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Transcriptional Regulation of Membrane Lipid Homeostasis in *Escherichia coli*[*]

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The biophysical properties of membrane phospholipids are controlled by the composition of their constituent fatty acids and are tightly regulated in *Escherichia coli*. The FabR (fatty acid biosynthesis repressor) transcriptional repressor controls the proportion of unsaturated fatty acids in the membrane by regulating the expression of the *fabB* (β-ketoacyl-ACP synthase I) and *fabA* (β-hydroxydecanoyl-ACP dehydratase/isomerase) genes. FabR binding to a DNA palindrome located within the promoters of the *fabB* and *fabA* genes required the presence of an unsaturated acyl-acyl carrier protein (ACP) or acyl-CoA and was antagonized by saturated acyl-ACP or acyl-CoA. The FabR-dependent repression of *fabB* and *fabA* by exogenous unsaturated fatty acids confirmed the role for FabR in responding to the acyl-CoA pool composition, and the perturbation of the unsaturated:saturated acyl-ACP ratio using a specific inhibitor of lipid A formation verified FabR-dependent regulation of *fabB* by the acyl-ACP composition *in vivo*. Thus, FabR plays a key role in controlling the membrane biophysical properties by regulating gene expression in response to the composition of the long-chain acyl-thioester pool. This mechanism ensures that a balanced composition of fatty acids is available for incorporation into the membrane via the PlsB/PlsC acyltransferases.

Bacterial growth optimization and survival depends on the ability of the organisms to control the biophysical properties of their phospholipids to match the permeability properties of the membrane to environmental conditions (for review, see Ref. 1). These biophysical properties are defined in large part by fatty acid structures that are incorporated into the component phospholipids. *Escherichia coli* primarily contains 16- and 18-carbon saturated fatty acids (SFAs)[3] and monounsaturated fatty acids (UFAs) that are produced from the anaerobic type II fatty acid biosynthetic pathway (2). FabA and FabB are the essential enzymes for UFA biosynthesis. FabA is a bifunctional dehydratase/isomerase that converts 3-hydroxydecanoyl-acyl carrier protein (ACP) to *cis*-3-decenoyl-ACP (3). FabB is then required to elongate the 3-*cis* intermediate to 3-keto-5-*cis*-dodecenoyl-ACP, which is subsequently elongated to form either 16:1Δ9 or 18:1Δ11 UFA-ACP (4). In *E. coli*, the transcription of the *fabA* and *fabB* genes is controlled by the opposing action of the FadR (fatty acid degradation repressor) transcriptional activator and the FabR transcriptional repressor (5–7). Based on the analysis of *fabA* and *fabB* transcript levels in *fadR* and *fabR* knock-out strains, FadR has the greatest influence on *fabA* expression, whereas FabR has the most pronounced effect on *fabB* expression. Regulation of FadR involves exogenous UFA and SFA, which enter the cell and are converted to CoA thioesters. These long-chain acyl-CoAs bind to FadR to induce a conformational change that releases FadR from its DNA binding site (8, 10). FadR was first discovered as a repressor of β-oxidation genes, but the FadR cognate sequence also exists in the −40 regions of the *fabA* and *fabB* fatty acid biosynthetic genes. In this context, FadR functions as an activator of *fabA* and *fabB* gene transcription, and its release from the DNA binding site by acyl-CoA reduces transcription from these promoters (5, 7). FabR is a repressor that binds downstream of the FadR sites on the *fabA* and *fabB* promoters, but the ligand that controls the binding of FabR to DNA is unknown (6). This report demonstrates that FabR regulates gene expression by monitoring the structure of fatty acids attached to either acyl-ACP or acyl-CoA thioesters. UFA-ACP or UFA-CoA are postulated to induce a conformational change that increases FabR binding to the *fabB* promoter repressing *fabB* and *fabA* transcription, whereas SFA-ACP or SFA-CoA block this conformational change from occurring and prevent FabR-dependent repression of *fabB* and *fabA* expression. Thus, FabR is a transcriptional regulator that responds to the composition of the long-chain acyl thioester pool available for membrane phospholipid synthesis and adjusts gene expression to properly balance production of UFA and SFA by the fatty acid biosynthetic pathway.

EXPERIMENTAL PROCEDURES

Materials and Bacterial Strains—Acyl-CoAs were purchased from Avanti Polar Lipids. Radiolabeled fatty acids were purchased from Amersham Biosciences. Acyl-ACPs were synthesized by the acyl-ACP synthetase method (11), ACP antibodies were as described (12), and FadR was purified as described previously (13). All other chemicals were reagent grade or better. The bacterial strains used in this study were derivatives of *E. coli* K12, and their relevant genotypes and source are listed in Table 1.

Cloning, Expression, and Purification of the Recombinant FabR—Gene fragment coding for the *fabR-yijD* operon was amplified by PCR using genomic DNA of *E. coli* strain UB1005 as the template. The operon was used because it exhibited better expression than *fabR* alone. The PCR product was cloned

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[3] The abbreviations used are: SFA, saturated fatty acid; ACP, acyl carrier protein; UFA, unsaturated fatty acid; FASII, type II fatty acid synthase system.

into pCR-Blunt-TOPO (Invitrogen) and sequenced. Plasmid containing the correct *fabR-yijD* sequence was digested with NheI and EcoRI, and the gel-purified DNA fragment was inserted into pET-28b restricted with the same enzymes. The resultant plasmid was used to transform Rosetta (Novagen) cells to overexpress FabR with an N-terminal His-tag. FabR expression was induced with 1 mM isopropyl-β-D-thiogalactopuranoside for 3 h at 37 °C. Cells were collected by centrifugation, resuspended in MCAC-0 buffer (20 mM Tris, pH 7.9, 0.5 M NaCl, 1 mM phenylmethylsulfonyl fluoride, 0.1% Triton X-100) and lysed with a French press. The inclusion bodies were precipitated by centrifugation at 10,000 × *g* for 30 min and washed with the same buffer once. The pellet was solubilized in denaturing buffer (100 mM NaH_2PO_4, 10 mM Tris, 8 M urea, pH 8.0) at room temperature for 1 h. The urea insoluble material was removed by centrifugation at 10,000 × *g* for 30 min. The solubilized inclusion bodies were mixed with pre-equilibrated cobalt resin (Clontech) at room temperature for 30 min. The mixture was loaded into an empty column and washed with 4 resin volumes of denaturing buffer, pH 6.3, then eluted with 2 volumes of denaturing buffer, pH 5.9, and 2 volumes of denaturing buffer, pH 4.5. All of the purification procedures were carried out at 4 °C. The fractions containing most of the FabR proteins were pooled and concentrated and dialyzed stepwise (>12 h) against buffer (50 mM HEPES, pH 7.4, 50 mM NaCl) containing 2 M urea.

