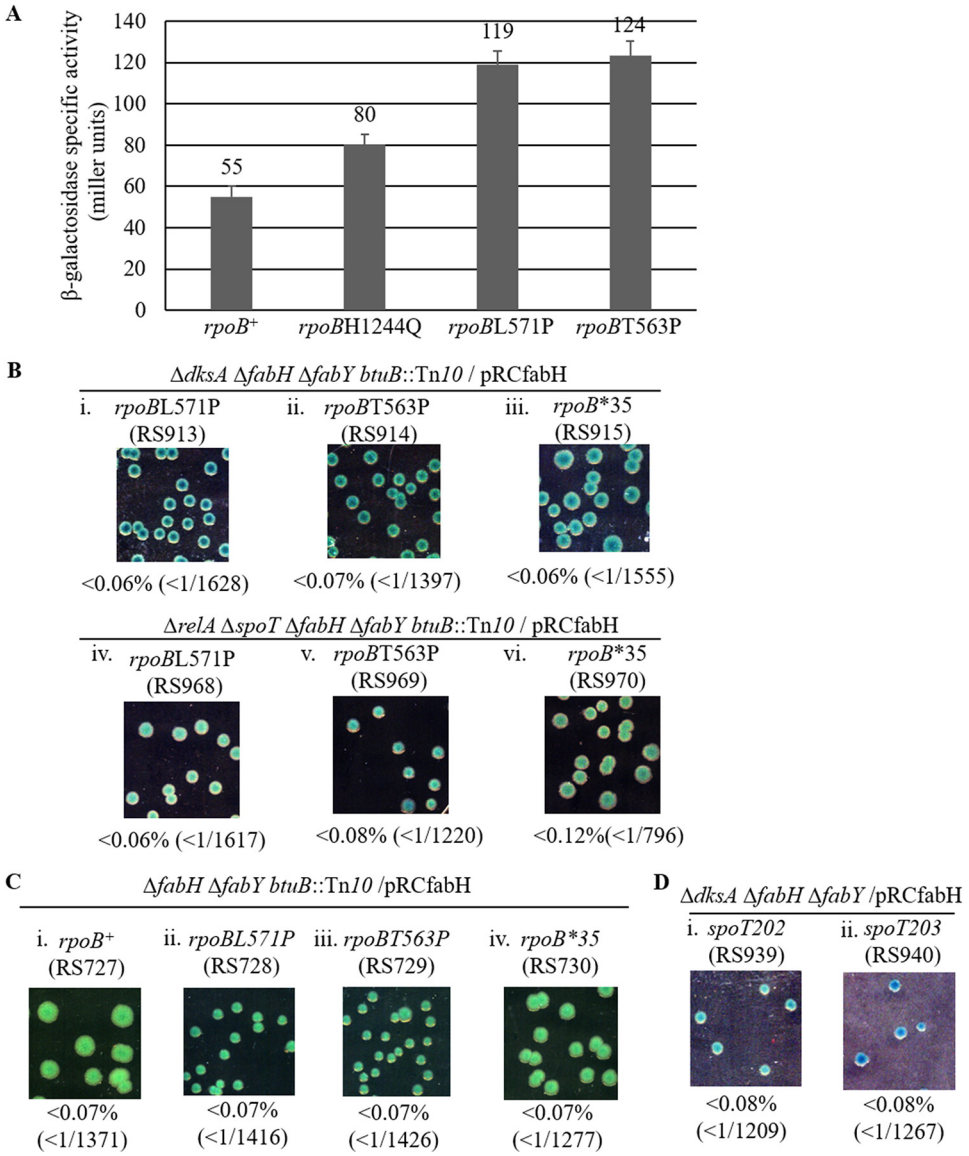

FIG 6 Suppression of *fabH*-associated synthetic lethality by the stringent *rpoB* mutants or by an increase in (p)ppGpp requires FabH function. (A) *fabY-lac* expression in the ppGpp^0 strain carrying the indicated stringent *rpoB* alleles. The *β*-galactosidase activity was measured at mid-log phase during growth in LB medium. The bars indicate the standard errors of the means calculated from three independent experiments. The strains are the Δ*relA* Δ*spoT btuB*::Tn*10 rpoB*$^+$ (RS892), Δ*relA* Δ*spoT btuB*::Tn*10 rpoBL571P* (RS893), Δ*relA* Δ*spoT btuB*::Tn*10 rpoBT563P* (RS894), and Δ*relA* Δ*spoT btuB*::Tn*10 rpoB*35* (RS895) strains. (B to D) Retention or loss of unstable plasmid pRCfabH was scored by the blue-white segregation assay in LB agar plates containing X-gal. The relevant genotype of the strains is indicated, and the percentages of white colonies were estimated as described in the legend to Fig. 1C.

