The FabR (YijC) Transcription Factor Regulates Unsaturated Fatty Acid Biosynthesis in *Escherichia coli*

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SUMMARY

Unsaturated fatty acid biosynthesis is a vital facet of *Escherichia coli* physiology and requires the expression of two genes, *fabA* and *fabB*, in the type II fatty acid synthase system. This study links the FabR (YijC) transcription factor to the regulation of unsaturated fatty acid content through the regulation of *fabB* gene expression. The *yijC* (*fabR*) gene was deleted by replacement with a selectable cassette and the resulting strains (*fabR::kan*) possessed significantly elevated levels of unsaturated fatty acids, particularly cis-vaccenate, in their membrane phospholipids. The altered fatty acid composition was observed in *fabR::kan fabF1* double mutant pinpointing *fabB* as the condensing enzyme responsible for the increased cis-vaccenate production. The *fabR::kan* strains had 4-8 fold higher levels of *fabB* and a 2-3 fold increase in *fabA* transcripts as judged by northern blotting, Affymetrix array analysis and real-time PCR. FabR did not regulate the enzymes of fatty acid β-oxidation. The elevated level of *fabB* mRNA was reflected by higher condensing enzyme activity in *fabR::kan fabF1* double mutants. Thus, FabR functions as a repressor that potently controls the expression of the *fabB* gene, which in turn, modulates the physical properties of the membrane by altering the level of unsaturated fatty acid production.
INTRODUCTION

Unsaturated fatty acid biosynthesis is required to maintain membrane structure and function in *Escherichia coli* and many other organisms. *E. coli* possesses a type II fatty acid synthase system and the double bond is introduced into the growing acyl chain at the ten-carbon β-hydroxydecanoyl-ACP intermediate (for reviews, see (1,2)). Early genetic studies identified two genes, *fabA* and *fabB*, that were essential for olefin formation. Inactivating mutations in either one of these genes lead to an absolute requirement for unsaturated fatty acids for growth (1). FabA introduces the double bond into the acyl chain through its dual activities as a β-hydroxydecanoyl-ACP dehydratase and a *trans*-2, *cis*-3-decanoyl-ACP isomerase (3). Since there is a second β-hydroxyacyl-ACP dehydratase (FabZ) that only produces the *trans*-2 isomer (4,5), unsaturated fatty acid formation also depends on the ability of the type II system to efficiently divert the *cis*-3 intermediate to the unsaturated branch of the elongation pathway. The FabB condensing enzyme fulfills this function presumably by efficiently catalyzing the elongation of *cis*-3-decenoyl-ACP (1,2). The *fabA* and *fabB* genes are always found together in bacterial genomes (6), supporting the idea that these two gene products work in tandem to generate unsaturated fatty acids.

FadR is a transcriptional regulator that modulates the expression of the *fabB* and *fabA* genes. FadR was discovered through the analysis of a mutation that results in the constitutive induction of the β-oxidation enzymes (7), and led to its characterization as a repressor of fatty acid β-oxidation genes (8). FadR is released from its DNA binding sites by long-chain acyl-CoAs (9-12), which bind to the carboxy terminus of the protein and release the amino terminal winged helix domain from the DNA (13-15). An interesting twist in the FadR story began with the observation that *fabA*(Ts) *fadR* double mutants were unable to grow at the permissive temperature without an unsaturated fatty acid supplement (16). This suggested a positive effect of FadR on *fabA* expression and it was soon demonstrated that FadR is a transcriptional activator that binds to the −40 region of the *fabA* gene, a site common for activators of σ70-responsive promoters (17,18). FadR is also a positive regulator of the *fabB* gene, although the changes in *fabB* expression in
fadR mutants are not as great as with fabA (19). Thus, FadR acts as a repressor of β-oxidation genes and an activator of the two genes required for unsaturated fatty acid synthesis (20).