FabR in buffered 2 M urea was applied to a Superdex™ 10/300 GL column (Amersham Biosciences) that was eluted with 50 mM Tris, pH 7.4, 500 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol, and 200 μl fractions were collected in a 96-well microtiter plate. The purity of the recombinant protein was assessed by SDS-PAGE. The identity of FabR protein was confirmed by mass spectrometry.

Electrophoretic Mobility Shift Assay—Biotin-labeled DNA probes were detected by chemiluminescence using a light shift chemiluminescent electrophoretic mobility shift assay kit (Pierce). All experimental procedures were performed according to the manufacturer's instructions, with the exception that the binding buffer condition was changed. The oligonucleotide probe contained only the FabR protein binding palindrome. The double-stranded probes were generated from two complementary primers (one labeled with biotin) by incubating at 94 °C for 2 min, followed by decreasing the temperature to 14 °C over the course of 4 h. Gel mobility shift assays were performed by incubating 20 pg of labeled probe with proteins at indicated concentration in binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 300 μg ml^{-1} bovine serum albumin, and 1 μg/μl poly(dI-dC)) at room temperature for 30 min. Then, 5 μl of 5× TBE (Tris borate EDTA) sample buffer was added to the 20-μl reaction before being loaded onto a 6% DNA retardation gel. After electrophoreses at 100 V for 1 h, the samples were transferred onto a positively charged nylon membrane (Hybond™ N^+) in 0.5× TBE at 40 V for 1 h. Transferred DNAs were cross-linked to the membrane at 120 mJ/cm^2 and detected using horseradish peroxidase-conjugated streptavidin (LightShift™ chemiluminescent EMSA kit) according to the manufacturer's instructions. The DNA band signal was visualized by expose to x-ray film for 1–4 min.

Measurement of mRNA Levels—The levels of *fabA* and *fabB* mRNA were measured by real-time PCR using oligonucleotide primers, and probes were synthesized at the Hartwell Center for Bioinformatics and Biotechnology at St. Jude Children's Research Hospital as described previously (6). Amplification and detection of real-time PCR product was performed with Taqman Universal PCR Master Mixes (Applied Biosystems) and ABI Prism 7700 sequence system (Applied Biosystems). Experiments were performed with three replicas for each cDNA, and negative-reverse transcriptase and no-template controls were run for each reaction on the same 96-well plate. *E. coli* strains were grown with or without 0.025% palmitoleic acid to mid-log phase, or strains were grown to mid-log phase and treated with 100 μg/ml cerulenin for 15 min. Cells were harvested by centrifugation, and total RNA was isolated using Ambion RNAqueous purification kit according to the manufacturer's instructions (Ambion). Aliquots of the reverse transcription reaction were added in the real-time PCR reaction containing 1× of Taqman Universal PCR Master Mixes (Applied Biosystems), 660 nM of each forward and reverse primer, and 150 nM of probe. Amplification and detection of specific products were performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems). All of the real-time values were compared using the ΔC_T method, where the amount of *fabA* or *fabB* cDNA ($2^{-\Delta\Delta C(T)}$) was calculated by normalizing C_T to the housekeeping gene *acpP* (ΔC_T) before being compared with the amount of *fabA*/*fabB* cDNA in strain SJ95 grown without palmitoleic acid ($\Delta\Delta C_T$), which was set as the calibrator at 1.0.

Fatty Acid Composition—Cultures (10 ml) of *E. coli* strains were grown to mid-log phase in LB medium, and the lipids were extracted (14). The fatty acid methyl esters were prepared and quantified using a Hewlett-Packard Model 5890 gas chromatograph as described previously (6).

Inhibition of Lipid A Synthesis with CHIR-090—E. coli strains were grown in minimal medium M9 containing glycerol as the carbon source to mid-exponential phase. The total 25 ml of culture was distributed into five flasks (5-ml each). At 0, 10, 20, 30, 40, and 50 min following the addition of CHIR-090, 5-ml aliquots of cell culture were labeled with 2 μCi/ml of [1-^{14}C]acetic acid for 10 min. As a control, 5 ml of mock-treated (dimethyl sulfoxide) cell culture was labeled with 2 μCi/ml of [1-^{14}C]acetic acid for 10 min. The cells were collected, the lipids were extracted (14), and the acyl chains were converted to methyl esters (6). The fatty acid methyl esters were separated into unsaturated and saturated fractions by chromatography on 20% silver nitrate-impregnated Silica Gel H thin layer plates. The plates were visualized using a PhosphorImager screen, and UFA and SFA were quantified using a Typhoon 9200 and ImageQuant 5.2 software (Amersham Biosciences).

Affymetrix Microarray Analysis—The Affymetrix *E. coli* genome 2.0 arrays were used to assess the effect of CHIR-090 treatment on the global gene expression. *E. coli* strain MG1655 was grown in Luria broth to mid-log phase with an OD at 600 nm of 0.6 before the cells were treated with either 1 μg/ml CHIR-090 (10× minimum inhibitory concentration) or an equal volume of dimethyl sulfoxide solvent control. After a 10-min treatment, the cells were harvested by centrifugation,

TABLE 1

FabR regulation of fatty acid composition

Strains were grown in Luria broth at 37 °C, harvested in the mid-log phase of growth, the lipids were extracted, fatty acid methyl esters were prepared, and the compositions were determined by gas-liquid chromatography as described under "Experimental Procedures." wt, wild-type.