To ask if transcripts originating upstream of the *yihW-yihX* intergenic region or from within the *yihX-yihY-dtd* open reading frames contribute to *fabY* expression, polar pKD3 insertions were made at four locations, as shown in Fig. S4. The insertions were made such that they do not disrupt the flanking ORFs. The plasmid segregation assay showed that the *dtd*::Cm insertion placed between the *dtd* and *fabY* ORFs was synthetically lethal with the Δ*fabH* mutation (Table S3) and decreased *fabY-lac* expression 6.5-fold (Fig. 7C), as expected for a polar insertion. As the insertion was progressively moved away from the *fabY* ORF, *fabY-lac* expression progressively increased (Fig. 7C). In the Δ*fabH*/pRCfabH strain, white colonies were recovered in the plasmid segregation assay, and the growth rate of the white colonies increased (Table S3). These results suggest that promoters located within the *yihX-yihY-dtd* ORFs contribute to *fabY* expression.

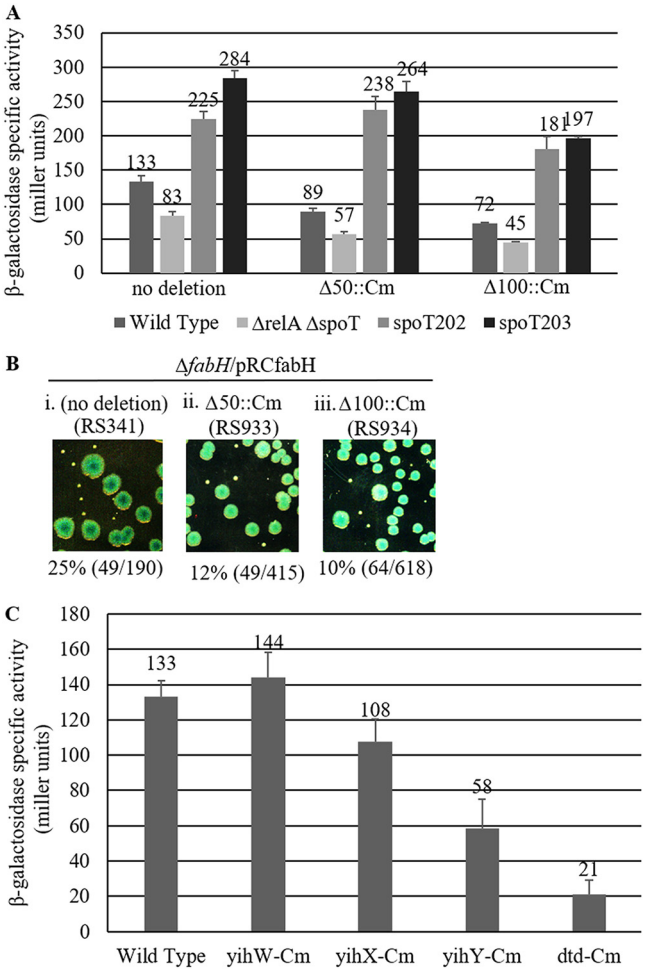

FIG 7 *fabH* expression is not driven from a single well-defined promoter. (A) *fabY-lac* expression in wild-type (RS198), Δ*relA* Δ*spoT* (RS224) (cured of pRC*spoT*), *spoT202* (RS243), and *spoT203* (RS245) strains with or without the 50-bp (Δ50::Cm) or 100-bp (Δ100::Cm) polar insertion-deletion in the *yihW-yihX* intergenic region (see Fig. S4 in the supplemental material), including Δ50::Cm (RS925), Δ*relA* Δ*spoT* Δ50::Cm (RS927), *spoT202* Δ50::Cm (RS929), *spoT203* Δ50::Cm (RS931), Δ100::Cm (RS926), Δ*relA* Δ*spoT* Δ100::Cm (RS928), *spoT202* Δ100::Cm (RS930), and *spoT203* Δ100::Cm (RS932) strains. β-Galactosidase activity was measured by collecting cells at mid-log phase during growth in LB medium at 37°C. The error bars indicate the standard errors of the means calculated from three independent experiments. (B) Retention or loss of unstable plasmid pRCfabH was scored by the blue-white plasmid segregation assay in LB agar plates containing X-gal. (C) *fabY-lac* expression was measured as described above in strains with an insertion at different positions in the operon (shown in Fig. S4), including wild-type (RS198), *yihW*-Cm (RS780), *yihX*-Cm (RS781), *yihY*-Cm (RS782), and *dtd*-Cm (RS783) strains.