The promoter regions of the fabA and fabB genes are highly related suggesting that their expression is similarly regulated. The FadR binding site is not the only region of sequence similarity in the fabB and fabA promoters and their alignment is shown in Fig. 1 with the sites of transcription initiation indicated by the arrows for both fabA (18) and fabB (21). Recently, McCue et al. (22) used a bioinformatic "phylogenetic footprinting" method to identify putative transcription factor binding sites in bacterial genomes and located a strongly predicted site in the promoter regions of the fabA and fabB genes. They went on to show that a protein, YijC, specifically bound to a DNA affinity column carrying the predicted transcription factor palindrome (Fig. 1). The oligonucleotide 5′-GGCGTACAAGTGTACGCT was used to isolate YijC protein from E. coli cell-free extracts (22). The YijC binding sequence on fabB consists of a central CGTACAXXTGTACG palindrome, whereas the predicted site in fabA has a two base pair mismatch (Fig. 1). Based on the location of these binding sites, McCue et al. (22) proposed renaming the yijC gene fabR for Fatty Acid Biosynthesis Regulator. However, there are no direct data demonstrating that FabR actually influences the levels of fabA or fabB mRNA or alters the production of unsaturated fatty acids by the pathway. The goal of this study was to determine whether FabR (YijC) regulates unsaturated fatty acid formation by examining the effects of deleting this gene on fatty acid metabolism.

**EXPERIMENTAL PROCEDURES**

Materials and bacterial strains—[α-32P]dCTP (3,000 Ci/mmol) and [2-14C]malonyl-CoA (55 mCi/mmole) were purchased from Amersham Pharmacia Biotech. Restriction enzymes and other molecular biology reagents were from Promega Life Science, Life Technologies and New England Biolabs. ACP was purchased from Sigma and myristoyl-ACP was prepared using the acyl-ACP synthetase method (23). The E. coli strains that we use in this study and their relevant genotypes
are listed in Table I. Plasmid pDM4 (24) expresses the \textit{fabB} gene was constructed by cloning \textit{fabB} with its promoter into pBR322 and plasmid pSJ21 expresses the \textit{fabA} gene and was constructed by cloning the \textit{fabA} into pBluescript. Transductions with P1 phage were performed as described in Miller (25). DNA sequencing, Affymetrix microarray analysis and oligonucleotide synthesis were performed by the Hartwell Center at St. Jude.

\textit{Generation of a \textit{fabR} deletion strain}—The gene targeting strategy shown in Figure 2A was used to generate a \textit{fabR} deletion strain of \textit{E. coli}. The 813-bp fragment upstream of \textit{fabR} was amplified by PCR with primers P\textsubscript{S} (5\textsuperscript{′}-CGCGAGCTCACATCTGTGGGATGATATGG, a \textit{Sacl} site is underlined) and P\textsubscript{Br} (5\textsuperscript{′}-CGCGGATCCATCACGATGTCTGAATCC, a \textit{BamHI} site is underlined). The \textit{Sacl-BamHI} digested 813-bp fragment was cloned into \textit{Sacl-BamHI} site of pUC19. The 788-bp fragment downstream of \textit{fabR} was amplified similarly by PCR with primers P\textsubscript{Br} (5\textsuperscript{′}-CGCGGATCCATGTGAAGGAGCACTGTAATG, a \textit{BamHI} site is underlined) and P\textsubscript{H} (5\textsuperscript{′}-CCCAAGCTTATAACAGATCGACTACGCTAC, a \textit{HindIII} site is underlined). Then the \textit{BamHI-HindIII} digested 788-bp fragment was inserted into the \textit{BamHI-HindIII} site of the plasmid harboring the 813-bp upstream fragment. The \textit{BamHI} digested kanamycin resistant gene fragment was cloned into the \textit{BamHI} site of the plasmid harboring both the upstream (813-bp) and downstream (788-bp) PCR fragments, yielding plasmid pUCFabRK. This plasmid was digested with \textit{Sacl} and \textit{AflIII}. The 3.3-kb fragment, containing both PCR fragments, kanamycin gene replacing \textit{fabR} and a 350-bp fragment of pUC19, was purified from 1\% agarose gel. The 3.3-kb linear DNA (25 ng) was transformed into strain PDJ1 (\textit{recD}) by electroporation and kanamycin-resistant transformants were isolated. The \textit{fabR::kan} strain grew with the same doubling time as the wild-type on both rich and minimal media.