Strain	Relevant genotype	Plasmid	Source	Fatty acid composition				
				16:0	18:0	16:1[a]	18:1[a]	UFA/SFA
UB1005	wt[b]		Ref. 37	48.1	2.6	28.2	21.0	0.97
KZ6[c]	*fabR*		This work	30.1	1.6	13.0	55.3	2.2
KZ7[c]	*yijD*		This work	48.0	1.5	32.2	18.1	1.0
UB1005	wt	pBlue[d]	This work	50.7	4.6	31.1	13.3	0.80
UB1005	wt	pFabR	This work	52.1	2.1	29.2	16.5	0.84
UB1005	wt	pYijD	This work	50.5	2.4	28.9	18.0	0.88
UB1005	wt	pFabR-YijD	This work	55.8	1.4	27.9	14.7	0.75
KZ6	*fabR*	pFabR	This work	51.7	1.9	27.4	18.8	0.86
BW25113	Keio[e]		Ref. 17	32.7	2.3	24.1	40.8	1.9
JW3935[f]	*fabR*		Ref. 17	30.4	1.3	23.8	44.1	2.1
JW3936[f]	*yijD*		Ref. 17	32.5	2.2	23.3	41.8	1.9
BW25113	Keio	pFabR	This work	54.0	1.7	20.2	23.5	0.78

[a] The contributions of the respective cyclopropane derivatives were included in these percentages.
[b] Strain UB1005 (*gyrA37*, *metB1*, *relA1*, *spoT1*, *LAM*$^-$, *LAM*^{R}, F$^-$) (37).
[c] Strains KZ6 and KZ7 were derivatives of strain UB1005.
[d] The pBluescript II KS (Stratagene) plasmid was used as empty vector control, and all of the other expression plasmids were constructed in this vector.
[e] Strain BW25113 (Δ*(araD-araB)567*, Δ*lacZ4787(::rrnB-3)*, *LAM*$^-$, *rph-1*, *hsdR514*, Δ*(rhaD-rhaB)568*, F$^-$) was the parent strain for the Keio collection.
[f] Strains JW3935 and JW3936 were derivatives of strain BW25113.

and total RNA was isolated using a Ambion RNAqueous purification kit according to the manufacturer's instructions (Ambion). The purified RNA was mixed with 0.5 volume of LiCl precipitation solution (Ambion) and incubated at −20 °C for 30 min. The precipitated RNA sample was treated with Turbo DNase (Ambion) at 37 °C for 30 min with 1 unit of DNase for 5 μg of RNA. Agarose gel electrophoresis and the Agilent Technologies 2100 Bioanalyzer lab-on-a-chip system were used to assess the integrity of the purified RNA before it was further processed for microarray experiments. The mRNA in the isolated total RNA sample was converted to cDNA using procedures recommended by Affymetrix. RNA was then removed by alkaline hydrolysis, and cDNA was purified using a MiniElute PCR purification kit (Qiagen). The cDNA was fragmented with DNase I (Amersham Biosciences) with 0.6 units/μg cDNA. The 3′ termini of fragmented cDNA were labeled with biotin using GeneChip DNA labeling reagent (Affymetrix), and the labeling efficiency was determined by a gel shift assay based on the retardation of the biotinylated cDNA upon addition of NeutrAvidin (Pierce). 1.5–4.0 μg of fragmented labeled cDNA was hybridized for 16 h at 50 °C to *E. coli* 2.0 genome arrays (Affymetrix). After washing, staining, and scanning of the arrays were performed according to the manufacturer's protocol (Affymetrix), the arrays were analyzed using the GeneChip operating software, and global scaling was used to normalize the data from different arrays with the target intensity value set at 500. Spotfire software was used to analyze the data from three independent samples from each treatment.

RESULTS

Fatty Acid Compositional Control by FabR, Not YijD—The insertional inactivation of FabR by the introduction of a transposon results in a substantial increase in the proportion of UFA in the total phospholipid fatty acids reflected by an increase in the amount of *cis*-vaccenate (18:1Δ11) (6) (Table 1). The *fabR* (*yijC*) gene in *E. coli* is the leading gene in an operon containing a second small gene, *yijD* (15). YijD is an inner membrane protein with its carboxy terminus facing the cytosol (16) and because *yijD* expression may be compromised by the insertional inactivation of *fabR*, it is not known whether it also functions in fatty acid composition control. Based on the examination of the 130 top scoring YijD homologs in the Integrated Microbial Genomes database, *yijD* always occurs as the second gene in a two-gene operon containing *fabR*. This strong genetic association of *fabR* and *yijD* suggests that they may both be involved in controlling fatty acid biosynthesis. However, neither the specific deletion in the *yijD* gene nor the introduction of a YijD-expressing plasmid altered the fatty acid composition of *E. coli* (Table 1). Thus, FabR was both necessary and sufficient for the regulation of the UFA:SFA ratio, and the importance of this highly conserved genetic partner of *fabR* remains unknown.

The Keio collection (17) of *E. coli* insertional inactivation mutants is widely used in the field for determining gene knock-out phenotypes. The *yijD* knock-out strain from this collection had a fatty acid composition that was consistent with a role for YijD in regulating fatty acid synthesis (Table 1). However, the parent strain and the *fabR* knock-out strain in the Keio collection also had the same fatty acid composition that is abnormally high in total UFA and *cis*-vaccenate, and thus, all of these strains were phenotypically similar to strain KZ6 (Δ*fabR*) (Table 1). DNA sequencing of the *fabR* gene in strain BW25113 showed that it contained a Gly-to-Thr missense mutation in the DNA binding domain 125 nucleotides from the start codon that predicted the expression of a FabR^{G42V} mutant protein. Gly^{42} is an invariant residue in FabR proteins, and this mutation likely inactivates FabR DNA binding. This conclusion was supported by the recovery of a normal fatty acid composition following the expression of a wild-type *fabR* gene in strain BW25113 (Table 1). Thus, derivatives of strain BW25113 cannot be used to study FabR regulation of fatty acid biosynthesis.

UFA-ACP and UFA-CoA Activate FabR DNA Binding—FabR expressed in *E. coli* was mainly found in inclusion bodies. This necessitated the development of a procedure for the solubilization and refolding of FabR described under "Experimental Procedures." Although the method was not efficient (<5%) with regard to the amount of soluble FabR recovered, the method