***fabY* expression does not rescue *fabB15*(Ts)- or Δ*fabF*-associated growth defects.** In addition to FabH, there are two β-ketoacyl-ACP synthases, namely, FabB and FabF, in *Escherichia coli* (1, 2). We asked if the deletion or increased expression of *fabY* affected the growth phenotypes of the *fabB15*(Ts) or Δ*fabF* mutant. The *fabB15*(Ts) allele does not grow at 42°C due to the loss of unsaturated fatty acid biosynthesis, and this can be overcome by oleic acid supplementation (48, 49) (Table S4). While *fabB* expression using pCAfabB rescued the growth defect, *fabY* expression did not (Table S4), indicating that it cannot compensate for the β-ketoacyl-ACP synthase I deficiency. The *fabF* function is dispensable for the growth of *E. coli* with FabB activity. Like the Δ*fabF* mutant, the Δ*fabF* Δ*fabY* double mutant showed no growth defect in LB medium (data not shown). The inactivation of *fabF* in the *fabB15*(Ts) genetic background blocks saturated and unsaturated fatty acid biosynthesis and confers a requirement of palmitate and oleate for growth at 42°C (50) (Table S4). Expression of *fabY* did not support the growth of this strain in the presence of oleate at 42°C, indicating that it cannot

compensate for the β-ketoacyl-ACP synthase II deficiency (Table S4). Thus, the *fabY*-encoded function seems to specifically substitute for FabH but not FabB or FabF. Like *fabY*, the expression of *fabH* from a plasmid did not rescue the growth defect of the *fabB15*(Ts) or *fabF fabB15*(Ts) strain (Table S4).

Expression of FabB or FabF does not rescue the *fabH*-associated growth defects. Biochemical studies had indicated that the residual initiation of fatty acid biosynthesis in the *fabH* mutant could arise from the inefficient side reactions performed by the FabB and FabF enzymes, namely, the synthesis of acetyl-ACP from malonyl-ACP through decarboxylation. If the slow growth of the *fabH* mutant is due to the reduced rate of initiation of fatty acid biosynthesis, it is possible that increasing the expression of FabB or FabF could increase the rate of initiation of fatty acid biosynthesis and improve the growth of the *fabH* mutant. Using pCAfabB and pCAfabF clones from the ASKA collection, which are functional (Table S4), we asked if the phenotypes of the *fabH* mutant were altered by the expression of *fabB* or *fabF*. Neither the growth nor the synthetic phenotypes of the *fabH* mutant were rescued with pCAfabB or pCAfabF (Table S5), suggesting that the reduced initiation of fatty acid biosynthesis in the *fabH* mutant was not alleviated by increased expression of FabB or FabF. The absence of rescue by *fabB* expression is noteworthy because overproduction of FabB imparted resistance to thiolactomycin (51), which inhibits FabH, FabB, and FabF.