\textit{Genomic analyses of \textit{fabR} deletion strain}—PCR and Southern blot analyses were used to confirm the genotype of the \textit{fabR} deletion strain. PCR experiments were performed with primer pair, P1 (5\textsuperscript{′}-GATCAGCTCCAGCACCATAG) and P2 (5\textsuperscript{′}-TTACTGGAGCTGTACTGCG) to amplify a fragment encompassing 1 kb both upstream and downstream of \textit{fabR}. The PCR products from...
both wild type and \(\textit{fabR::kan}\) strains were digested with enzymes \(Nco\), \(Bam\)HI and \(Hind\)III individually. Two other primer pairs, \(P_1\) and \(P_{K1}\) (5'-ATCTTGTGCAATGTAACATCAGAG), \(P_{K2}\) (5'-AGTCAGCAACACCTTCTTCACG) and \(P_2\), were used to amplify two 1 kb fragments in \(\textit{fabR::kan}\) strain. The PCR products were purified from 1% agarose gel and DNA sequencing was performed in the Hartwell Center with ABI Prism 3700 DNA analyzer. Southern blots were performed on genomic DNA isolated from both wild type and \(\textit{fabR::kan}\) strains by phenol/chloroform/isoamyl alcohol extraction method (26). Three different restriction enzymes, \(NdeI\), \(EcoRI\) and \(Scal\), were used to digest 5 \(\mu\)g of genomic DNA. The digested genomic DNA was separated by electrophoresis on a 0.8% agarose gel. The probe was the 230-bp \(Clal-Scal\) fragment downstream of \(\textit{fabR}\) (Fig. 2A), and was labeled with \([\alpha-32P]\)dCTP. Southern transfer, hybridization and washing were carried out by standard procedures (27). Bands were detected with a Molecular Dynamics Storm 860 imager and their intensity was determined using the ImageQuant 5.1 program.

\textbf{RNA analyses of \(\textit{fabR::kan}\) deletion strain—}Total RNA was isolated from exponentially growing cells, both wild type and \(\textit{fabR::kan}\), using MasterPure RNA purification kit (Epicentre Technologies). The same RNA sample was used for northern blot, Affymetrix array and TaqMan RT-PCR analyses. Briefly, cell pellets from 40-ml cultures were resuspended in lysis buffer (containing 0.17 mg/ml proteinase K) and incubated at 65\(^\circ\)C for 15 min. After removing the debris and proteins by centrifugation, the RNA was precipitated with isopropanol. The remaining DNA was removed by treating RNA preparations with DNase I (0.025 U/\(\mu\)l) at 37\(^\circ\)C for 20 min. RNA samples were isopropanol-precipitated, washed twice with 75% ethanol, and redissolved in TE.

Northern blots were performed with 10 \(\mu\)g of total RNA separated by electrophoresis on a 1% agarose formaldehyde gel. The probes were the 320-bp \(Hincl-MunI\) fragment of plasmid pSJ21 (for \(\textit{fabA}\)) and the 280-bp \(EcoRI-Clal\) fragment of pDM4 (for \(\textit{fabB}\)). Northern transfer, hybridization and washing were performed using standard procedures (28). Detection and quantitation of the blot was performed using a Molecular Dynamics Storm 860 imager. Staining
the membrane with methylene blue assessed equal loading of the samples and the intensity of the bands was consistent in all lanes.