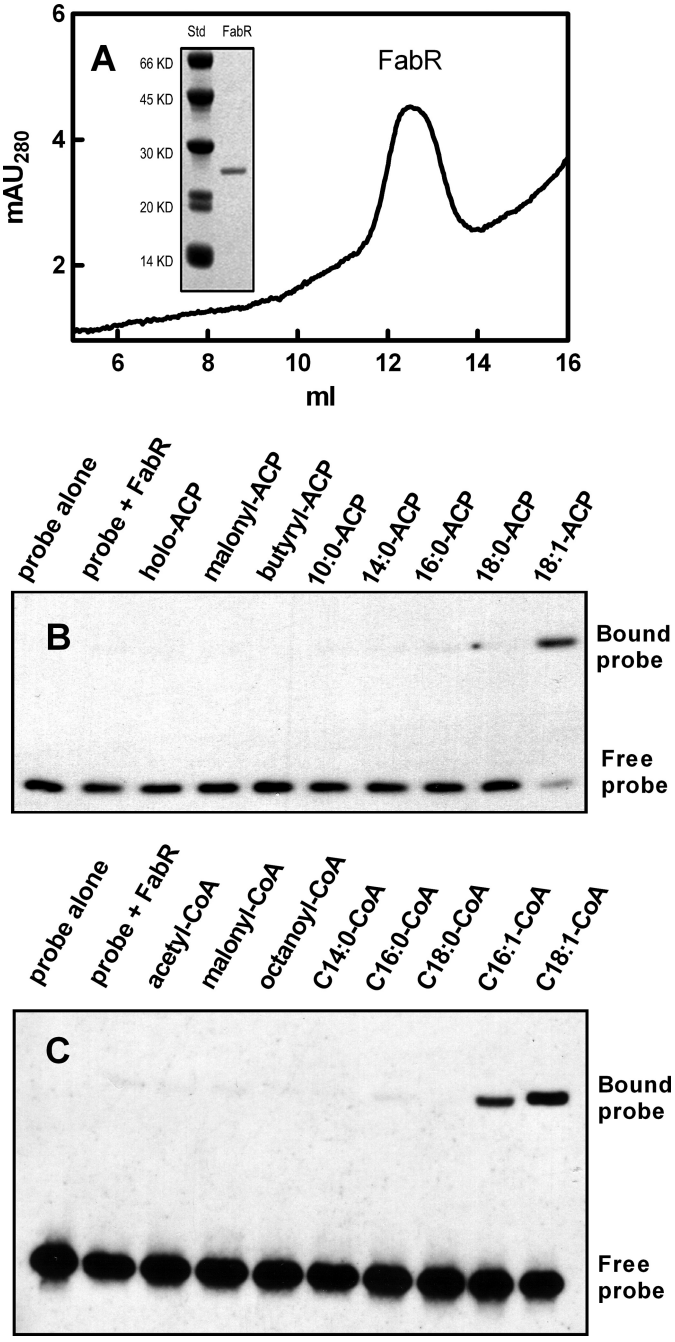

FIGURE 1. **Ligand screen for FabR binding to its DNA palindrome.** *A*, FabR was solubilized in urea, purified by affinity-chromatography and renatured as described under "Experimental Procedures." The refolded FabR protein was subjected to gel filtration chromatography to isolate correctly assembled dimers mAU_{280}, milliabsorbance units. The stained SDS gel in the *inset* illustrates the purity of the final FabR preparation. *B*, a panel of ACP derivatives were screened for their ability to promote FabR DNA binding. *C*, shown is a screen of CoA thioesters tested for their ability to promote FabR DNA binding. The ligand concentrations were 1 μM.

yielded sufficient quantities of pure protein for the biochemical analysis of FabR interactions with DNA and ligands. The final step was gel filtration chromatography to isolate correctly folded protein and remove the bulk of the urea (Fig. 1*A*). The fraction containing FabR was collected and concentrated, and the buffer was exchanged to yield the pure preparation (Fig. 1*A*, *inset*).

FabR functions as a repressor of *fabA* and *fabB in vivo* based on the increased expression of these genes (6) and the subsequent increase in the UFA content of the phospholipids in *fabR* knock-out strains (Table 1). The reintroduction of FabR into strain KZ6 (Δ*fabR*) using a FabR expression plasmid restored the normal fatty acid composition; however, the presence of the FabR expression plasmid in the wild-type strain UB1005 did not significantly repress the synthesis of UFA (Table 1). This finding led to the idea that FabR was not a classical bacterial repressor that binds DNA in the absence of ligand, but, rather, requires the presence of a limiting intracellular ligand for DNA binding *in vivo*. Therefore, we analyzed a selection of candidate intermediates in fatty acid synthesis to identify potential FabR regulatory ligands that promoted DNA binding to the FabR site within the *fabB* promoter (Fig. 1*B*). Of the acyl-ACPs tested, 18:1Δ11-ACP was effective in promoting the binding of FabR to DNA, whereas saturated and short-chain acyl-ACP were not (Fig. 1*B*). A screen of CoA thioesters revealed that the only ligands that promoted FabR-DNA interaction were the unsaturated acyl-CoAs (Fig. 1*C*). FabR binding to the palindrome present in the *fabA* promoter exhibited the same pattern of ligand dependence (data not shown). These data pinpoint UFA thioesters of ACP and CoA as ligands that interact with FabR, resulting in its tight binding to the *fabB* promoter.

Increased FabR binding in the presence of 18:1Δ11-ACP or 18:Δ9-CoA, but not with 16:0-ACP or 16:0-CoA, was also observed when a dialyzed crude cell lysate of the wild-type strain UB1005 was used as the protein source (data not shown). The UFA-ACP-dependent gel shift activity was not detected in extracts from a *fabR*-null strain. These observations suggested that FabR was the only protein that bound to the DNA palindrome in the *fabB* promoter in *E. coli* in a UFA thioester-dependent manner.

Formation of FabR·acyl-ACP·DNA Complexes—An ACP antibody (12) was used to determine whether the FabR·DNA complex detected in the gel shift experiments consisted of a ternary complex between FabR, 18:1Δ11-ACP, and DNA (Fig. 2*A*). The presence of the ACP antibody resulted in a supershift of the FabR·DNA complex when it was formed in the presence of 18:1Δ11-ACP, but not when 18:1Δ9-CoA was used as the ligand. Also, the FabR·UFA-CoA·DNA complex migrated slightly faster than the FabR·UFA-ACP·DNA complex, consistent with its lower molecular weight. These data showed that the band arising in the presence of acyl-ACP consisted of a FabR·acyl-ACP·DNA ternary complex.

