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# Reassessment of the Genetic Regulation of Fatty Acid Synthesis in *Escherichia coli*: Global Positive Control by the Functional Dual Regulator FadR

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**ABSTRACT**

**In *Escherichia coli*, the FadR transcriptional regulator represses the expression of fatty acid degradation (*fad*) genes. However, FadR is also an activator of the expression of *fabA* and *fabB*, two genes involved in unsaturated fatty acid synthesis. Therefore, FadR plays an important role in maintaining the balance between saturated and unsaturated fatty acids in the membrane. We recently showed that FadR also activates the promoter upstream of the *fabH* gene (L. My, B. Rekoske, J. J. Lemke, J. P. Viala, R. L. Gourse, and E. Bouveret, J Bacteriol 195:3784–3795, 2013, doi:10.1128/JB.00384-13). Furthermore, recent transcriptomic and proteomic data suggested that FadR activates the majority of fatty acid (FA) synthesis genes. In the present study, we tested the role of FadR in the expression of all genes involved in FA synthesis. We found that FadR activates the transcription of all tested FA synthesis genes, and we identified the FadR binding site for each of these genes. This necessitated the reassessment of the transcription start sites for *accA* and *accB* genes described previously, and we provide evidence for the presence of multiple promoters driving the expression of these genes. We showed further that regulation by FadR impacts the amount of FA synthesis enzymes in the cell. Our results show that FadR is a global regulator of FA metabolism in *E. coli*, acting both as a repressor of catabolism and an activator of anabolism, two directly opposing pathways.**

**IMPORTANCE**

**In most bacteria, a transcriptional regulator tunes the level of FA synthesis enzymes. Oddly, such a global regulator still was missing for *E. coli*, which nonetheless is one of the prominent model bacteria used for engineering biofuel production using the FA synthesis pathway. Our work identifies the FadR functional dual regulator as a global activator of almost all FA synthesis genes in *E. coli*. Because FadR also is the repressor of FA degradation, FadR acts both as a repressor and an activator of the two opposite pathways of FA degradation and synthesis. Our results show that there are still discoveries waiting to be made in the understanding of the genetic regulation of FA synthesis, even in the very well-known bacterium *E. coli*.**

Fatty acid (FA) degradation and synthesis are two central metabolic pathways involved in energy production and in biogenesis of membranes and various secondary metabolites, respectively. FA synthesis begins with the activation of acetyl coenzyme A (acetyl-CoA) into malonyl-CoA by the acetyl-carboxylase complex, encoded by the *accABCD* genes in *Escherichia coli* (Fig. 1A). A series of condensation, reduction, and dehydration reactions performed by the products of the *fab* genes then elongate the acyl chain carried by the small acyl carrier protein (ACP). FA synthesis consumes a lot of energy; therefore, both FA degradation and synthesis must be tightly controlled. All of the biochemical steps of FA synthesis and their allosteric control are very well described in *E. coli* (1). The key regulators are the long-chain acyl-ACP end products, which exert a negative regulatory feedback on key enzymes of the FA synthesis pathway, such as the acetyl-CoA carboxylase, FabH, and FabI. This negative feedback coordinates FA synthesis with the incorporation of fatty acids in membrane biogenesis (1). However, the transcriptional regulation of this process is much less understood. Only the expression of *fabA* and *fabB* genes, involved specifically in the synthesis of unsaturated FA, have been shown to be regulated. Expression of *fabA* and *fabB* is repressed by FabR, which binds a site overlapping their promoters, and is activated by FadR, which binds a consensus site located around the −40 position (2–4). However, recent studies of FA synthesis regulation in other bacteria and especially in Gram-positive bacteria have shown that *E. coli* is far from being the usual case. Usually, most bacteria possess a regulator for controlling all the genes of FA synthesis and not just those involved in unsaturated FA synthesis (5, 6). In general, the gene coding for this regulator is located upstream of a gene cluster that contains all the genes for FA synthesis. In contrast, the *fabHDG-acpP-fabF* gene cluster of *E. coli* does not contain any gene coding for a dedicated transcriptional regulator, the genes coding for the acetyl-CoA carboxylase are scattered around the chromosome (Fig. 1B), and no global regulator of all these FA synthesis genes had been described so far. FadR, whose principal role first was discovered to be a

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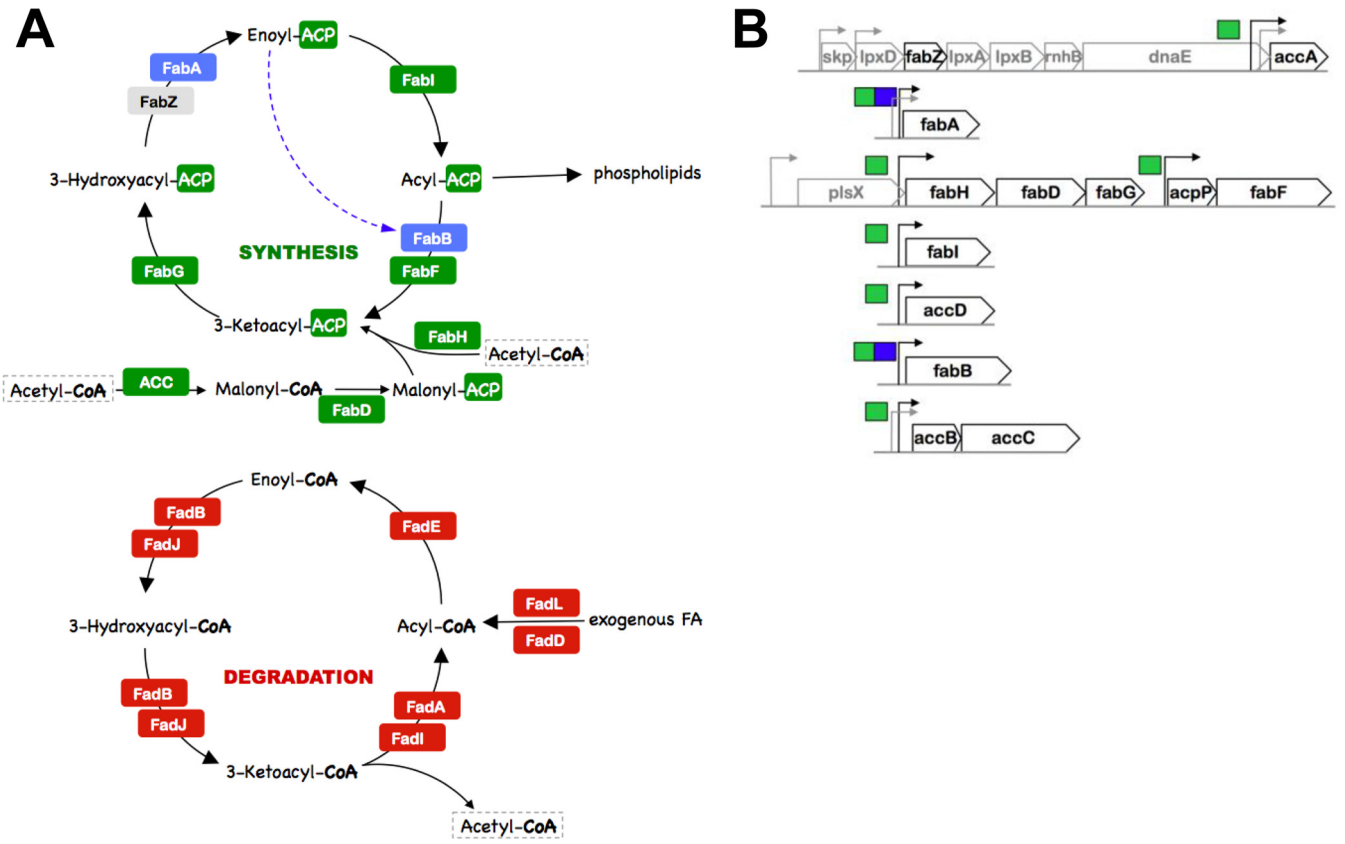


**FIG 1** Transcriptional regulation of FA synthesis and degradation genes in *E. coli*. (A) FA synthesis and degradation pathways. FA degradation enzymes, colored in red, are coded by genes repressed by FadR (4). The FadL and FadD proteins involved in the uptake and activation of exogenous FA also are repressed by FadR. FadI and FadJ serve functions parallel to those of FadA and FadB under anaerobic conditions (33). Finally, FadM, which is a long-chain acyl-CoA thioesterase involved in the β-oxidation of oleic acid, also is repressed by FadR but is not depicted here. FA synthesis enzymes colored in green are coded by genes activated by FadR (8; this paper). FabA and FabB enzymes, colored in blue, are involved in unsaturated FA synthesis. *fabA* and *fabB* genes are repressed by FabR and activated by FadR (5). ACC, acetyl-CoA carboxylase, composed of AccABCD proteins. (B) Organization of the transcription units. The FadR binding box is indicated by a green box. The FabR binding box is indicated by a blue box. The transcription start sites are indicated by arrows, which are black for the promoters activated by FadR and gray for the alternative promoters.

repressor of FA degradation genes in *E. coli*, later was shown to activate the expression of *fabA* and *fabB* genes involved in unsaturated FA synthesis. Indeed, a *fadR* mutant contains about one-third fewer unsaturated fatty acids (7). However, binding of FadR to its operator is prevented by the binding of either saturated or unsaturated fatty acyl-CoA indistinctively. Therefore, researchers have always wondered why FadR would be involved specifically and only for *fabA* and *fabB* activation (3). *E. coli* also might need to have a mechanism for tuning the expression of all of the FA synthesis genes, as is the case in other bacteria.