Affymetrix array analyses were performed using messenger RNA species enriched by the specific degradation of the 16S and 23S ribosomal RNAs, which constitute 90% of the total prokaryotic RNA population. Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Epicentre Technologies) and primers specific to 16S and 23S rRNA were used to synthesize the complementary cDNAs. Then rRNAs were removed enzymatically by treatment with RNase H. The cDNA molecules were degraded by DNase I digestion and the enriched mRNAs were purified using Qiagen RNeasy columns. The RNA was fragmented by heat and ion-mediated hydrolysis and the 5′-end RNA termini were enzymatically modified by T4 polynucleotide kinase and ATPγS. A biotin group was then conjugated to the thiolated RNA. After purification of the product (using the RNA/DNA mini kit, Qiagen), the efficiency of the labeling was assessed using a gel-shift assay based on the retardation of the biotinylated RNA upon addition of NeutrAvidin molecules. 1.5 to 4.0 µg of fragmented RNA (biotin-labeled target) was hybridized for 16 hr at 45°C to E. coli oligonucleotide arrays (Affymetrix) containing all known E. coli genes together with more than 2,700 probe sets designed to intergenic sequences. Arrays were washed at 25°C with 6 × SSPE (0.9 M NaCl, 60 mM NaH2PO4, 6 mM EDTA and 0.01% Tween 20) followed by a stringent wash at 50°C with 100 mM MES, 0.1 M NaCl, 0.01% Tween 20. The arrays were then stained with phycoerythrin conjugated streptavidin (Molecular Probes) and the fluorescence intensities were determined using a laser confocal scanner (Hewlett-Packard). The scanned images were analyzed using Microarray software (Affymetrix). Sample loading and variations in staining were standardized by scaling the average of the fluorescent intensities of all genes on an array to constant target intensity for all arrays used. The expression data were analyzed as previously described (29). The signal intensity for each gene was calculated as the average intensity difference, represented by \[ \frac{\Sigma (PM - MM)}{\text{number of probe pairs}} \], where PM and MM denote perfect-match and mismatch probes.
Oligonucleotide primers and probes for real-time RT-PCR were designed with Primer Express 1.0 software (ABI Prism; Perkin-Elmer/Applied Biosystems) and the probes were purchased from Applied Biosystems. The probes consisted of an oligonucleotide labeled at the 5′ ends with the reporter dyes, 3FAM or VIC, and at the 3′ ends with the quencher dye TAMRA (PE Applied Biosystems) and are listed in Table II.

The reverse transcription was performed on total RNA prepared as above after a second step of DNaseI treatment (DNA-free, AMBION). The RT mixture (20 µl) contained 500 ng of total RNA, 10 ng/µl random hexamers (Life Technologies), 33 units of RNAguard Ribonuclease inhibitor (Amersham) and 20 U/µl of Superscript II reverse transcriptase (Life Technologies). Aliquots (1 µl) of the reverse transcription reaction were added to the real-time PCR reaction (30 µl) containing 600 nM of each forward and reverse primer and 166 nM of probe. Amplification and detection of specific products was performed with the ABI Prism 7700 Sequence Detection System (PE Applied Biosystems) with the following profile: 1 cycle at 50°C, 1 cycle at 95°C for 10 min, 40 cycles at 95°C for 15s, and 60°C for 1 min. The critical threshold cycle (C_T) is defined as the cycle at which the fluorescence becomes detectable above background and is inversely proportional to the logarithm of the initial template molecules. The C_T values were used to calculate the relative number of fabA and fabB cDNA molecules in wild-type and fabR::kan mutant. The quantity of cDNA for each experimental gene was normalized to the quantity of acpP cDNA in each sample.

Fatty acid composition analysis—Cultures (10 ml) of E. coli strains were grown to mid-log phase in M9 minimal medium (25) supplemented with 0.4% glucose, 0.01% methionine, 0.0005% thiamin, and harvested by centrifugation. The cell pellet was suspended in 1 ml of water and the lipids were extracted as described by Bligh and Dyer (30) and fatty acid methyl esters were prepared by the addition of 2 ml of HCl/methanol to the dry extract. The fatty acid methyl esters were fractionated using a Hewlett-Packard Model 5890 gas chromatograph equipped with a flame ionization detector and a glass column (2 m by 4 mm, internal diameter) containing 3% SP2100
coated on Supelcoport (100/120 mesh) operated at 190°C. Fatty acid methyl esters were identified by comparing their retention times with standards (Matreya).