Simultaneous Binding of FabR and FadR to the fabB Promoter—Previous studies on the regulation of *fabB* gene expression indicated that both FadR and FabR contributed to its regulation (6). We determined that FadR bound to the *fabB* promoter and was released from its binding site by acyl-CoA *in vitro* (Fig. 2*B*). As shown earlier, unsaturated acyl-CoA or acyl-ACP triggered FabR binding. The complex formed between the *fabB* probe and FadR migrated slightly faster than the FabR·probe complex. In the presence of FadR plus FabR, only the FadR·DNA complex formed. However, in the presence of 18:1Δ11-ACP, we detected a FadR·DNA complex, a FabR·acyl-ACP·DNA complex, and a third slower migrating complex representing a FadR·FabR·acyl-ACP·DNA complex (Fig. 2*B*). These data support the idea that

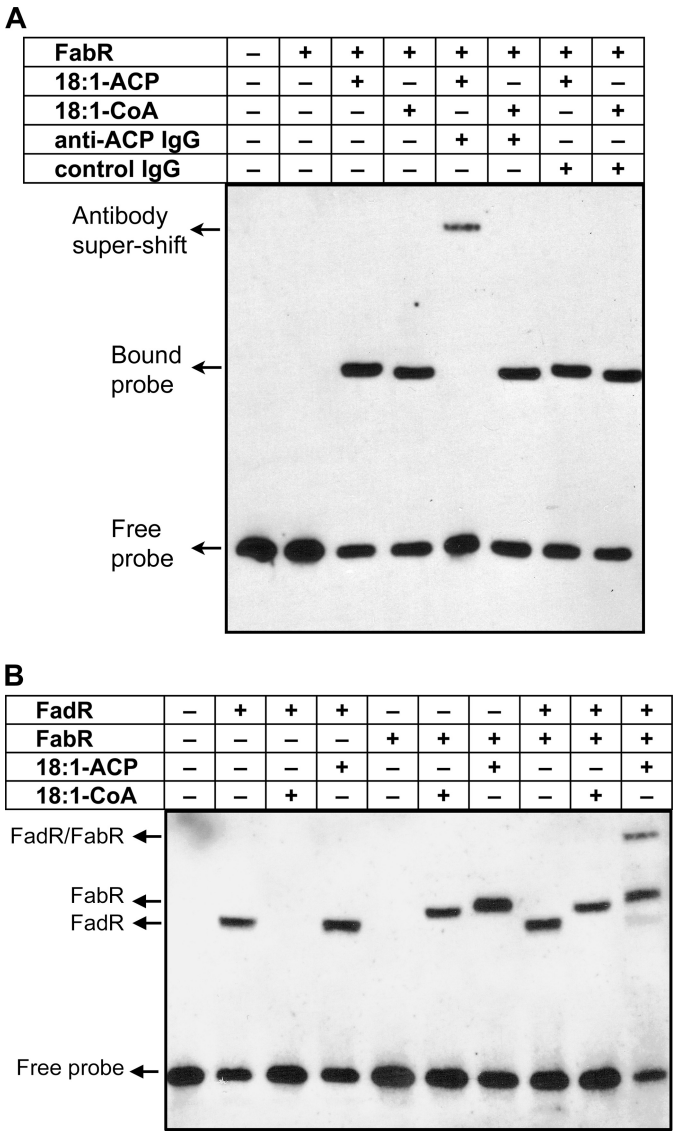

FIGURE 2. **Formation of acyl-ACP·FabR·DNA and acyl-ACP·FadR·FabR·DNA complexes.** *A*, electrophoretic mobility gel shifts were performed using the reagents listed *above each lane* to illustrate the supershift of the acyl-ACP-dependent FabR DNA gel shift in the presence of anti-ACP IgG. *B*, FabR and FadR simultaneously bind to the *fabB* promoter. Electrophoretic mobility gel shifts were performed using the reagents listed *above each lane* in the presence of FabR, FabR, or both transcription factors.

FadR and FabR were able to simultaneously associate with the promoter regions of the *fabA* and *fabB* genes.

SFA Thioesters Antagonize FabR DNA Binding—The inability of 16:0-ACP to promote FabR DNA binding (Fig. 1*A*) suggested that SFA-ACP either does not bind to FabR or antagonizes the UFA-ACP-induced FabR association with DNA. This model was evaluated by determining if SFA thioesters acted as inhibitors of UFA thioester-dependent FabR DNA binding. The UFA-CoA-dependent binding of FabR to the *fabB* promoter was blocked by increasing concentrations of SFA-CoA (Fig. 3*A*). Similarly, the presence of SFA-ACP antagonized the UFA-ACP-dependent FabR binding (Fig. 3*B*). These data were consistent with both UFA and SFA thioesters binding to FabR with relatively similar affinities. These data were consistent with a model where binding of UFA ligands induced a conformational change that increases FabR affinity for DNA, but the binding of SFA ligands failed to trigger the conformational change to a high affinity DNA binding protein.

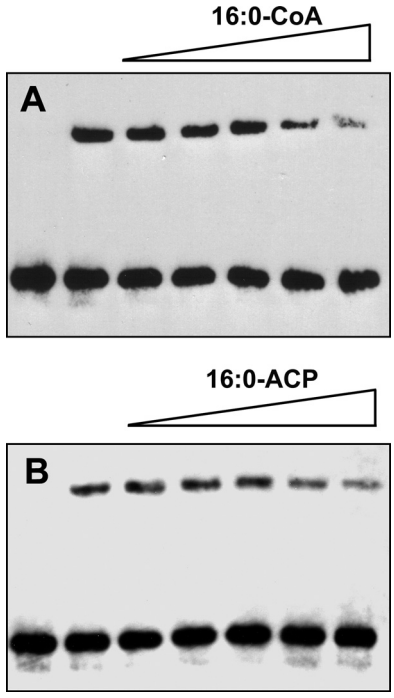

FIGURE 3. **Antagonism of UFA-dependent DNA binding by the presence of SFA ligands.** *A*, competition of 16:0-CoA with 18:1Δ9-CoA. *Lane 1*, FabR only. *Lanes 2–7*, FabR DNA binding was activated by the presence of 1 μM 18:1Δ9-CoA and the completion experiment was performed with 16:0-CoA concentrations from 0 to 3.2 μM. *B*, competition between 18:1Δ11-ACP and 16:0-ACP. *Lane 1*, FabR alone. *Lanes 2–7*, FabR DNA binding was activated by 0.1 μM 18:1Δ11-ACP and antagonized with 16:0-ACP using concentrations between 0 and 0.32 μM.

Regulation of fabA and fabB Expression by Exogenous UFA—The *in vitro* results suggested that FabR mediated the regulation of *fabB* gene expression by exogenous UFA that enter the cell and are converted to UFA-CoA. This was tested using real-time PCR to quantitate the levels of *fabA* and *fabB* mRNA in the presence and absence of exogenous 16:1Δ9 in wild-type and *fadR*- and *fabR*-null strains (Table 2). The transcription of both *fabA* and *fabB* were strongly repressed by exogenous UFA in the *fadE*-null strain. FadE deletion blocks the metabolism of acyl-CoAs via fatty acid β-oxidation (18). The elimination of the transcriptional activation by FadR led to a decrease in *fabA* expression in the absence of UFA, but there was little change in the presence of 16:1, consistent with FadR being the dominant regulator of *fabA* (4–6). The repression of *fabB* transcription by exogenous 16:1 in the *fadR*-null strain SJ201 was consistent with increased DNA binding of FabR in the presence of increased levels of UFA-CoA. The levels of *fabA* and *fabB* mRNA were higher in the *fabR*-null strain, and both levels were modestly reduced by exogenous UFA consistent with the removal of the activating effect of FadR on both promoters. There was no UFA-dependent regulation of either *fabA* or *fabB* in the *fadR fabR* double knock-out strain.