Indeed, we previously showed that FadR also activates the promoter just upstream of *fabH*, thereby contributing to the increase in *fabHDG-acpP-fabF* expression (8). Furthermore, a global transcriptome study of a strain overproducing FadR evidenced a global increase in the expression of FA synthesis genes (9). Finally, sequences matching the FadR binding consensus had been spotted before in *acpP* and *fabI* promoters, and they are even located at a position compatible with activation by FadR (4, 10). However, *acpP* was reported at that time not to be regulated by FadR (11), and the results for *fabI* were contradictory (4, 10). Therefore, although it long has been postulated that FadR only activates the *fabA* and *fabB* genes, we suspected that FadR is the missing global regulator of FA synthesis genes in *E. coli*. FadR is not essential for growth, and FA synthesis genes may be expressed from several promoters, as has been shown for *fabA* (12). In consequence, only adjustments in the expression levels can be performed by FadR, which explains why potential FadR regulatory effects had been overlooked until now. Importantly, we showed before that the abundance of FadR protein itself varies depending on growth condition (8). Therefore, a global regulation of FA synthesis by FadR also would be important to tune and coordinate the protein amounts of the FA synthesis machinery with growth.

In this study, we screened systematically the effect of *fadR* deletion or FadR overproduction on the expression of all of the genes involved in FA synthesis except for *fabZ*. We found that they all were directly activated by FadR, and we mapped the binding sites upstream of the respective promoters. For this, we had to reassess the nature of the transcription start sites of *accA* and *accB* genes described previously (13). As a consequence, we describe here a complex genetic control of these genes, each possessing one FadR-dependent promoter and another independent one. This multiple promoter organization may be a common feature of FA synthesis genes in *E. coli*. Finally, we showed that the global activation of FA

**TABLE 1** Plasmids

| Laboratory code | Name[c] | Description[a] | Limits of the transcriptional fusions[b] | Reference or source |
|---|---|---|---|---|
| pEB1209 | pET-6His-Tev-FadR | Amp$^r$, pBR322 ori, T7 promoter, *fadR* | | 8 |
| pEB0227 | pBAD24 | Amp$^r$, pBR322 ori, $P_{BAD}$ promoter | | 38 |
| pEB1210 | pBAD-FadR | Amp$^r$, pBR322 ori, $P_{BAD}$ promoter-*fadR* | | 8 |
| pEB1489 | pET-6His-FcsA | Amp$^r$, pBR322 ori, T7 promoter-*fcsA* | | 19 |
| pEB0898 | pUA66 | Kan$^r$, p15A ori, MCS-*gfp* | | 14 |
| pEB1179 | pUA-*fabH* | | −473/−189 | 8 |
| pEB1298 | pUA-*fabH** | | | 8 |
| pEB1235 | pUA-*fabA* | | −154/+70 | 8 |
| pEB1386 | pUA-*fabB* | | −224/+29 | 8 |
| pEB1234 | pUA-*fadR* | | −373/+40 | 8 |
| | pUA-*acpP* | | −292/+61 | 14 |
| pEB1567 | pUA-*acpP** | ebm1074/1075 on pUA-*acpP* | | This work |
| pEB1531 | pUA-*fabI* | ebm1069/1070 in pUA66 | −232/+26 | This work |
| pEB1568 | pUA-*fabI** | ebm1076/1077 on pEB1531 | | This work |
| pEB1556 | pUA-*accD* | ebm1101/1102 in pUA66 | −256/+60 | This work |
| pEB1578 | pUA-*accD** | ebm1138/1139 on pEB1556 | | This work |
| pEB1632 | pUA-*accA* | ebm1218/1100 in pUA66 | −402/+44 | This work |
| pEB1635 | pUA-*accA** | ebm1220/1221 on pEB1632 | | This work |
| pEB1630 | pUA-*accAP1* | ebm1217/1100 in pUA66 | −295/+44 | This work |
| pEB1631 | pUA-*accAP2* | ebm1218/1219 in pUA66 | −402/−270 | This work |
| pEB1636 | pUA-*accAP2** | ebm1220/1221 on pEB1631 | | This work |
| | pUA-*accB* | | −917/+63 | 14 |
| pEB1597 | pUA-*accB** | ebm1173/1174 on pUA-*accB* | | This work |
| pEB1643 | pUA-*accBP1* | ebm1078/1240 in pUA66 | −461/−287 | This work |
| pEB1673 | pUA-*accBP1** | ebm1173/1174 on pEB1643 | | This work |
| pEB1640 | pUA-*accBP2* | ebm1236/1237 on pUA-*accB* | | This work |
| pEB1718 | pUA-*accBP2** | ebm1390/1391 on pUA-*accB* | | This work |

[a] A full description is given only for the vectors of reference. MCS, multiple cloning site; ori, origin of replication. For the new constructs, the oligonucleotides used either for amplification of the insert or for directed mutagenesis are indicated.
[b] Limits of the transcriptional fusions are given from the initiation codon of the corresponding gene (given the presence of multiple promoters for some genes, numbering from the transcription start nucleotide would have been ambiguous).
[c] An asterisk indicates a mutation in the FadR binding site.

synthesis genes by FadR plays a role in tuning the amounts of the enzymes encoded by them in the cell.

## MATERIALS AND METHODS

**Media and chemicals.** *E. coli* cells were grown at 37°C in lysogeny broth (LB) medium unless otherwise stated. The plasmids were maintained with ampicillin (100 μg/ml), chloramphenicol (50 μg/ml), or kanamycin (50 μg/ml). The minimal medium used to test the carbon sources contained the following: 1× M9 salts, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 2 μg/ml vitamin $B_1$, 0.2% Casamino Acids. Sodium oleate was purchased from Sigma. A stock solution of sodium oleate was prepared at 200 mg/ml in 10% NP-40 and then diluted to 2 mg/ml in the growth medium.

**Plasmids.** Gene expression was monitored using transcriptional fusions with *gfp* using the pUA66 and pUA139 plasmids (Table 1) (14). The transcriptional fusions with promoters of *acpP* and *accB* were available in the *E. coli* promoter library obtained from Open Biosystems (14). The other intergenic regions were amplified by PCR with different primer pairs (see Table S1 in the supplemental material) using purified genomic DNA of *E. coli* MG1655 for the template. PCR products then were digested by BamHI/XhoI restriction enzymes and cloned into pUA139 or pUA66 depending on the desired orientation (14).

**Strains.** The deletion mutant strains were obtained from the Keio collection (15). The sequential peptide affinity (SPA)-tagged strains were obtained from the collection of strains described in reference 16 and obtained from Open Biosystems. For both types of strains (Table 2), the recombinant genes were transferred to the desired strain background by

**TABLE 2** Strains

| Laboratory code | Name | Description | Reference |
|---|---|---|---|
| | DY330 series | Collection of strains with SPA tag on the chromosome | 16 |
| EB944 | MG1655 | | |
| EB929 | AccA-SPA strain | MG1655 *accA-SPA*-Kan$^r$ | This work |
| EB930 | AccC-SPA strain | MG1655 *accC-SPA*-Kan$^r$ | This work |
| EB969 | AccD-SPA strain | MG1655 *accD-SPA*-Kan$^r$ | This work |
| EB744 | FabA-SPA strain | MG1655 *fabA-SPA*-Kan$^r$ | This work |
| EB745 | FabB-SPA strain | MG1655 *fabB-SPA*-Kan$^r$ | This work |
| EB931 | FabI-SPA strain | MG1655 *fabI-SPA*-Kan$^r$ | This work |
| EB584 | MG1655Δ*fabR* | | 8 |
| EB586 | MG1655Δ*fadR* | | 8 |
| EB933 | Δ*fadR*/AccA-SPA strain | MG1655Δ*fadR accA-SPA*-Kan$^r$ | This work |
| EB934 | Δ*fadR*/AccC-SPA strain | MG1655Δ*fadR accC-SPA*-Kan$^r$ | This work |
| EB970 | Δ*fadR*/AccD-SPA strain | MG1655Δ*fadR accD-SPA*-Kan$^r$ | This work |
| EB751 | Δ*fadR*/FabA-SPA strain | MG1655Δ*fadR fabA-SPA*-Kan$^r$ | This work |
| EB746 | Δ*fadR*/FabB-SPA strain | MG1655Δ*fadR fabB-SPA*-Kan$^r$ | This work |
| EB935 | Δ*fadR*/FabI-SPA strain | MG1655Δ*fadR fabI-SPA*-Kan$^r$ | This work |

P1 transduction (17). When required (for transformation with the transcriptional fusion plasmids carrying resistance to kanamycin), the gene for resistance to kanamycin was removed using the pCP20 plasmid (18).