Phospholipid deficiency is not solely responsible for the growth defect of the *fabH yiiD* mutant. Our data are consistent with the idea that the initiation of fatty acid biosynthesis in the absence of FabH activity was *fabY* mediated and that the growth defects of the Δ*fabH* Δ*fabY*, ppGpp^0 Δ*fabH*, and Δ*dksA* Δ*fabH* strains arise from the abrogation of the initiation of fatty acid biosynthesis, specifically the loss of acetoacetyl-ACP synthesis. In addition to phospholipids, acetoacetyl-ACP is required for the synthesis of essential molecules such as lipid A and coenzyme lipoic acid (1, 52). Therefore, if the synthesis of acetoacetyl-ACP was blocked in the Δ*fabH* Δ*fabY*, ppGpp^0 Δ*fabH*, or Δ*dksA* Δ*fabH* mutant, we expected that the growth defect of these strains could not be rescued by palmitate and oleate. The plasmid segregation assay showed that palmitate and oleate did not support the growth of these strains (Fig. 8A) but supported the growth of the parental strains under these conditions. On the other hand, palmitate and oleate supplementation weakly supported the growth of the *fabB15*(Ts) Δ*fabF* Δ*fabH* strain in LB medium at 42°C. This suggested that a limited synthesis of acetoacetyl-ACP and the essential intermediates from this molecule may be possible in the absence of the three known ketoacyl-ACP synthases (Fig. 8B). Possibly, the *fabY*-encoded function weakly supported the synthesis of acetoacetyl-ACP, lipoic acid, and lipid A in the absence of FabB, FabF, and FabH. Consistent with this idea, growth of the *fabB15*(Ts) Δ*fabF* Δ*fabH* mutant improved following the expression of *fabY* from a plasmid (Fig. 8B).

DISCUSSION

In this study, we have identified genetic changes that conferred synthetic lethality in the *fabH* background and an overexpression suppressor of the synthetic lethal phenotype. Based on the genetic evidence presented in this study, we propose that the *yiiD* (renamed *fabY*)-encoded function compensates for the loss of *fabH* by supporting the biochemical activity required for the synthesis of acetoacetyl-ACP. One long-standing question in the area of fatty acid biosynthesis has been regarding the source of the "primer" carbon atoms (the last two carbons of the fatty acid chain by chemical nomenclature) (1). The current understanding is that the majority of these carbon atoms are incorporated directly from acetyl-coA through the FabH (β-ketoacyl-ACP synthase III)-catalyzed acetoacetyl-ACP synthase reaction and that a minor fraction is incorporated indirectly from acetyl-coA following the synthesis of acetyl-ACP. The synthesis of acetoacetyl-ACP using acetyl-ACP is catalyzed by FabB (β-ketoacyl-ACP synthase I) and FabF (β-ketoacyl-ACP synthase II). Redundancy in the synthesis of acetoacetyl-ACP, the four-carbon precursor essential for fatty acid synthesis, was one possible explanation for the nonessentiality of FabH function in *Escherichia coli* (29, 32). However, our results indicate that, even though FabB and FabF catalyzed acetoacetyl-