The expression of *fabB* was also increased in a FabR-dependent manner by the antibiotic cerulenin. Cerulenin irreversibly blocks the activity of the elongation condensing enzymes leading to the cessation of fatty acid biosynthesis (19). Treatment of

TABLE 2

Regulation of *fabA* and *fabB* gene expression by FadR, FabR, exogenous UFA, and cerulenin

Strains were grown in glycerol minimal medium with or without 0.025% 16:1, or 100 μg/ml cerulenin, and the amount of *fabA* and *fabB* mRNA were quantified by quantitative reverse transcription-PCR as described under "Experimental Procedures." Strain SJ95 (*gyrA37*, *metB1*, *relA1*, *spoT1*, *fadE*, *zaf-2*::Tn*10*, *LAM*$^-$, *LAM*^{R}, F$^-$) was a *fadE* (acyl-CoA dehydrogenase-defective) derivative of strain UB1005 (37) constructed to eliminate acyl-CoA utilization by β-oxidation. Strain SJ201 was constructed by transducing the *fadE* mutation into a *fadR* derivative of strain UB1005. Strains ANS9 and ANS11 were constructed by transduction of *fabR* (6) into strains SJ95 and SJ201, respectively.

Strain	Relevant genotype	mRNA expression ratios					
		Strain/strain SJ95		16:1/control		Cerulenin/control	
		fabA	*fabB*	*fabA*	*fabB*	*fabA*	*fabB*
SJ95	*fadE*	1.0	1.0	0.29	0.15	2.1	4.3
SJ201	*fadE fadR*	0.4	1.1	1.0	0.29	1.6	4.1
ANS11	*fadE fabR*	4.7	5.1	0.43	0.45	0.9	1.0
ANS9	*fadE fadR fabR*	0.4	4.6	1.1	0.92	0.9	1.0

cells with cerulenin led to an increase in *fabB* mRNA levels that was dependent on the presence of FabR (Table 2). This result is understood based on the fact that the cerulenin inhibition of *de novo* fatty acid biosynthesis prevented the formation of long-chain acyl-ACP end products (20). Because FabR lacks high affinity DNA binding in the absence of a ligand (Fig. 1), the depletion of acyl-ACP was consistent with the activation of *fabB* transcription.

Perturbation of Fatty Acid Synthesis by CHIR-090—The data suggested that FabR responds to the UFA:SFA ratio rather than the absolute concentration of a UFA ligand. To validate this idea *in vivo*, we used CHIR-090 to perturb the UFA:SFA ratio of fatty acids produced by the pathway. CHIR-090 is a potent and selective inhibitor of LpxC, a deacetylase that catalyzes the committed step in lipid A biosynthesis (21–23). LpxC inhibition efficiently blocks the incorporation of β-hydroxy14:0-ACP from the fatty acid synthesis cycle into lipid A. Normally, lipid A synthesis extracts ~33% of the total saturated acyl chains produced by the fatty acid biosynthesis cycle as β-hydroxy14:0-ACP. Thus, inhibition at this step blocks the diversion of SFA to lipid A allowing it to be elongated to long-chain SFA-ACP, which is available to the glycerol phosphate acyltransferase. Strains UB1005 and KZ6 (Δ*fabR*) were treated with 1 μg/ml CHIR-090 and the SFA:UFA ratio of the fatty acids synthesized during a 10-min [^{14}C]acetate pulse was determined (Fig. 4). In the wild-type strain UB1005, the UFA:SFA ratio was ~1.1 in the absence of drugs. The UFA:SFA ratio declined and then recovered over the 50-min time course of the experiment (Fig. 4*A*). These data showed that the inhibition of LpxC led to an increase in the SFA-ACP produced by fatty acid synthesis for incorporation into phospholipid and that this overproduction of SFA diminished with time. The treatment of strain (Δ*fabR*) with CHIR-090 also led to an abrupt decrease in the UFA:SFA ratio, but in the absence of FabR, the system did not recover, and the UFA:SFA ratio remained depressed throughout the course of the experiment (Fig. 4*B*).

These data were consistent with a role for FabR in sensing the long-chain acyl-ACP composition available to the acyltransferases and adjusting the expression of *fabA* and *fabB* to bring the UFA:SFA ratio back into balance. This conclusion was verified by examining the effects of CHIR-090 on global gene expression by Affymetrix array analysis using strain UB1005. There were only a few genes that were significantly regulated in response to CHIR-090 inhibition of LpxC (Table 3). The *fabA* and *fabB* genes were significantly up-regulated following CHIR-090 treatment, explaining the recovery of the UFA:SFA ratio and suggested that the derepression of *fabA* and *fabB* gene expression by FabR. The regulation of *fabA* and *fabB* by FabR was corroborated using quantitative reverse transcription-PCR (Fig. 4*C*). Both genes were up-regulated in response to CHIR-090 treatment in the wild-type strain UB1005, and there was no change in the expression of these genes in strain KZ6 (Δ*fabR*). These data showed FabR-dependent regulation of gene expression in response to a change in the UFA:SFA ratio of acyl chains produced by the fatty acid biosynthetic pathway for membrane phospholipid biosynthesis.