**Measure of expression using transcriptional fusions with GFP.** The *E. coli* MG1655 wild-type strain or isogenic mutant strains were transformed with plasmids carrying the *gfp* transcriptional fusions (14) and maintained with kanamycin. For cotransformation, compatible plasmids (pBAD24 and derivatives) were used with ampicillin for their maintenance. Selection plates were incubated at 37°C for 16 h. Six hundred microliters of LB medium supplemented with the required antibiotics, and with 0.05% arabinose when necessary for $P_{BAD}$-driven expression, was inoculated (4 to 6 replicates each assay) and grown for 16 h at 30°C in 96-well polypropylene plates of 2.2-ml wells under aeration and agitation. Fluorescent intensity measurement was performed in a Tecan infinite M200. One hundred fifty microliters of each well was transferred into a black Greiner 96-well plate for reading optical density at 600 nm ($OD_{600}$) and fluorescence (excitation, 485 nm; emission, 530 nm). The expression levels were calculated by dividing the intensity of fluorescence by the $OD_{600}$. These results are given in arbitrary units, because the intensity of fluorescence is acquired with an optimal and variable gain; hence, the absolute values cannot be compared between different types of experiment and growth conditions.

**Mapping of the transcription start sites by 5′-RACE experiments.** For 5′ rapid amplification of cDNA ends (RACE), total RNAs were prepared using the PureYields RNA Midiprep system from Promega on 10-ml bacterial cultures of strains MG1655, EB586 (Δ*fadR*), and MG1655/pEB1210 (FadR overproduction) grown at 37°C in LB until the $OD_{600}$ reached 2. For overproduction of FadR, the MG1655 strain transformed with pEB1210 plasmid was grown to an $OD_{600}$ of 0.5 and then induced with 0.05% arabinose until an $OD_{600}$ of 2. The transcription start sites (+1) then were determined using the FirstChoice RLM-RACE kit from Ambion. We followed the instructions from the manual exactly, except for the last step of reverse transcription, for which we used the RT Superscript III kit (Invitrogen) with random hexamers. Oligonucleotides used for outer and inner nested PCRs are listed in Table S1 in the supplemental material.

**EMSA.** For electrophoretic mobility shift assay (EMSA), we purified FadR and FcsA proteins, which were produced using the pEB1209 and pEB1489 plasmids, respectively, as described previously (8, 19). Octanoyl-CoA and oleyl-CoA were synthesized from octanoate or oleate and coenzyme A (all purchased from Sigma) using the fatty-acyl CoA synthetase FcsA enzyme (19) as described previously (8). Fatty acid (50 μM) and CoA (50 μM) were added to a reaction buffer containing 50 mM HEPES buffer (pH 7.5), 1 mM dithiothreitol (DTT), 5 mM $MgCl_2$, and 1 mM MgATP. FcsA was added at a final concentration of 1 μM to catalyze the ligation at 30°C for 60 min. Two microliters of purified FadR at 10 μM then was preincubated with 4 μl of the acylation reaction mixture at 37°C for 10 min; therefore, acyl-CoAs are estimated to be 10-fold in excess of FadR. The EMSA then was performed by mixing 2 μl of purified FadR at 10 μM, untreated or preincubated with acyl-CoA, with 20 nM PCR fragment in a 20-μl final reaction buffer containing 25 mM Tris-HCl buffer (pH 7.2), 10 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5 mM EDTA, 50 mM KCl, and 5% glycerol. The mix was incubated for 30 min at 20°C. The reactions then were analyzed by native PAGE. DNA was stained with GelRed (FluoProbes). In Fig. 6B, for each experiment, the white line separates different parts of the same image, which was edited before as a whole. The following primers were used to amplify the promoter regions: *accB*, ebm1078/1079; *accD*, ebm1101/1102; and *fabI*, ebm1069/1070 (see Table S1 in the supplemental material).

**SDS-PAGE, Western blotting, and protein relative quantification.** SDS-PAGE, electrotransfer onto nitrocellulose membranes, and Western blot analyses were performed as previously described (20). Monoclonal anti-Flag M2, used for SPA tag detection, was purchased from Sigma. The relative amounts of FA synthesis enzymes fused to the SPA tag were quantified by 10% SDS-PAGE and Western blotting using anti-Flag antibody. The amounts produced then were quantified using Alexa Fluor 680–goat anti-mouse IgG fluorescent secondary antibodies (Invitrogen) on an Odyssey Fc imager from LI-COR Biosciences.

## RESULTS

**FadR activates the global expression of fatty acid synthesis genes.** Our initial finding that the *fabH* promoter was directly activated by FadR (8), the global increase in the expression of FA synthesis genes in a strain overproducing FadR (9), and the potential presence of a consensus sequence for FadR binding in the promoters of FA synthesis genes other than *fabA* and *fabB* were strong indications that FadR activates the transcription of genes additional to those previously reported. Therefore, we decided to screen the effect of *fadR* deletion or FadR overproduction on the transcription of all of the genes involved in FA synthesis. We first used transcriptional fusions with GFP (8, 14). The transcriptional fusions we needed were either available from a library (14) or were constructed if missing (Table 1). In total, in addition to the already described *fabA* and *fabB* genes, we tested transcriptional fusions with the upstream regions of the following genes: *accA*, *accBC*, *accD*, *fabHGD*, *acpP-fabF*, and *fabI*. Only the transcriptional fusion for testing *fabZ* was missing, due to the complex genetic organization of *fabZ* in cluster with genes involved in lipopolysaccharide synthesis (Fig. 1B) and to the lack of a described specific promoter for *fabZ* (21). It has to be noted that the *accA* transcription unit lies just downstream of this complex operon (Fig. 1B). In the Δ*fadR* mutant, the measured activities of all of the transcriptional fusions in late exponential phase were reduced, compared to those of the wild type, at various levels (Fig. 2A). First, expression from the *fabH* promoter was totally abolished in the *fadR* mutant, as we have described before (8). The expression of *accD*, *acpP*, and *fabI* fusions was significantly reduced but not abolished. The expression of *accA* and *accB* fusions was only mildly reduced, but, as will be described below, in the case of *accA* this could be explained by the presence of multiple promoters. In reverse, in a strain where FadR was overproduced using the pBAD-FadR plasmid, all constructs displayed a drastic increase of expression (Fig. 2B). In addition to the proximal promoter of *fabH* and the promoters of *fabA* and *fabB* already described, this suggested that *accA*, *accBC*, *accD*, *fabI*, and *acpP-fabF* genes also were activated by FadR.

Because we observed a global effect of FadR on the expression of FA synthesis genes, we also decided to test the effect of a *fabR* deletion. In this case, we did not observe any change in FA synthesis gene expression apart from the expected activation of *fabA* and *fabB* expression (see Fig. S1 in the supplemental material).

**Identification of the FadR binding site in the promoters of FA synthesis genes.** We analyzed the promoter regions of all of the genes studied as described above. For all of the genes activated by FadR, we were able to spot a sequence matching the FadR binding consensus sequence, including the ones already mentioned for *fabI* and *acpP* (Fig. 3). The conservation is not very good, especially the left half of the dyad, which might explain why the *accA*, *accB*, and *accD* sites were not spotted before. However, the sites were located at distances ranging from −32 to −41 nucleotides relative to the transcription start sites (+1) for the genes *acpP*, *fabI*, and *accD*, which is in agreement with the action of FadR as an activator, with a distance similar to what has been described for *fabA* and *fabB* genes (3, 12). The potential FadR binding site in the *fabI* promoter was mentioned two times in review papers, but the experimental and contradictory data were never published (4, 10).