Condensing enzyme assay—The condensing enzyme activity assay was essentially the same as described previously (31). Cultures (20 ml) of E. coli strains (fabF1 and fabR::kan fabF1) were grown to mid-log phase in M9 minimal medium (25) supplemented with 0.4% glucose, 0.01% methionine, 0.0005% thiamin, and harvested by centrifugation. The cell pellet was suspended in 5 ml of 20 mM Tris-HCl, pH 7.6, containing 1 mM EDTA and 1 mM DTT. Cells were lysed by two passages through a French press cell at 20,000 psi. The total cell lysate was centrifuged at 50,000 rpm at 4°C for 1 hour. The cell-free extract, supernatant from ultracentrifugation, was saved for the assay. Protein content in the cell-free extract was determined with the Bradford assay using γ-globulin as the standard (32). The condensation assay contained 45 µM myristoyl-ACP, 50 µM [2-14C]malonyl-CoA, 100 µM ACP, 25 ng FabD (malonyl-CoA:ACP transacylase) and the indicated concentrations of cell-free extract (0.1-1 µg) in a final volume of 40 µl. The mixtures were incubated at 37°C for 15 min and then reduced with borohydride, extracted into toluene and quantititated by scintillation counting. One unit of condensing enzyme activity corresponds to the formation of 1 µmole of β-[14C]ketohexadecanoyl-ACP per min.

RESULTS

Characterization of the fabR deletion strain—A targeting construct was prepared that replaced the fabR (yijC) gene with a kanamycin-resistance cassette as described under “Experimental Procedures” and the linearized fragment was transformed into strain PDJ1 (recD). Kanamycin-resistant transformants were selected and a combination of PCR and Southern blot analysis were used to verify that the kanamycin gene was inserted in place of fabR in the correct position in the genome. Primers P1 and P2 were designed outside the sequence that was used in the targeting construct for linear transformation (Fig. 2A). Substitution of the kanamycin gene for fabR increased the size of the PCR product from these two primers by 600-bp. Thus, the PCR
product from the fabR::kan strain with P1 and P2 was 3 kb in length (Fig. 2B, lane 2) compared to the 2.4 kb product from wild type cells (Fig. 2B, lane 1). When these two PCR products were digested with three restriction enzymes (Fig. 2B; lanes 3 and 6, NcoI; lanes 4 and 7, BamHI; and lanes 5 and 8, HindIII), the products were of the predicted sizes (Fig. 2B). Sequence results of the PCR products read through the junction regions where the kanamycin gene was inserted (data not shown). Pk1 and Pk2 were designed from sequence of kanamycin gene. PCR reactions with the two primer pairs, P1 and Pk1, Pk2 and P2, produced products of the correct size (1 kb) in the fabR::kan strain indicating the presence of the kanamycin gene in the correct locations, whereas no products were detected with these two primer sets in wild type cells (data not shown).

Results from Southern blot analysis with the 230-bp Clal-ScaI fragment corroborated the PCR results confirming that the kanamycin gene replaced fabR gene in strain MWF1 in the correct location without compromising the neighboring genes. The probe hybridized with a 650-bp fragment of wild type genomic DNA digested with Ndel (Fig. 2C, lane 5). The Ndel restriction site in the fabR gene disappeared in the fabR deletion strain, resulting in the probe hybridizing with a 3.2-kb fragment (Fig. 2C, lane 6). The probe also recognized bands of the correct sizes in the EcoRI and ScaI digests (Fig. 2C). In the case of the fabR::kan strain, the fragments were larger by 600 bp due to the presence of the kanamycin gene in place of fabR.