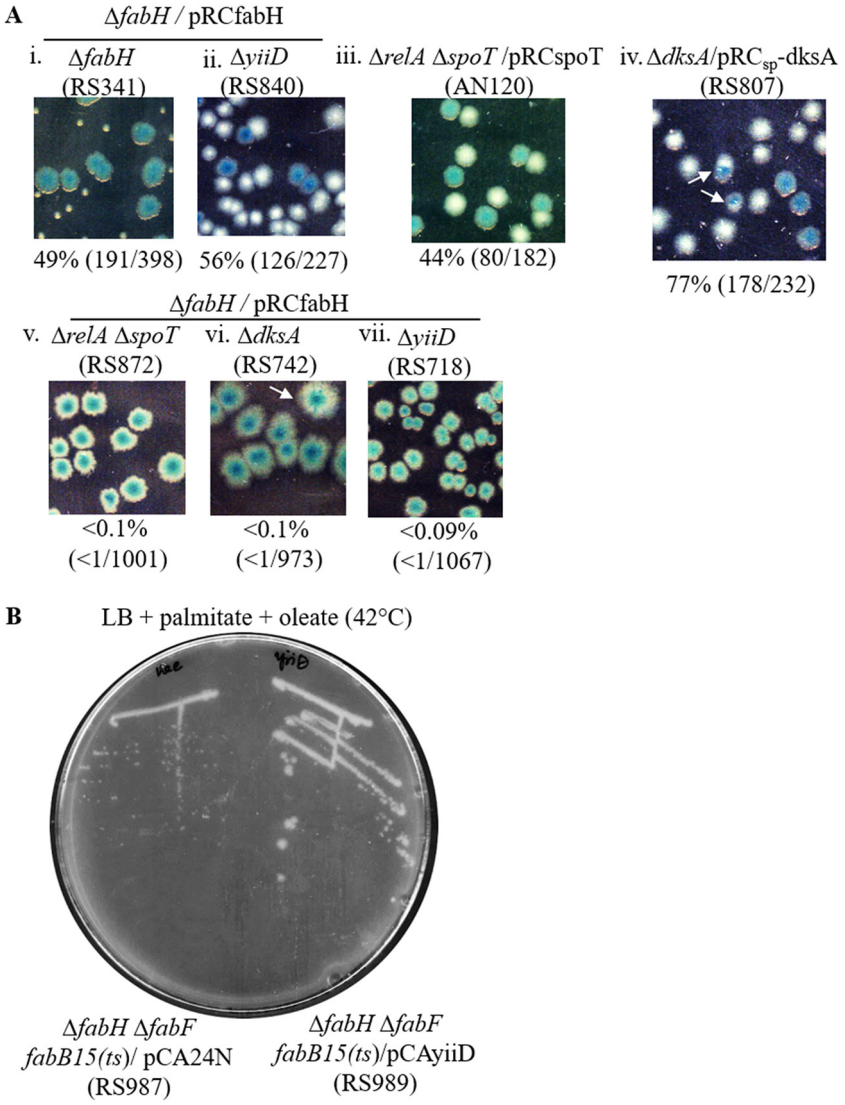

FIG 8 Oleic acid and palmitic acid do not rescue the *fabH*-associated synthetic lethalities, but *yiiD* expression rescues the growth defect of the Δ*fabH* Δ*fabF fabB15* strain at 42°C. (A) Retention or loss of unstable plasmid pRCfabH was studied by the blue-white plasmid segregation assay in LB agar plates containing oleic acid, palmitic acid, and X-gal. The relevant genotype for each strain is indicated, and the percentages of white colonies were estimated as described in the legend to Fig. 1. The arrows indicate sectored colonies. (B) Strains were streaked on LB agar plates supplemented with 0.01% oleic acid and 0.05% palmitic acid and incubated at 42°C for 24 h.

ACP synthesis *in vitro*, this activity was unavailable or insufficient to support the growth of *Escherichia coli* lacking FabH and that the *fabY*-encoded activity was required to support the growth of the *fabH* mutant. Consistent with a role in fatty acid biosynthesis, *fabY* expression rescued the altered fatty acid profile of the *fabH* mutant. *fabY* expression completely rescued the cell size defect of the *fabH* mutant, and this is consistent with the idea that the fatty acid biosynthetic capacity of the cell is an important determinant of cell size in *E. coli*.

No amino acid sequence similarity can be observed between FabY and either FabH, FabB, or FabF. The database of protein families (Pfam) showed that *yiiD* has two domains, an N-terminal acetyltransferase domain and a C-terminal thioesterase domain. It is possible that FabY catalyzes the synthesis of acetoacetyl-ACP by cleaving the acetyl moiety of acetyl-CoA using the thioesterase activity and transfers it to malonyl-ACP utilizing the acetyltransferase activity. Although we propose that FabY might carry

out acetoacetyl-ACP synthesis, we think that it is unlikely to directly substitute for the biochemical reaction carried out by FabH. This is because FabY does not share functional domains with FabH. FabH, FabB, and FabF represent two classes of decarboxylating Claisen condensation enzymes. While the FabB and FabF enzymes have Cys-His-His active sites, FabH has a Cys-His-Asn active-site triad. These differences are reflected in the rest of the primary sequences of the proteins. FabB and FabF are about 37% identical, whereas alignment of either FabB or FabH with FabH gives scattered alignments of very low quality (see reference 1 and references therein). Is it possible that FabY catalyzed the transfer of the acetyl moiety from acetyl-CoA to ACP to make acetyl-ACP, which is then used for acetoacetyl-ACP synthesis by FabB/FabF? We do not favor this idea, because the growth defect of the *fabB15*(Ts) Δ*fabF* Δ*fabH* strain improved following *yiiD* expression (Fig. 8B).