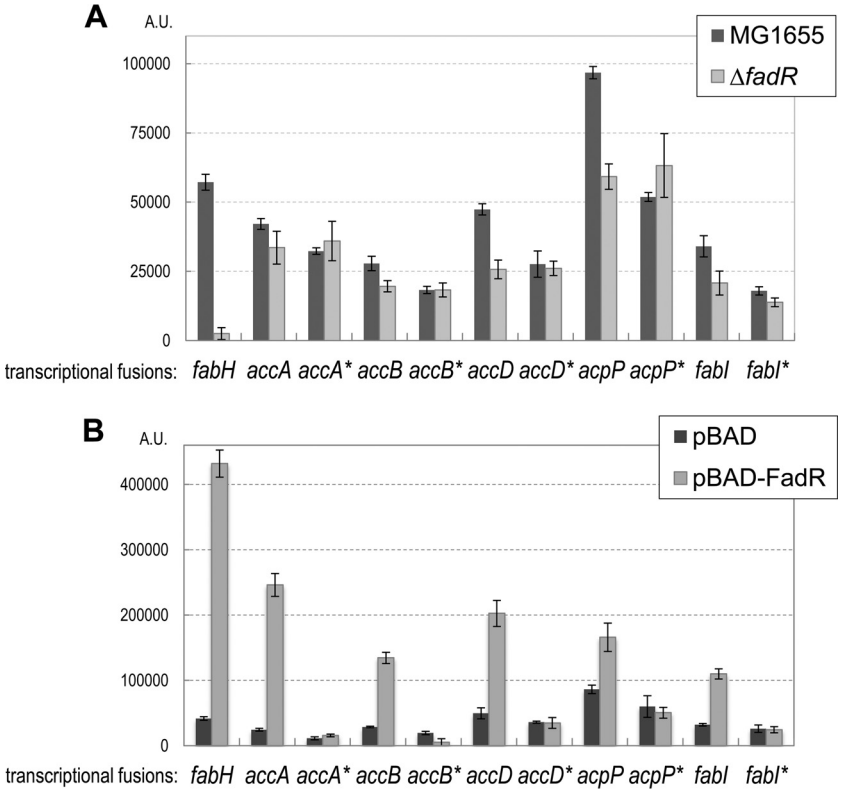


**FIG 2** Global activation of FA synthesis gene expression by FadR. (A) Comparison of transcriptional fusion activity in wild-type MG1655 and in the *fadR* mutant EB586 strains grown at 37°C in LB until late exponential phase (6 h of growth). (B) Transcriptional fusion activity when FadR protein is overproduced. MG1655 strains transformed by the indicated transcriptional fusions and the pBAD24 or pBAD-FadR (pEB1210) plasmid were incubated overnight at 37°C in LB supplemented with 0.05% arabinose. The asterisk indicates that a mutation (see Table S1 in the supplemental material) was introduced in the FadR binding site. The activities correspond to the ratio between GFP fluorescence and the $OD_{600}$ of 4 replicates and are given in arbitrary units (A.U.). The error bars stand for standard deviations.

First, we determined experimentally the +1 site of *fabI* by a 5′-RACE experiment (see Fig. S2 in the supplemental material) and confirmed that the FadR binding sequence was at the −40 position relative to this +1 site (Fig. 3). Oddly, for *accA* and *accB*, the location of the potential FadR binding site did not fit with the +1 site of transcription described previously (13) (see below).

The sensitivity of the transcriptional fusions to the presence of FadR strongly suggested the direct activation of all of these promoters by FadR. In order to prove this and the existence of the FadR binding sites, we performed mutagenesis on the transcriptional fusions. We introduced mutations at the distal and less conserved part of the FadR binding motifs that we identified and farther upstream from the −35 position in order to avoid the complete destruction of the promoters. Indeed, these mutations did not abolish the expression of the transcriptional fusions (Fig. 2). However, the mutations decreased the activities to a level similar to the one obtained with the Δ*fadR* mutation, and these mutant constructions were not affected anymore by the *fadR* deletion (Fig. 2A). This is especially clear for the *accD*, *acpP*, and *fabI* promoters for which the decreased activity was significant. Finally, for all the promoters, the mutation totally abolished the activation by FadR overproduction (Fig. 2B).

**Dissection of the *accA* and *accB* promoter regions.** Because the FadR binding site location within the promoters of *accA* and *accB* was not logically consistent with an activation effect, we had to reassess the promoter organization of these two genes. First, we mapped the +1 site of transcription using the 5′-RACE experiment by using the wild-type and the FadR-overproducing strains. In both cases, we defined a new +1 site about 35 nucleotides downstream of the FadR potential binding site (noted as P2 in Fig. 4 and 5; also see Fig. S2 in the supplemental material). These 2 sites correspond to strong promoter prediction using the BProm server (22) and also to high-throughput studies that mapped transcription start sites in *E. coli* (23). The goal then was to determine if the previously described promoters were erroneous or if two (or more) distinct transcription start sites were present for the transcription of *accA* and *accBC* genes. To answer this question, we constructed truncated or mutated transcriptional fusions.

For *accA*, we were able to separate two distinct promoter regions, both active in the wild-type strain (Fig. 4A and C). The proximal $accAp_1$ fusion contained the promoter described previously (13), while the distal $accAp_2$ fusion contained the FadR-activated promoter for which we had identified the +1 site by 5′-RACE in the wild-type strain (Fig. 4). We asked whether the FadR-independent transcription start site identified previously could be detected in the absence of FadR. We mapped again the +1 position by a 5′-RACE experiment, but this time a *fadR* deletion mutant was used. Indeed, in the *fadR* mutant, we were able to detect an additional and smaller band corresponding to the $accAp_1$ promoter (Fig. 4B). Consistently, the activity of the $accAp_2$ transcriptional fusion was drastically reduced in the *fadR* mutant, while the $accAp_1$ fusion conserved the same activity (Fig. 4C).

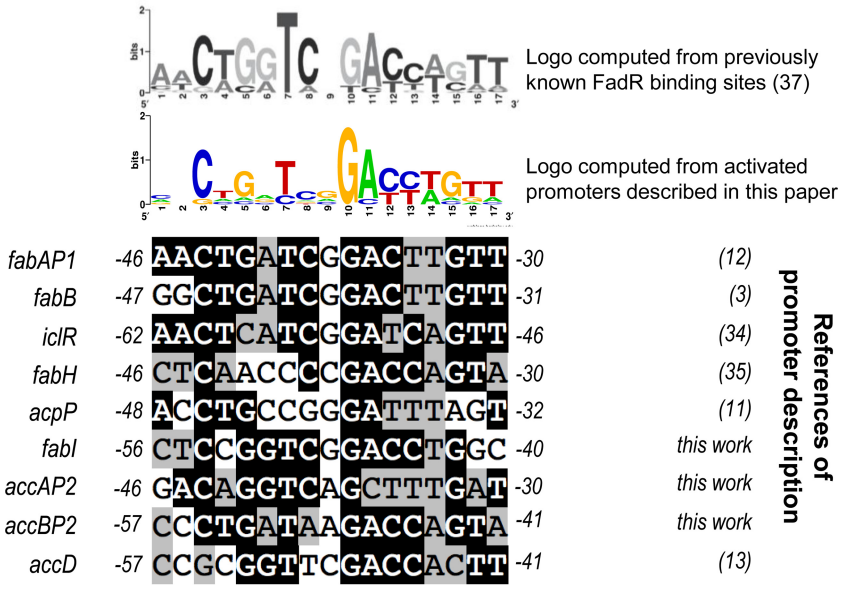


**FIG 3** FadR binding sites in the promoters of fatty acid synthesis genes. Sequences corresponding to the genes activated by FadR were aligned. The left and right positions are given relative to the corresponding transcription start site (+1). The references for the identification of the transcription start nucleotide are given at the right. A logo corresponding to this alignment of activated promoters then was computed only using the WebLogo generator (36) and is shown in color at the top. Shaded letters in the alignment indicate a match to the computed consensus sequence motif described before for FadR binding sites in *Enterobacteriales*, which is shown at the top in grayscale (37). Black is used for highly conserved bases, and gray is used when the base was in the consensus but at a lower frequency.

However, the $accAp_2$ promoter activity was not totally abolished, as shown by the detection of both the P2 and P1 transcripts by 5′-RACE in the *fadR* mutant (Fig. 4B). Finally, as expected, the mutation introduced in the FadR binding site abolished the activation of the $accAp_2$ transcriptional fusion by FadR overproduction (Fig. 4C and D).