**Deletion of fabR leads to the overproduction of unsaturated fatty acids**—The effect of FabR deletion on the production of the major membrane fatty acids of _E. coli_ was determined. Strain PDJ1 synthesized slightly more saturated fatty acids (SFA) than unsaturated fatty acids (UFA). The UFA:SFA ratio was 0.87 and the amount of cis-vaccenate (C18:1Δ11) and palmitoleate (C16:1Δ9) was approximately the same (Table III). Strain MWF1 (fabR::kan) produced significantly more UFA increasing the UFA/SFA ratio to 1.77. Unlike the wild type cells, C18:1Δ11 was the predominant UFA species in the fabR::kan strain. A similar alteration of fatty acid composition was observed in cells that expressed fabB from a multicopy plasmid (pDM4) (Table III). In both cases, the 18:1/16:1 ratio was significantly increased (Table III). The similarity between the fatty acid
composition alterations due to FabB overexpression (Table III and Ref. (24)), and the composition of the \textit{fabR::kan} strain pointed to an increased level of \textit{fabB} expression as the underlying cause for the increased UFA and altered 18:1/16:1 ratio in strain MWF1. In contrast, increased expression of the \textit{fabA} gene actually slightly elevated the amounts of SFA in the membranes (Table III) consistent with previously reported results (33). The activity of FabF regulates the cellular content of 18:1\(\Delta11\) (24,34,35), therefore, we transduced the \textit{fabR::kan} allele into strain SJ109 (\textit{fabF1}) and examined the fatty acid composition to test if the regulation of FabB alone was responsible for the elevation in UFA. As expected, the \textit{fabF1} mutant had reduced amounts of C18:1\(\Delta11\). The 18:1\(\Delta11\) levels in the \textit{fabR::kan fabF1} double mutant were elevated compared to the \textit{fabF} control and were similar to the amount of C18:1\(\Delta11\) in the wild type cells (Table III). These compositional data strongly support the conclusion that FabR acts as a repressor of \textit{fabB}, which in turn, modifies the membrane fatty acid composition.

The relationship between FabR and FadR regulation was investigated by determining the fatty acid composition in strain PDJ14 (\textit{fadR}) and ANS4 (\textit{fadR fabR}) (Table III). As reported earlier (16), the \textit{fadR} strain PDJ14 had a higher level of saturated fatty acids compared to the control strain. The \textit{fadR fabR} double mutant strain ANS4 had essentially the same composition as the \textit{fadR} strain with a similar UFA/SFA and 18:1/16:1 ratios. These data illustrate that the regulation of fatty acid composition by \textit{fabR} requires a functional \textit{fadR} gene.

\textit{Steady-state levels of fabB and fabA mRNA in the fabR deletion strain}—Since \textit{fabA} and \textit{fabB} are the two essential genes required for UFA synthesis, northern blot experiments with \textsuperscript{32}P-labeled probes specific for \textit{fabB} or \textit{fabA} were used to evaluate the levels of their respective mRNAs (Fig. 3). The mRNA levels for both genes were elevated in the \textit{fabR::kan} strain (Fig. 3), although the magnitude of induction of \textit{fabB} was significantly greater than \textit{fabA}. The northern blots revealed that deletion of \textit{fabR} gene resulted in a 7-fold elevation of \textit{fabB} compared to a 3-fold elevation of \textit{fabA} mRNA (Fig. 3).
The fabR strain has elevated condensing enzyme activity—The RNA analysis data suggest that fabR::kan strains will have higher levels of FabB protein and condensing enzyme activity. We tested this idea by performing condensing enzyme assays on cell-free extracts from strain ANS7 (fabR::kan fabF1). These experiments showed that the condensing enzyme specific activity in the cell-free extracts of strain ANS7 (fabR::kan fabF1) was 2.5 times higher than that of the strain SJ109 (fabF1) extracts using myristoyl-ACP as the substrate (Fig. 4). Since strain ANS7 did not have a functional FabF, we concluded that the increased condensing enzyme activity was due to higher amounts of FabB, which is consistent with the increased fabB mRNA levels. We did not perform a similar experiment with FabA due to the lack of the appropriate specific substrates to measure activity in crude extracts; however, previous work demonstrated a direct relationship between fabA mRNA level and FabA activity (33).