DISCUSSION

Our work uncovers the mechanism that controls the DNA binding of the FabR transcriptional repressor and leads to the regulatory model for UFA/SFA homeostasis illustrated in Fig. 5. The *fabB* and *fabA* genes are negatively regulated by FabR, a transcriptional repressor that senses fatty acid structure. All long-chain acyl-ACP or acyl-CoA ligands bind to FabR. The binding of UFA thioesters increases the affinity of FabR for the palindrome present in the *fabB* and *fabA* promoters, thereby repressing transcription, whereas the binding of SFA thioesters to FabR does not trigger DNA binding. The finding that *fabR* deletion strains have increased expression of *fabA* and *fabB* (6) and higher levels of UFA (Table 1) illustrates the active repression of these genes by a pool of UFA-ACP during normal growth. The acyl-ACP pool available for the acyltransferase system is estimated at 6–12% or the total ACP in growing cells (24), and the total cellular content of ACP is ~4 pmol/10^8 cells or 40 μM (25, 26). These measurements translate into an intracellular acyl-ACP concentration of ~2.5–5 μM. This concentration coupled with the affinities of acyl-ACP for FabR reported in this study, suggests not only that there is sufficient acyl-ACP present *in vivo* to regulate FabR, but that FabR may be saturated with acyl-ACP *in vivo*. The fact that FabR senses the UFA:SFA ratio, rather than the absolute amount of UFA- or SFA-ACP, is an important aspect of the model because it ensures that FabR adjusts gene expression to balance the composition of UFA- and SFA-ACP, regardless of the absolute level of acyl-ACP, which does vary with growth rate and carbon source (20). CHIR-090 treatment leads to a reduction in the UFA:SFA ratio in acyl-ACP produced for membrane phospholipid formation due to the block in the diversion of SFA to lipid A synthesis. *E. coli* rapidly responds to this increase in SFA by increasing the expression of *fabA* and *fabB* in a FabR-dependent manner, which leads to a normalization of the UFA:SFA ratio.

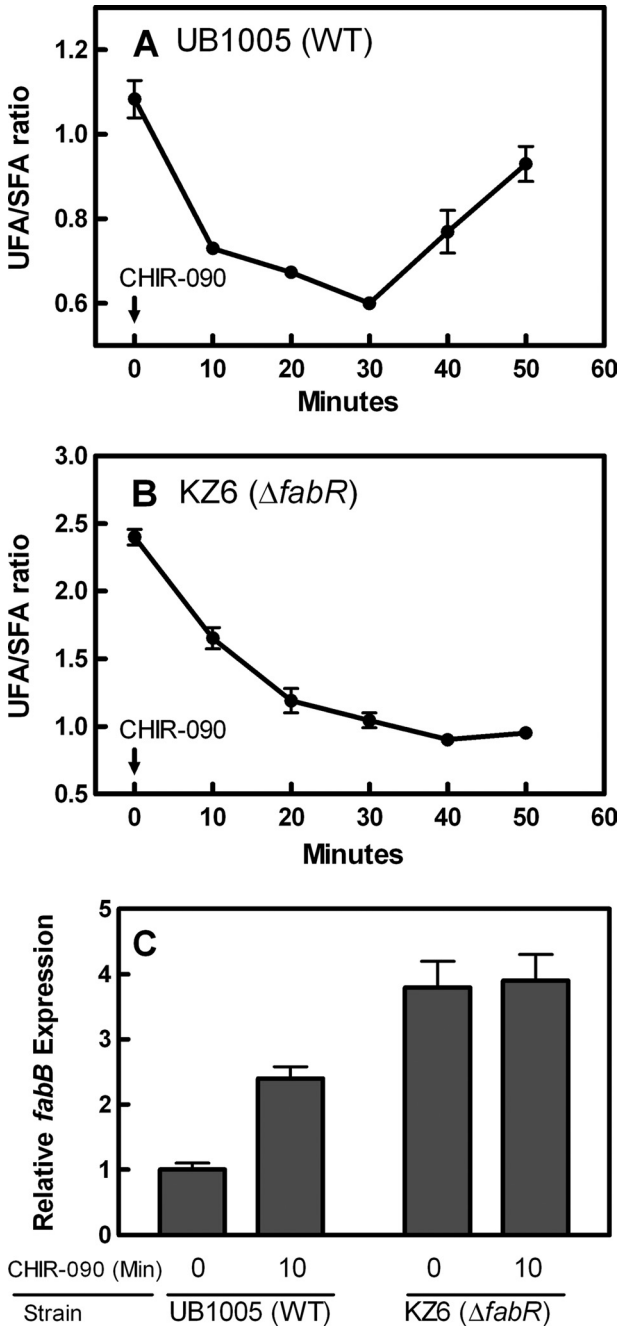

FIGURE 4. **Response of wild-type and *fabR*-null strains to the inhibition of lipid A synthesis with CHIR-090.** Strains were pulsed with [^{14}C]acetate for 10 min prior to CHIR-090 treatment to establish the normal UFA:SFA ratio in the fatty acid produced by this strain. Next, CHIR-090 was added, and samples were removed at 0, 10, 20, 30, and 40 min and pulsed with [^{14}C]acetate for 10 min, and the UFA:SFA ratio was determined following the preparation of methyl esters, separation by argentation thin layer chromatography, and quantification of the ratio of labeled UFA and SFA as described under "Experimental Procedures." *A*, strain UB1005. *B*, strain KZ6 (Δ*fabR*). *C*, increased *fabB* gene expression following a 10-min treatment with CHIR-090. The levels of *fabB* expression was determined by quantitative reverse transcription-PCR before and after CHIR-090 treatment as described under "Experimental Procedures." Transcript levels were normalized to *acpP*, and the level of *fabB* mRNA in the wild-type, untreated strain was set as 1.0 to calculate the comparisons. The mean of triplicate experiments ± S.E. is plotted.

TABLE 3

Gene expression changes following treatment with CHIR-090

Triplicate Affymetrix *E. coli* 2.0 arrays were used to determine the effect of a 10-min treatment with 1 μg/ml CHIR-090 compared to a dimethyl sulfoxide (DMSO) control on gene expression as described under "Experimental Procedures." These data were selected from the complete data set deposited in the NCBI Gene Expression Omnibus (9) and are accessible through GEO Series accession number GSE18623.

Gene[a]	Function	CHIR/DMSO[b]	*p* value
fabB	β-Ketoacyl-ACP synthase I	3.40	0.019
cspB	Cold shock protein	2.90	0.015
yedA	Hypothetical transporter	2.71	0.021
fabA	β-Hydroxydecanoyl-ACP dehydratase	2.54	0.023
lpxP	Palmitoleoyl-ACP acyltransferase	2.25	0.019
yacA	Regulator of *secA* translation	2.14	0.033
wbbH	*O*-Antigen polymerase	2.10	0.022
pssA	Phosphatidylserine synthase	2.10	0.016
yqfA	Hypothetical oxidoreductase	2.08	0.006
mltE	Murein transglycosylase	0.41	0.008
ycbM	Aliphatic sulfonate permease	0.35	0.011

[a] All genes that were up or down-regulated 2-fold or greater with a *p* value <0.05 are reported in the table.

[b] Ratio of expression levels in cells treated with CHIR-090 to the dimethyl sulfoxide control.