In the case of *accB*, we identified two overlapping promoters (Fig. 5A) with an organization very similar to what has been described for *fabA* (12). The activity of the distal $accBp_1$ transcriptional fusion confirmed the existence of the promoter described previously (Fig. 5C) (13). However, because of the close overlap of the two promoters, we could not simply separate the $accBp_2$ region from the $accBp_1$ promoter. We circumvented the problem by mutating the −10 region of the $accBp_1$ promoter. This enabled us to show that the $accBp_2$ promoter was active and activated by FadR (Fig. 5D). In addition, we observed that overproduction of FadR repressed $accBp_1$ (Fig. 5D), which is expected, given that the FadR binding site lies on top of the $accBp_1$ promoter (Fig. 5A). As expected, the mutation in the binding site of FadR prevented both the repression of $accBp_1$ by FadR (compare *accBP1** to *accBP1* in Fig. 5D) and the activation of $accBp_2$ by FadR (compare *accBP2** to *accBP2* in Fig. 5D). However, in contrast to *accA*, the $accBp_2$ promoter always appeared to be preferred to the $accBp_1$ promoter, even in the absence of FadR. Indeed, the $accBp_2$ promoter clearly was activated when FadR was overproduced (Fig. 5D), yet it was not particularly affected in the *fadR* mutant (Fig. 5C), and we were not able to detect the +1 position from the $accBp_1$ promoter by 5′-RACE even in the *fadR* mutant (Fig. 5B).

**FadR directly binds to the promoters of FA synthesis genes, which is dissociable in an acyl-CoA-dependent manner.** The results described above were strong evidence that FadR directly activates all of the studied promoters. However, we wanted to unambiguously demonstrate the direct binding of FadR to the identified motifs. Furthermore, it was important to show that this regulation depended on the presence or absence of fatty acyl-CoA. Indeed, FadR recognizes and binds its operator in its apo form, without ligand, while the fixation of long-chain fatty acyl-CoA on FadR triggers its dissociation (Fig. 6A). Therefore, we performed EMSA by using purified 6His-Tev-FadR protein and DNA fragments obtained by PCRs that comprised the binding sites for FadR. A specific FadR binding was obtained with DNA fragments containing the promoters of *accB*, *accD*, and *fabI* genes (Fig. 6B, second lane of each panel). Furthermore, the binding was abolished when long-chain oleoyl-CoA ($C_{18:1}$) was added to the reaction (Fig. 6B, third lane of each gel), whereas the binding was not affected by the addition of the short-chain octanoyl-CoA (Fig. 6B, fourth lanes). We also performed the same experiments using PCR fragments containing mutations in the FadR sites as before, and we could not detect any band shift (data not shown). Therefore, despite the fact that we could not obtain a total displacement of the DNA band, the binding was highly specific. The weak binding might be explained by a weak affinity of FadR for its operators in activated genes. Indeed, even for the well-described *fabB* gene, the affinity was reported to be 20 times weaker than that for the *fad* genes, and an affinity 200 times weaker was mentioned for *fabI* (4).

**Regulation by FadR affects the amounts of fatty acid synthesis enzymes in the cell.** The previous experiments clearly demonstrated that FadR directly activates the expression from all of the promoters that we studied. However, due to the complex organization of the genes, with multiple promoters in some cases and the long mRNA untranslated regions, it had to be proven that these promoters were indeed controlling the production of the enzymes, and that the FadR regulation had a significant impact on the amounts of the enzymes in the cell. In order to answer these questions, we used a series of recombinant strains that produce

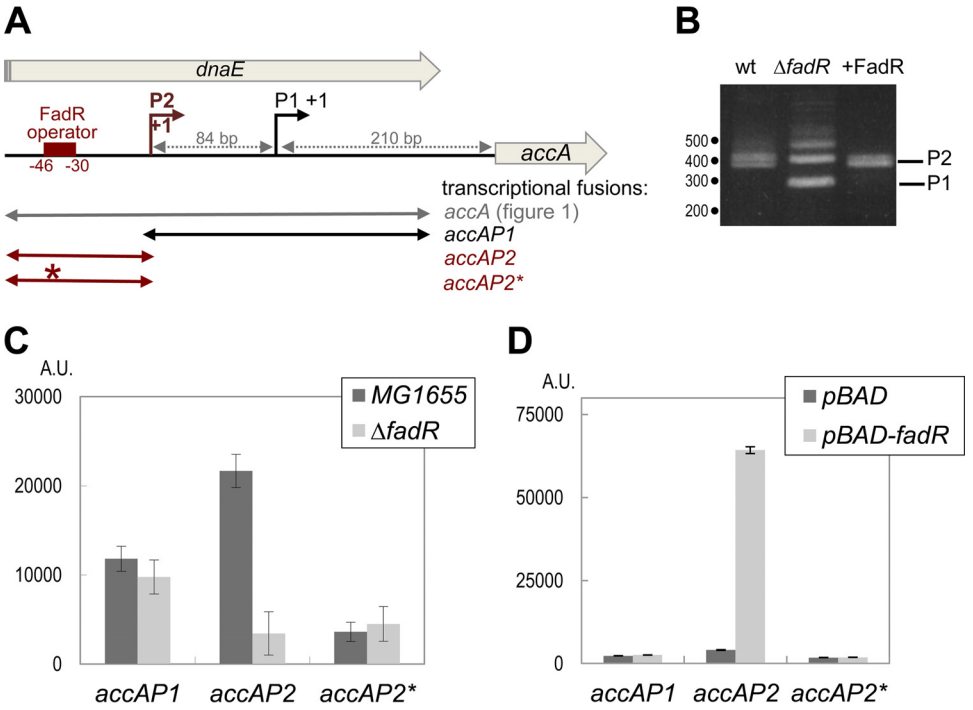


**FIG 4** Dissection of the *accA* promoter region. (A) The P1 promoter corresponds to the promoter described in reference 13, and the P2 promoter corresponds to the one activated by FadR and identified in our study. The distances to the +1 position are given from the initiation codon of *accA*. The position of the FadR box is given relative to the P2 promoter. The limits of the transcriptional fusions are indicated below. The red star indicates the mutation introduced in the FadR binding site. (B) 5′-RACE experiments were performed on a wild-type strain, a *fadR* mutant, or a strain with overproduction of FadR. The result of the last inner nested PCR with oligonucleotide ebm1100 is shown. The DNA ladder is indicated at the left (in base pairs). The bands other than the annotated P1 and P2 were aspecific PCR contaminants that did not correspond to *accA* transcripts. (C) Comparison of transcriptional fusion activities in the wild type and in the *fadR* mutant, performed as described in the legend to Fig. 2A. (D) Comparison of transcriptional fusion activities with or without overproduction of FadR protein, performed as described in the legend to Fig. 2B.

the FA synthesis enzymes fused at their C termini with the SPA tag (16, 24). These recombinant proteins were produced at their natural level, as they were expressed from their wild-type promoter(s). Apart from AccD and FabA, we could detect an increase of all the proteins when FadR was overproduced using the pBAD-FadR plasmid, with the strongest effect observed for AccA and AccC (3- to 4-fold) (Fig. 7A). Similar results were obtained before for the FabH, -D, and -G enzymes, and, to a lesser extent, for ACP and FabF (8). The absence of an increase in FabA-SPA level is consistent with our previous observation that overproducing FadR does not increase *fabA* transcription very much (8).

Only a small 2-fold decrease could be observed in the levels of the enzymes in the Δ*fadR* mutant compared to that in the wild-type strain (Fig. 7B). This was expected, as there already was not a very strong decrease of the transcriptional fusions in the Δ*fadR* mutant (Fig. 2A), and even for FabA-SPA, whose expression is strongly dependent on FadR (25), the decrease was only 2-fold (Fig. 7B). Furthermore, several promoters in addition to the promoter activated by FadR might be responsible for the production of the FA synthesis enzymes, as has been shown for *fabA* (12), *fabH* (8), and *accA* and *accB* (described above).

Finally, we compared the amounts of enzymes when strains were grown with glucose or with oleate as the sole carbon source. The import of oleate and its activation to oleyl-CoA in the cell triggers the dissociation of FadR from the DNA (Fig. 6A). We obtained small but reproducible decreases comparable to the levels obtained in the *fadR* mutant (Fig. 7C). These results show that the FadR regulation that we observed on the activity of the promoters indeed has a consequence on the physiological amounts of the corresponding enzymes.