Another line of evidence supporting a role for *fabY* in fatty acid biosynthesis was its reported interaction with AcpP in a study that looked at the protein-protein interaction network in *E. coli* (53). Based on our results, we propose that the interaction could be between FabY and malonyl-ACP. Our results have important implications for the choice of FabH as an antimicrobial drug target (54, 55). It was reported that fast-growing bypass suppressors accumulate in the *fabH* mutant, and these were suspected to arise from mutations in the *fabB* and *fabF* genes (1) and were expected to reduce the effectiveness of drugs that inhibit FabH. The *fabH* mutant used here gave rise to a slow-growth phenotype, but the strain was genetically stable. Accumulation of suppressors was not evident in either the *fabH* mutant or the *fabH fabY* double mutant (data not shown). Our results indicate that complete inhibition of the initiation of fatty acid biosynthesis can be achieved following the inactivation of *fabH* and *fabY* in *E. coli*. Identification of a molecule(s) that can inhibit the two activities could lead to the development of effective antimicrobials, and understanding the structure and biochemical properties of FabY would be crucial for the development of such molecules. We predict that the need to inhibit both enzymes will not be limited to *E. coli*, since a taxonomic profile generated using the EGGNOG database for proteins carrying the acetyltransferase and thioesterase domains showed that the distribution of such proteins was seen primarily across proteobacteria (94%) and, to a lesser extent, in other phyla such as firmicutes, chlamydia, fusobacteria, chloroflexi, and bacteroidetes. Although FabY and FabH are unrelated by sequence, it is possible that they are structurally related.

Evidence presented here suggests that in the absence of *fabH* function, *fabY* expression is positively regulated by (p)ppGpp and DksA, possibly at the level of transcription. This is supported by RNAP site 1^- and site 2^- *fabH* and *dksA fabH* synthetic lethality (Fig. 1B and Fig. 2A, panel iv) and the reduction in *fabY-lac* activity in the *fabH* mutant lacking ppGpp or *dksA* (Fig. 5D). It is not clear if this regulation is direct or indirect. From transcriptional profiling, it was observed that *yiiD* and *fabH* expression levels are 3-fold decreased and elevated, respectively, in the $ppGpp^0$ strain compared to the wild-type strain (21). This indicates that transcriptional regulation by (p)ppGpp and DksA could be direct.

Since the growth of the *fabH* mutant was dependent on DksA function (Fig. 1B) but not site 2 (Fig. 2A, panel iii), it may be argued that the potentiation of DksA function by (p)ppGpp is not important and that (p)ppGpp-independent regulation by DksA (through the RNAP secondary channel) is required for growth. Our results are consistent with the following model. In the absence of (p)ppGpp regulation through site 1, synergistic regulation by (p)ppGpp and DksA is necessary for the growth of the *fabH* mutant. In the absence of site 2, (p)ppGpp regulation through site 1 together with (p)ppGpp-independent regulation by DksA are necessary for the growth of the *fabH* mutant.

Interestingly, the expression of *fabH*, whose function is compensated for by the expression of *fabY*, is negatively regulated by (p)ppGpp and DksA (24). This suggests that the cellular (p)ppGpp pool can modulate the amount of acetoacetyl-ACP synthesized through FabH and FabY; under stress, the increase in the (p)ppGpp content would

favor FabY-dependent initiation of fatty acid biosynthesis, and under fast-growth conditions, when the basal (p)ppGpp pool is small, FabH-dependent biosynthesis would be favored. This idea is supported by the finding that the growth rate of the *fabH* mutant was similar to that of the wild-type strain under slow-growth conditions where the basal (p)ppGpp level is elevated (see Fig. S1B in the supplemental material). The physiological relevance of such regulation needs to be addressed.

A polar insertion-deletion of the *yihW-yihX* intergenic region reduced *fabY-lacZ* expression slightly (Fig. 7A), and moving this insertion away from the *fabY* gene progressively increased *fabY-lacZ* expression (Fig. 7C). These results suggest that promoters within the *yihX-yihY-dtd* ORFs could contribute to the expression of *fabY*. Further studies to understand the expression of *fabY* are in progress. Identifying the promoter(s) would be helpful for understanding if the (p)ppGpp- and DksA-dependent regulation of *fabY* transcription is direct or indirect.