Gene expression in fabR and fadR strains—A comparison of the global transcription effect of FabR was addressed using the Affymetrix array technology to examine the changes in all transcripts. Our analysis of 4 sets of global gene expression profiles comparing mRNA levels in strain ANS8 (fabR::kan) to the wild type revealed that fabB and fabA were elevated 4- and 2-fold, respectively (Fig. 5A). The analogous array experiment comparing wild-type and a fadR mutant showed that fabA transcription was significantly reduced in the absence of FadR (Fig. 5A). We did not observe any reduction in fabB transcript levels in fadR strains using this technology (Fig. 5A). In the fadR fabR double mutant, fabA mRNA was reduced to the same extent as in the fadR mutant (Fig. 5A). In this experiment, fabB transcripts were also lower in the double mutant compared to the fabR strain indicating that in the absence of FabR, FadR had a significantly greater activating effect on fabB expression.

The array experiments also revealed the upregulation of the genes involved in β-oxidation in fadR and fadR fabR double mutants (Table IV), as expected for fadR null strains (20). The exceptions were the yfcX and yfcY genes that were not known to be involved in fatty acid β-oxidation, but whose predicted functions strongly point to a role for these gene products in β-
oxidation (Table IV and Discussion). We did not detect other fatty acid biosynthesis, fatty acid β-oxidation or additional known or unknown genes that were significantly regulated by FabR in the array experiments.

The TaqMan real-time PCR method is a sensitive and quantitative technology and was used to validate and extend the findings from the array analysis (Fig. 5B). The threshold cycle number ($C_T$) for each gene was determined and normalized to the $C_T$ determined for the acpP gene in the same RNA sample. In these experiments, both the fabB and fabA mRNA levels were elevated in strain ANS8 (fabR::kan) 5-fold and 3-fold, respectively, compared to the wild-type strain UB1005 confirming that FabR represses the mRNA levels of both genes. In the fadR mutant, the levels of fabA mRNA were significantly reduced, as anticipated. The level of fabB mRNA remained essentially the same in the fadR mutant strain. The levels of fabA mRNA in the fadR fabR double mutant were reduced to levels comparable to the fadR single mutant (Fig. 5B). Also, we observed a significant reduction in the fabB mRNA in the double mutant compared to the fabR::kan strain, supporting the idea that FadR has an activating effect on fabB transcription (19), especially in the absence of FabR.

Taken together these expression analyses point to FabR repression as a potent regulator of fabB expression and to a lesser extent a regulator of fabA expression. On the other hand, FadR activation is most important for the regulation of fabA expression, but also contributes to fabB regulation.

**DISCUSSION**

Our study demonstrates the function of the FabR transcription factor in controlling UFA production through the regulation of fabB in the *E. coli* type II fatty acid synthase system. FabR controls the UFA production and thus directly influences the physical properties of the membrane bilayer (35,36). Also, the increased FabB activity leads to the increased production of 18:1Δ11. The formation of 18:1Δ11 was originally hypothesized to be a unique property of the FabF (β-
ketoacyl-ACP synthase II) based on the reduced amount of this fatty acid characteristic of the *fabF*
mutant phenotype (31,34). Later, the enforced overexpression of FabB from a multicopy plasmid
was demonstrated to drive 18:1\(\Delta11\) synthesis (24), illustrating that the substrate specificity of the
condensing enzymes is not absolute. Our work validates this point by showing that the
physiological regulation of FabB levels by FabR is also an important determinant of the cellular
content of 18:1\(\Delta11\). The regulation of *fabB* is the most important function of FabR in determining
fatty acid composition, and this discovery is a unique example of a transcription factor that
exclusively regulates the expression of the enzymes of a type II fatty acid synthase.