## DISCUSSION

The first indication that FadR was a transcriptional activator of FA synthesis was that a double mutant containing the *fabA*$^{ts}$ and *fadR* mutations required supplementation with unsaturated FA for growth even at low temperatures (7). It was later demonstrated that FadR directly activates the transcription of *fabA* and *fabB* genes by binding its consensus sequence located −30 bp from the transcription start sites (2, 3), and that FadR binding to DNA is prevented by the binding of long-chain fatty acyl-CoA (26). A *fadR* mutant is viable, but its ratio between unsaturated and saturated FA is altered, suggesting a specific involvement of FadR for regulating unsaturated FA synthesis. However, we recently showed that FadR is required for the activity of the promoter just upstream of the *fabH* gene (8). In addition, recent data (9) and unpublished data on the *fabI* gene mentioned in a review paper (4) suggested that FadR activates the transcription of the majority of FA synthesis genes.

Therefore, in this paper we reassessed the regulation of fatty acid synthesis gene expression by the functional dual regulator FadR in *E. coli* and showed that FadR activates all fatty acid synthesis genes that we tested. Only the expression of *fabZ* was not directly tested. However, we did not identify any potential FadR binding site, and we did not observe any effect of FadR overpro-

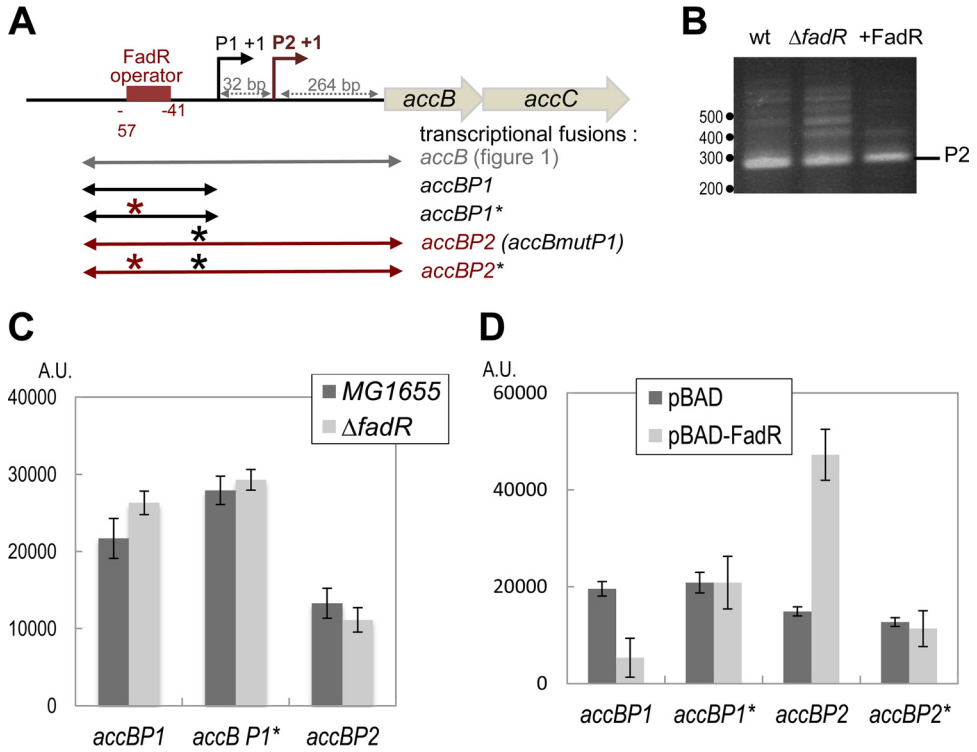


**FIG 5** Dissection of the *accB* promoter region. (A) The P1 promoter corresponds to the promoter described in reference 13, and the P2 promoter corresponds to the one activated by FadR and identified in our study. The distances to the position +1 are given from the initiation codon of *accB*. The position of the FadR box is given relative to the P2 promoter. The limits of the transcriptional fusions are indicated below. The red star indicates the mutation introduced in the FadR binding site, and the black star indicates the mutation introduced in the −10 position of the P1 promoter in order to kill it in the *accBp*$_2$ construction (designated *accB*mutP1). (B) 5′-RACE experiments were performed on a wild-type strain, a *fadR* mutant, or a strain with overproduction of FadR. The result of the last inner nested PCR with oligonucleotide ebm1180 is shown. (C) Comparison of transcriptional fusion activities in the wild type and in the *fadR* mutant, performed as described in the legend to Fig. 2A. (D) Comparison of transcriptional fusion activities with or without overproduction of FadR protein, as described in the legend to Fig. 2B.

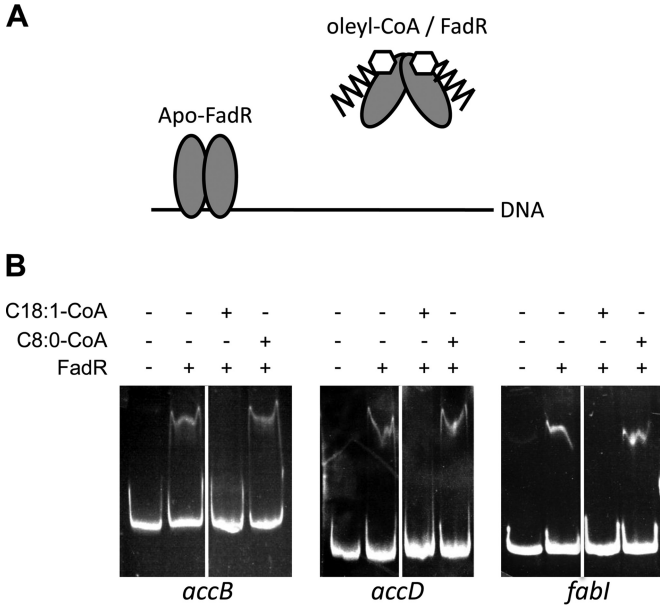


**FIG 6** Acyl-CoA-dependent fixation of FadR on the promoters of *accB*, *accD*, and *fabI*. (A) In its apo form, FadR binds its operator. In the presence of long-chain acyl-CoA, FadR dissociates from its operator. (B) EMSAs were performed using purified 6His-Tev-FadR and PCR products containing the *accB*, *accD*, or *fabI* promoters and in the presence or absence of oleyl-CoA ($C_{18:1}$-CoA) or octanoyl-CoA ($C_{8:0}$-CoA).

duction on FabZ protein amount (data not shown), in agreement with previous results (9), which suggested that this gene was regulated independently from the others. Therefore, in *E. coli*, FadR alone is responsible for controlling the expression of the two opposite pathways of FA degradation and FA synthesis (Fig. 1A). In other bacteria, two distinct regulators are used for the two functions (5, 6). However, our results show that in all bacteria studied so far, a regulator is present to control and coordinate the expression of the fatty acid synthesis genes. In addition to this global genetic control of FA synthesis genes, the unsaturated-to-saturated FA ratio is controlled by various mechanisms in bacteria. In *E. coli*, the balance between unsaturated and saturated FA is sensed by the FabR repressor, which controls the expression of *fabA* and *fabB*, which are specifically required for the synthesis of unsaturated FA (25) (Fig. 1). We showed here that FabR does not impact the expression of other FA synthesis genes.

The molecular mechanism controlling FadR binding to its operator is the same for the promoters of FA degradation and of FA synthesis genes. However, the binding strength is clearly lower for FA synthesis genes. This is reflected by the difficulty of finding evidence for the binding of FadR to the promoters of synthesis genes by classical EMSAs, whereas the binding is easily detected on promoters of FA degradation genes. This was already well shown before by quantitative measurements of FadR affinity for *fabA*, *fabB*, and *fabI* promoters, listed here by decreasing affinity, well behind the *fad* genes (4). Certainly for these reasons, we were not