MATERIALS AND METHODS

Growth conditions. Strains were grown in LB medium (1% tryptone, 0.5% yeast extract, and 1% NaCl) or minimal A medium. The growth temperature was 37°C unless indicated otherwise. The final concentrations of the antibiotic used are 15 μg/ml chloramphenicol (Cm), 25 μg/ml kanamycin (Kan), 10 μg/ml tetracycline (Tet), 50 μg/ml ampicillin (Amp), and 12.5 μg/ml spectinomycin (Sp). Isopropyl-β-D-thiogalactopyranoside (IPTG) was used at a final concentration of 1 mM unless mentioned otherwise. 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) was added at 50 μg/ml. Oleic acid and palmitic acid solutions were prepared in a 10% solution of Brij 58 and were present at a final concentration of 0.01% unless indicated otherwise.

Strains and plasmids. All strains were constructed in the MG1655 background, and mutations were introduced by phage P1 transduction or recombineering (see below). Strains, plasmids, and primers used in the study are listed in Table S1 in the supplemental material. The genetic techniques for cloning, P1 transduction, and other genetic manipulations were performed as described previously, with minor modifications (56, 57). Gene deletions have been sourced from the Keio collection (32), and whenever required, the Kan cassette was removed using the FLP recombinase expressed from a pCP20 plasmid (58). Plasmid clones have been sourced from the ASKA collection (59). Plasmids pRCspoT, pRCdksA, and pRC$_{sp}$-spoT were constructed from the single-copy plasmid pRC7 (33) and have been described previously (60). The *dksA* or *spoT* gene was cloned immediately upstream of the *lacZ* gene in these plasmids and expressed from the *lac* promoter but with its native ribosome binding site (RBS) and start codon. The *fabH* gene with its promoter was cloned into pRC7 between the BamHI and HindIII sites using the primers JGU*fabH*-98bp and JGU*fabH*stop+12bp to construct pRCfabH. The *fabH* and *lacZ* genes in pRCfabH are expressed from the *fabH* promoter, and therefore, IPTG is not required for *lacZ* expression. The plasmids conferring spectinomycin resistance, pRC$_{sp}$-fabH and pRC$_{sp}$-dksA, were made from pRCfabH and pRCdksA, respectively, by replacing the *bla* gene with *aadA* by recombineering as described previously (60). The *yiiD* (*fabY*) gene was cloned into the pBAD24 vector under the control of the *araBAD* promoter between the EcoRI and HindIII sites using the primers JGUEcoRIyiiDFW and JGUHindIIIyiiDRV. pΔdksA was constructed from pJK537 (49) by PvuII digestion, followed by religation of the plasmid; this plasmid is identical to pJK533 (49), leading to a truncation of DksA after the 36th amino acid. Insertions-deletions in the upstream regulatory sequence or insertions between the ORFs in the *yihX-yihY-dtd-yiiD* operon were made by recombineering using appropriate primers and replaced with the Cm cassette from a pKD3 plasmid (47). The junctions were sequenced to confirm that no unexpected sequence change was introduced. The transcriptional fusion *yiiD-lac* was constructed at the chromosomal *yiiD* locus using the *yiiD*::FRT allele generated from the Keio collection and plasmid pKG137 using a previously described protocol (42). The new junctions generated were verified by sequencing. The *lac* fusion is followed by a Kan cassette, and this was used for selections during phage P1 transduction. In order to screen for multicopy suppressors of ppGpp^0 Δ*fabH* synthetic lethality, an *E. coli* genomic library constructed in the plasmid pACYC184 was used, and the genes present in the plasmid clones were identified by sequencing as described previously (61).