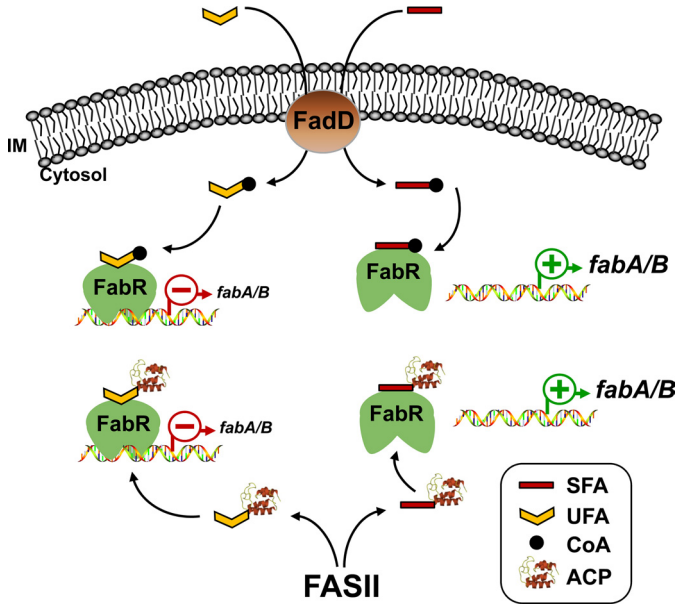

FIGURE 5. **Model for FabR regulation of the UFA:SFA ratio.** The level of *fabB*, and to a lesser extent *fabA*, expression controls the proportion of UFA produced by the FASII pathway. The FabR repressor is envisioned as having two conformations: one conformation binds DNA poorly, whereas the other conformation has a high affinity for its DNA palindrome located in the both *fabB* and *fabA* promoters. FabR binds to the entire pool of long-chain acyl-ACP end-products of FASII and/or to any acyl-CoAs that arise from the uptake of exogenous fatty acids. SFA thioesters form complexes that stabilize the FabR conformation with a low affinity for DNA allowiwng increased transcription of *fabB* and *fabA*. UFA thioester complexes stabilize a FabR conformation with a high affinity for DNA repressing *fabB* and *fabA* expression. FabR senses the acyl-ACP and acyl-CoA thioester pool composition available to the glycerophospholipid acyltransferases and modulates *fabB* and *fabA* gene expression to balance the UFA:SFA ratio of acyl chains produced by FASII. *IM*, inner membrane.

The ability of FabR to sense acyl-CoA thioesters as well as acyl-ACP provides a mechanism to adjust the proportion of UFA formed by *de novo* fatty acid synthesis to match the composition of fatty acids arriving from the environment. *E. coli* is capable of converting exogenous fatty acids to acyl-CoAs that can be used for phospholipid synthesis, thus sparing energetically expensive *de novo* fatty acid synthesis (27) These long-chain acyl-CoAs may also be used by the fatty acid β-oxidation system, but the ability of the PlsB and PlsC acyltransferases to efficiently use either acyl-CoAs or acyl-ACPs means that the intracellular acyl-CoA pool formed in the

presence of exogenous fatty acids competes with the endogenous acyl-ACP pool for the acyltransferases. The ability of FabR to respond to the structure of the acyl-CoAs in the same manner as the acyl-ACPs means that gene expression will be adjusted to match the overall composition of the long-chain thioester pool. Although the FabR system is an elegant mechanism to control fatty acid composition, the regulatory system can be overwhelmed. In cells containing an inactivated acyl-CoA dehydrogenase (FadE), the first step in fatty acid degradation, exogenous palmitate is not tolerated (28). Under these conditions, the cells are flooded with so much SFA-CoA that they are unable to incorporate enough UFA into phospholipids to support growth.

FabR is a member of the growing list of bacterial transcriptional regulators that respond to fatty acid structure. The control of FabR by the UFA:SFA thioester ratio is most similar to DesT, a transcriptional repressor in *Pseudomonas aeruginosa* that controls the expression of an operon responsible for the oxidative desaturation of SFA-CoA (29). FabR and DesT are highly related proteins that bind to similar DNA palindromes. DesT binds both UFA- and SFA-CoAs with equal affinity, but the binding of UFA-CoA is postulated to induce a conformational change that promotes DNA binding, whereas the binding of SFA-CoA prevents this conformational change from occurring (30). Thus, DesT plays a key role in sensing the composition of acyl-CoA pool derived from exogenous fatty acids and adjusting desaturase gene expression to ensure that the cell is not overloaded with SFA. FabT of *Streptococcus pneumoniae* is a transcriptional repressor that controls the transcription of the entire *fab* gene set in this organism (31). FabT DNA binding also requires an acyl-ACP ligand, but in this case, both long-chain UFA- and SFA-ACPs promote FabT DNA binding, whereas medium-chain acyl-ACPs do not (32). The lack of selectivity of FabT toward acyl-ACP unsaturation in this case is consistent with FabT functioning as a regulator of the entire set of *fab* genes with the exception of *fabM*, the gene encoding the isomerase component of the type II fatty acid synthase in this organism (31). FapR is a global transcriptional repressor of the *fab* gene set in Gram-positive bacteria like *Bacillus subtilis* and *Staphylococcus aureus* (33). However, this factor is regulated differently than FabR, DesT, and FabT. Malonyl-CoA is the ligand that releases FapR from its repressor sites in the promoters of the *fab* genes (34, 35). It is easy to understand how feedback regulation based on the composition of the acyl-ACP end-products of the biosynthetic pathway functions to appropriately control gene expression by FabR, DesT, and FabT, but the FapR case is fundamentally different. The increased expression of the pathway enzymes based on the concentration of the first intermediate (malonyl-CoA) in fatty acid biosynthesis is the opposite of feedback regulation that occurs in the other examples. This regulatory system places pathway control at the acetyl-CoA carboxylase step by a yet unknown mechanism.

Our findings explain the regulatory role of transcription factors in controlling bacterial responses to pathway inhibitors. The induction of condensing enzyme gene expression is a characteristic marker for drugs that block type II fatty acid synthesis. FASII inhibitors block acyl-ACP formation leading to the induction of *fabB* transcription in *E. coli* (Table 2) and *fabF* transcription in Gram-positive bacteria (33, 36). Our data explain this result by the absence of acyl-ACP, thus decreasing the repressor activity of FabR. However, FASII inhibitors also trigger the accumulation of malonyl-CoA, which is the explanation for the increased expression of *fabF* in *B. subtilis* (34, 35). Because the disappearance of long-chain acyl-ACP end-products and the accumulation of malonyl-CoA are coupled in cells treated with FASII inhibitors, experiments with these drugs must be interpreted with caution.

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