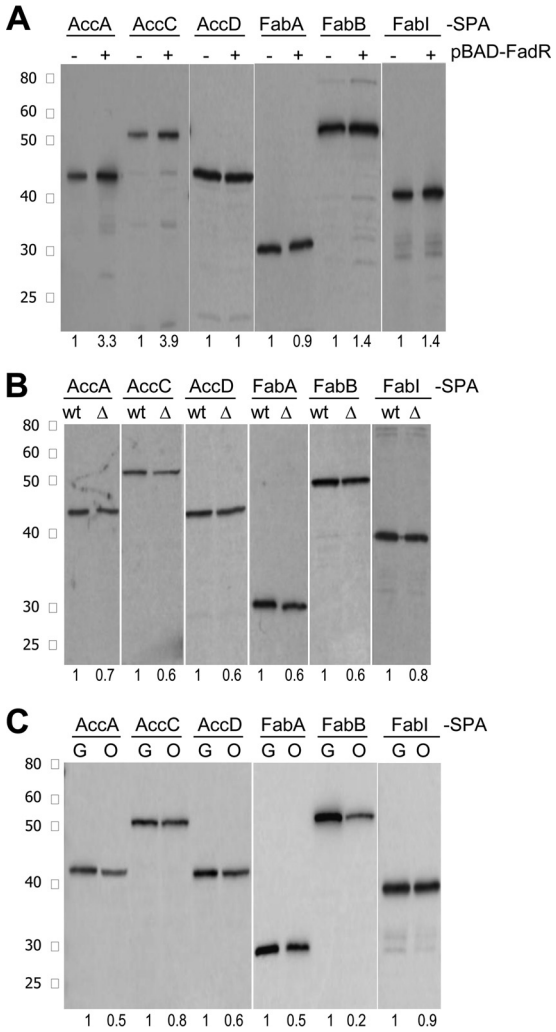


**FIG 7** Impact of FadR regulation on the abundance of FA synthesis enzymes. The six indicated strains producing SPA-tagged enzymes (EB929, EB930, EB969, EB744, EB745, and EB931) were grown under the indicated different conditions, and the amount of enzymes produced then was analyzed by 10% SDS-PAGE and Western blotting using anti-Flag antibody to detect the SPA tag. The relative protein amounts are indicated at the bottom of the images. These experiments were repeated at least 3 times independently with the same results. The molecular mass ladder (in kilodaltons) is indicated on the left. (A) The strains were transformed by pBAD24 or pBAD-FadR (pEB1210) plasmid and grown in LB at 37°C. Starting in exponential growth phase, the overproduction of FadR (+) was induced by 0.2% arabinose during 3 h. (B) The six strains from panel A (wt) plus the corresponding strains containing the Δ*fadR* deletion (Δ) (EB933, EB934, EB970, EB751, EB746, and EB935) were grown in LB at 37°C until stationary phase. (C) The six strains from panel A were grown at 37°C during 6 h in minimal medium containing 0.2% glucose (G) or 0.2% oleate (O) as the sole carbon source.

able to detect *in vitro* the binding of FadR on the *acpP* and *accA* promoters. Similarly, this explains why the *fadR* deletion had a small effect on transcription (Fig. 2A), while overproduction of FadR strongly increased expression (Fig. 2B). Using low-affinity targets, limiting levels of FadR protein ensures a modulating role of FadR on FA synthesis. Despite this low binding affinity, the results obtained with the transcriptional fusions containing mutations in the FadR binding site clearly demonstrated the direct activation of all of the tested fusions. The low conservation of the binding site consensus, in which the left part of the palindrome seems degenerated, certainly is responsible for the weak binding affinity and might be related to the function of recruiting RNA polymerase. The difference in binding affinities also might explain why FadR acts as an on/off switch on *fad* genes, whereas it only subtly tunes FA synthesis gene expression, in the manner of a dimmer switch. This behavior can be rationalized by the fact that otherwise the presence of any specific long-chain FA in the medium (for example, unsaturated fatty acids) would slow down expression, while FA synthesis in general should not be shut off. Therefore, this regulation has to be viewed as a way of managing the amount of enzymes in the cell for optimal allocation of protein resources in response to environmental changes rather than a way of directly controlling synthesis activity. Such a concept of resource allocation has been observed and explained before for central metabolism processes (27). In this context, we do not expect to observe any effect on the flux of FA synthesis if this global activation by FadR would be missing. Indeed, given that the flux magnitude is controlled mainly by allosteric enzyme regulation (1), the decrease in enzyme amounts observed in the absence of FadR (Fig. 7) certainly could not impact FA synthesis activity.

For the reasons just explained, because we do not expect to see an effect of FadR directly on FA synthesis activity, it might be difficult to demonstrate the importance of this regulation on the physiology of the bacteria. However, several results of our experiments clearly demonstrate the global regulation of FA synthesis gene expression by FadR under physiological conditions. First, both the decreased expression and the decrease in protein amounts in the Δ*fadR* mutant compared to those of the wild-type strain show that under wild-type conditions, FadR does activate the expression of the FA synthesis genes. Second, the switch in the +1 starting site used for the expression of *accA* from the P2 promoter in the wild-type strain to the P1 promoter in the Δ*fadR* mutant demonstrates that the P2 promoter is used and activated by FadR under physiological conditions (Fig. 4).

Finally, our results highlight the complexity of promoter organization of FA synthesis genes. As it was already shown for *fabA* (12) and for *fabH* (8), the expression of several FA synthesis genes appears to be driven by multiple promoters, with one of the promoters being activated by FadR (*fabA*, *accA*, *accB*, and *fabHGD*). The organization of the promoters of *accB* (Fig. 5) is strikingly similar to the organization previously reported for *fabA*, with two overlapping promoters and the downstream promoter being activated by FadR (12). This complex organization might be correlated with the scattering of FA synthesis genes on the chromosome, which might require elaborate mechanisms to ensure the coordination of expression of all the genes. The *fabZ* gene, inserted in the middle of an operon encoding genes involved in envelope biogenesis (Fig. 1B), is a most extreme case and might be involved in the coordination of FA synthesis with envelope biogenesis in general.

Individually, some promoters highly rely upon FadR, such as the promoter upstream of *fabH* (8) (Fig. 2A), the *accD* promoter, and the $accAp_2$ promoter described here (Fig. 4C). This pattern of activation is similar to the one described for *fabA* (2, 8, 28). The strong dependency of the $accAp_2$ promoter on FadR is highlighted by the observed shift of the transcription start site in the Δ*fadR* mutant. Similarly, a shift from the *fabA* promoter controlled by FadR to an upstream one has been observed in a *fadR* mutant (12). On the other hand, other promoters are less affected by FadR absence, such as promoters of *accB* or *fabI* (Fig. 2A). Despite the

confirmation of the presence of two possible promoters for *accB*, $accBp_1$, which was previously described (13), and $accBp_2$, which is activated by FadR (Fig. 5A), we were not able to detect the P1 transcript by 5′-RACE experiments, even in the *fadR* deletion strain (Fig. 5B). We suspect that various and complex regulation mechanisms, depending on the strains and growth conditions, explain these different observations.

This complexity might be only the tip of the iceberg, and many more mechanisms of the expression of the regulation of FA synthesis genes might be waiting to be discovered. For example, it has been observed that AccB acts as an autoregulator of *accBC* operon transcription by a still-unknown mechanism (29). Furthermore, it was already noted before that some genes, such as *accA* and *accB*, have very long mRNA leader sequences (300 bases long), with the presence of potential regulatory sequences (13). This leaves room for numerous additional post-transcriptional regulation mechanisms. It was suggested that AccA protein inhibits *accA* and *accD* translation (30), yet this result has been refuted recently (31). A different and most tempting prediction is that small noncoding RNAs control mRNA translation regulation.

There is a very high interest in engineering the FA synthesis pathway in *E. coli* for biofuel production. However, it appears that if one wants to do synthetic biology that really works, it is crucial to understand the regulation and countereffects that may take place in the cell (32). Even if the biochemistry of FA synthesis in *E. coli* is now very well known and mastered, our results show that there is still some room for progress in the understanding of the genetic regulation of FA synthesis and for discoveries of new mechanisms, even in *E. coli*.