Plasmid segregation assay. The synthetic lethality between mutations was studied using the "blue-white" plasmid segregation assay (33) that is based on the rationale that an essential gene function provided from an unstable plasmid would stabilize the plasmid. All strains used in this assay carry the Δ*lacZYAI*::FRT allele and additionally the mutation indicated. The ability of the strains carrying the mutation(s) indicated to grow following the loss of the unstable single-copy plasmids encoding β-galactosidase and carrying either *spoT* (pRCspoT or pRC$_{sp}$-spoT), *dksA* (pRC$_{sp}$-dksA), or *fabH* (pRCfabH or pRC$_{sp}$-fabH) was studied. Blue and white colonies represent the retention and loss of the plasmid, respectively. The stabilization of the plasmid (no white colonies) indicated that the plasmid-encoded function was essential for growth. Plasmid loss during growth in the plate can give rise to "sectored" colonies with blue and white coloring (these colonies are indicated with an arrow in some of the figures). Strains carrying the indicated unstable plasmid were grown overnight in the presence of an appropriate antibiotic. Cultures were washed and serially diluted using minimal A buffer with 10 mM $MgSO_4$ and serially diluted. Appropriate dilutions were spread on a plate containing X-gal and IPTG to obtain ~200 to 300 colonies per plate. IPTG was not added when pRCfabH or pRC$_{sp}$-fabH was used since *lacZ* is expressed from the *fabH* promoter in these plasmids. Plates were generally scored after 24 h at 37°C. For

the slow-growing strains carrying the *fabH* mutation, incubation was continued up to 48 h to distinguish white from blue colonies; when white colonies were not visible, incubation was continued up to 72 h before scoring. Representative white colonies taken from the plates with which segregation assays were performed were streaked onto fresh plates to confirm that they could grow and are therefore viable; the percentage of white colonies was calculated from the ratio of white colonies to the total number of colonies scored (white plus blue).

β-Galactosidase assay. Cultures were grown overnight in appropriate medium and diluted 100-fold in the same medium. At mid-log phase (A_{600} of 0.4 to 0.6), cultures were assayed for β-galactosidase activity (56). The values, reported as Miller units, are the means from three or more independent experiments. The standard deviations are represented as error bars.

Measurement of doubling time and cell viability. LB cultures grown overnight were diluted 100-fold in 10 ml of LB medium in a 100-ml conical flask and incubated in a water bath shaker at 37°C with shaking (200 rpm). The optical density (A_{600}) of the cultures was measured every 30 min and plotted on a log scale against time. The rate of increase in the A_{600} value between 0.2 and 0.6 was determined from the slope and used to calculate the doubling time. For each strain, the doubling times reported are the means from three independent experiments. The standard deviations are indicated as error bars. Viability was determined by spotting 10 μl of the cultures that were serially diluted, and the CFU was determined after incubation for 24 h at 37°C.

Estimation of cell size by microscopy. *E. coli* cells were cultured in LB medium or LB medium plus Cm (30 μg/ml) overnight and then subcultured 1:100 in the same medium. At an A_{600} of between 0.2 and 0.3, 2 ml of cells was pelleted and resuspended in an equal volume of a 3.7% formaldehyde solution in 1× phosphate-buffered saline (PBS). The cell suspensions were centrifuged, washed with 1× PBS twice, and finally resuspended in 20 to 50 μl in 1× PBS. Two microliters of the culture was mounted on 1% agarose pads and visualized using a 100× objective of a Nikon Eclipse Ti microscope. Cell length and width were measured from phase-contrast images using tools of the ROI manager in ImageJ software. Length and width measurements were done for each strain using 50 dividing cells with an "8" cell morphology. The length was calculated by drawing a straight line from the pole to the point of constriction, and the width was calculated by drawing a straight line in the middle of the cell. The area was calculated for each cell by multiplying the length and the width. Means and standard deviations were calculated using Microsoft Excel.

Analysis of fatty acid content. RS339, RS340, and RS688 were grown on LB agar plates containing chloramphenicol at 37°C, and cells from single colonies on plates incubated for 16 h were used for analysis. For each strain, cells were taken from three separate plates and processed in triplicate. Fatty acid methyl ester (FAME) extraction and analysis were carried out as previously described (62). Lipids were saponified in sodium hydroxide and methanol, methylated in acidified methyl alcohol, extracted in hexane and methyl tertiary butyl ether, and analyzed by using a gas chromatograph equipped with a flame ionization detector. The extraction efficiency of the protocol and authenticity of fatty acid peaks were verified using *Stenotrophomonas maltophilia* ATCC 13637^T (with a known fatty acid profile) as a positive control. Peaks were identified based on the retention time of a standard run under a similar set of conditions, using the software and database (RTSBA6) of MIS (MIDI Inc., Newark, DE).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00354-19.

SUPPLEMENTAL FILE 1, PDF file, 0.9 MB.

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We declare that we have no conflict of interest.

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