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## REFERENCES

1. **Chan DI, Vogel HJ.** 2010. Current understanding of fatty acid biosynthesis and the acyl carrier protein. Biochem J **430:**1–19. http://dx.doi.org/10.1042/BJ20100462.
2. **Henry MF, Cronan JEJ.** 1991. *Escherichia coli* transcription factor that both activates fatty acid synthesis and represses fatty acid degradation. J Mol Biol **222:**843–849. http://dx.doi.org/10.1016/0022-2836(91)90574-P.
3. **Campbell JW, Cronan JEJ.** 2001. *Escherichia coli* FadR positively regulates transcription of the fabB fatty acid biosynthetic gene. J Bacteriol **183:**5982–5990. http://dx.doi.org/10.1128/JB.183.20.5982-5990.2001.
4. **DiRusso CC, Black PN, Weimar JD.** 1999. Molecular inroads into the regulation and metabolism of fatty acids, lessons from bacteria. Prog Lipid Res **38:**129–197. http://dx.doi.org/10.1016/S0163-7827(98)00022-8.
5. **Fujita Y, Matsuoka H, Hirooka K.** 2007. Regulation of fatty acid metabolism in bacteria. Mol Microbiol **66:**829–839. http://dx.doi.org/10.1111/j.1365-2958.2007.05947.x.
6. **Zhang YM, Rock CO.** 2010. A rainbow coalition of lipid transcriptional regulators. Mol Microbiol **78:**5–8.
7. **Nunn WD, Giffin K, Clark D, Cronan JEJ.** 1983. Role for *fadR* in unsaturated fatty acid biosynthesis in *Escherichia coli*. J Bacteriol **154:**554–560.
8. **My L, Rekoske B, Lemke JJ, Viala JP, Gourse RL, Bouveret E.** 2013. Transcription of the *Escherichia coli* fatty acid synthesis operon *fabHDG* is directly activated by FadR and inhibited by ppGpp. J Bacteriol **195:**3784–3795. http://dx.doi.org/10.1128/JB.00384-13.
9. **Zhang F, Ouellet M, Batth TS, Adams PD, Petzold CJ, Mukhopadhyay A, Keasling JD.** 2012. Enhancing fatty acid production by the expression of the regulatory transcription factor FadR. Metab Eng **14:**653–660. http://dx.doi.org/10.1016/j.ymben.2012.08.009.
10. **Cronan JEJ, Subrahmanyam S.** 1998. FadR, transcriptional co-ordination of metabolic expediency. Mol Microbiol **29**(4):937–943. http://dx.doi.org/10.1046/j.1365-2958.1998.00917.x.
11. **Zhang Y, Cronan JEJ.** 1996. Polar allele duplication for transcriptional analysis of consecutive essential genes: application to a cluster of *Escherichia coli* fatty acid biosynthetic genes. J Bacteriol **178:**3614–3620.
12. **Feng Y, Cronan JE.** 2009. *Escherichia coli* unsaturated fatty acid synthesis: complex transcription of the *fabA* gene and in vivo identification of the essential reaction catalyzed by FabB. J Biol Chem **284:**29526–29535. http://dx.doi.org/10.1074/jbc.M109.023440.
13. **Li SJ, Cronan JEJ.** 1993. Growth rate regulation of *Escherichia coli* acetyl coenzyme A carboxylase, which catalyzes the first committed step of lipid biosynthesis. J Bacteriol **175:**332–340.
14. **Zaslaver A, Bren A, Ronen M, Itzkovitz S, Kikoin I, Shavit S, Liebermeister W, Surette MG, Alon U.** 2006. A comprehensive library of fluorescent transcriptional reporters for *Escherichia coli*. Nat Methods **3:**623–628. http://dx.doi.org/10.1038/nmeth895.
15. **Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, Datsenko KA, Tomita M, Wanner BL, Mori H.** 2006. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol Syst Biol **2:**2006.0008. http://dx.doi.org/10.1038/msb4100050.
16. **Butland G, Peregrin-Alvarez JM, Li J, Yang W, Yang X, Canadien V, Starostine A, Richards D, Beattie B, Krogan N, Davey M, Parkinson J, Greenblatt J, Emili A.** 2005. Interaction network containing conserved and essential protein complexes in *Escherichia coli*. Nature **433:**531–537. http://dx.doi.org/10.1038/nature03239.
17. **Miller JH.** 1992. A short course in bacterial genetics: a laboratory manual and handbook for *Escherichia coli* and related bacteria. Cold Spring Harbor Laboratory Press, Plainview, NY.
18. **Cherepanov PP, Wackernagel W.** 1995. Gene disruption in *Escherichia coli*: TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. Gene **158:**9–14. http://dx.doi.org/10.1016/0378-1119(95)00193-A.
19. **Crosby HA, Pelletier DA, Hurst GB, Escalante-Semerena JC.** 2012. System-wide studies of N-lysine acetylation in *Rhodopseudomonas palustris* reveal substrate specificity of protein acetyltransferases. J Biol Chem **287:**15590–15601. http://dx.doi.org/10.1074/jbc.M112.352104.
20. **Gully D, Moinier D, Loiseau L, Bouveret E.** 2003. New partners of acyl carrier protein detected in *Escherichia coli* by tandem affinity purification. FEBS Lett **548:**90–96. http://dx.doi.org/10.1016/S0014-5793(03)00746-4.
21. **Dartigalongue C, Missiakas D, Raina S.** 2001. Characterization of the *Escherichia coli* sigma E regulon. J Biol Chem **276:**20866–20875. http://dx.doi.org/10.1074/jbc.M100464200.
22. **Solovyev VV, Salamov A.** 2011. Automatic annotation of microbial genomes and metagenomic sequences, p 61–78. *In* Li RW (ed), Metagenomics and its applications in agriculture, biomedicine and environmental studies. Nova Science Publishers, Hauppauge, NY.
23. **Mendoza-Vargas A, Olvera L, Olvera M, Grande R, Vega-Alvarado L, Taboada B, Jimenez-Jacinto V, Salgado H, Juarez K, Contreras-Moreira B, Huerta AM, Collado-Vides J, Morett E.** 2009. Genome-wide identification of transcription start sites, promoters and transcription factor binding sites in *E. coli*. PLoS One **4:**e7526. http://dx.doi.org/10.1371/journal.pone.0007526.
24. **Zeghouf M, Li J, Butland G, Borkowska A, Canadien V, Richards D, Beattie B, Emili A, Greenblatt JF.** 2004. Sequential peptide affinity (SPA) system for the identification of mammalian and bacterial protein complexes. J Proteome Res **3:**463–468. http://dx.doi.org/10.1021/pr034084x.
25. **Feng Y, Cronan JE.** 2011. Complex binding of the FabR repressor of bacterial unsaturated fatty acid biosynthesis to its cognate promoters. Mol Microbiol **80:**195–218. http://dx.doi.org/10.1111/j.1365-2958.2011.07564.x.
26. **Henry MF, Cronan JEJ.** 1992. A new mechanism of transcriptional regulation: release of an activator triggered by small molecule binding. Cell **70:**671–679. http://dx.doi.org/10.1016/0092-8674(92)90435-F.
27. **Chubukov V, Gerosa L, Kochanowski K, Sauer U.** 2014. Coordination of microbial metabolism. Nat Rev Microbiol **12:**327–340. http://dx.doi.org/10.1038/nrmicro3238.
28. **DiRusso CC, Metzger AK, Heimert TL.** 1993. Regulation of transcription of genes required for fatty acid transport and unsaturated fatty acid biosynthesis in *Escherichia coli* by FadR. Mol Microbiol **7:**311–322. http://dx.doi.org/10.1111/j.1365-2958.1993.tb01122.x.
29. **James ES, Cronan JE.** 2004. Expression of two *Escherichia coli* acetyl-CoA

carboxylase subunits is autoregulated. J Biol Chem **279:**2520–2527. http://dx.doi.org/10.1074/jbc.M311584200.
30. **Meades GJ, Benson BK, Grove A, Waldrop GL.** 2010. A tale of two functions: enzymatic activity and translational repression by carboxyltransferase. Nucleic Acids Res **38:**1217–1227. http://dx.doi.org/10.1093/nar/gkp1079.
31. **Smith AC, Cronan JE.** 2014. Evidence against translational repression by the carboxyltransferase component of *Escherichia coli* acetyl coenzyme A carboxylase. J Bacteriol **196:**3768–3775. http://dx.doi.org/10.1128/JB.02091-14.
32. **Lennen RM, Pfleger BF.** 2012. Engineering *Escherichia coli* to synthesize free fatty acids. Trends Biotechnol **30:**659–667. http://dx.doi.org/10.1016/j.tibtech.2012.09.006.
33. **Campbell JW, Morgan-Kiss RM, Cronan JEJ.** 2003. A new *Escherichia coli* metabolic competency: growth on fatty acids by a novel anaerobic beta-oxidation pathway. Mol Microbiol **47:**793–805. http://dx.doi.org/10.1046/j.1365-2958.2003.03341.x.
34. **Gui L, Sunnarborg A, LaPorte DC.** 1996. Regulated expression of a repressor protein: FadR activates iclR. J Bacteriol **178:**4704–4709.
35. **Podkovyrov SM, Larson TJ.** 1996. Identification of promoter and stringent regulation of transcription of the *fabH*, *fabD* and *fabG* genes encoding fatty acid biosynthetic enzymes of *Escherichia coli*. Nucleic Acids Res **24:**1747–1752. http://dx.doi.org/10.1093/nar/24.9.1747.
36. **Crooks GE, Hon G, Chandonia JM, Brenner SE.** 2004. WebLogo: a sequence logo generator. Genome Res **14:**1188–1190. http://dx.doi.org/10.1101/gr.849004.
37. **Kazakov AE, Rodionov DA, Alm E, Arkin AP, Dubchak I, Gelfand MS.** 2009. Comparative genomics of regulation of fatty acid and branched-chain amino acid utilization in proteobacteria. J Bacteriol **191:**52–64. http://dx.doi.org/10.1128/JB.01175-08.
38. **Guzman LM, Belin D, Carson MJ, Beckwith J.** 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose $P_{BAD}$ promoter. J Bacteriol **177:**4121–4130.