Regulation of UFA content by the FabR repressor requires the presence of the FadR
activator. FabR binds to a sequence found in the promoter regions of the *fabA* and *fabB* genes
from *E. coli* (Fig. 1). The location of the predicted binding sequence and the data discussed above
suggests that FabR regulates by repression and antagonizes the action of the FadR activator. Our
experiments point to the level of FabB as a determinant of UFA synthesis and the potent regulation
of the *fabB* gene by FabR underscores a central role for this transcription factor in controlling
membrane fluidity in bacteria that produce UFA using the FabA/FabB combination. However,
FabR cannot accomplish this regulation without the participation of FadR. The fact that the UFA
content is not increased in a *fadR fabR* strain compared to a *fadR* strain illustrates that the level of
*fabA* expression, supported by the transcriptional activation by FadR is required for UFA regulation
by FabR. In the absence of FadR activation, we conclude that FabA activity is limiting for UFA
synthesis, whereas in the presence of FadR activation, the level of FabB activity controls UFA
production.

There are important questions that remain concerning the role of the FabR repressor in cell
physiology. One important aim will be to determine the mechanism that controls its binding to
DNA. FabR is a member of the TetR superfamily (Pfam00440; (37)) of bacterial regulatory
proteins, and by analogy to the known mechanisms used by these factors, it seems reasonable to
postulate that a regulatory ligand binds to the carboxy terminal domain of the protein to release the
amino terminal DNA-binding domain from its target sequence. FadR has a carboxy terminal domain that is homologous to the regulatory domains of the TetR family members (14). The FadR factor controls fabA and fabB expression in response to long-chain acyl-CoA derived from exogenous fatty acids (9,17) and it is tempting to speculate that FabR is controlled by a ligand that is an integral component of endogenous de novo fatty acid biosynthesis. A pathway intermediate such as a saturated long-chain acyl-ACP is an example of such a candidate ligand. However, we cannot rule out other mechanisms, such as phosphorylation. Another part of the FabR story that deserves attention is defining the mechanism(s) that controls the expression of fabR. Possibly, the fabR gene is controlled by another factor that ties UFA synthesis to other aspects of bacterial physiology in a fashion analogous to the control of acetate metabolism by FadR by virtue of its ability to control the expression of iclR, a transcriptional regulator of the glyoxylate bypass (38).

The Affymetrix array analysis extended the number of genes directly controlled by FadR. Previous work has cataloged a substantial list of genes controlled by FadR that are either related to fatty acid metabolism or survival in stationary phase (20,39,40). Our experiments do not address stationary phase, but the new information in this report expands the repertoire of β-oxidation genes subject to FadR regulation. The fadH and fadE genes are involved in β-oxidation and the prediction that they would be derepressed by FadR deletion is confirmed in this study (Table IV). We also found two new genes, yfcX and yfcY, which are also predicted to be involved in fatty acid β-oxidation (Table IV). The yfcX gene encodes a protein with predicted multifunctional capabilities (enoyl-CoA hydratase, β-hydroxyacyl-CoA dehydrogenase and β-hydroxybutyryl-CoA epimerase), and is thus similar in function to FadB and termed β-oxidation complex II. The difference between the two complexes is that complex I contains Δ3-cis-Δ2-trans-enoyl-CoA isomerase activity, which is predicted to be absent from complex II. The yfcY gene is predicted to be a β-ketoacyl-CoA thiolase based on its similarity to fadA, and is thus termed thiolase II. The reason for these two sets of similar β-oxidation genes is unknown, but may reflect differences in substrate specificity of the respective enzymes. Both of these genes have putative FadR binding
sites located in regions downstream of the predicted transcriptional start site, consistent with a role for FadR in repressing their expression. Further genetic and biochemical characterization of these two genes will be required to establish their substrate specificity and roles in fatty acid β-oxidation.

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FOOTNOTES

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§Both authors contributed equally to the work.

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1Abbreviations used are: ACP, acyl carrier protein; FabA, β-hydroxydecanoyl-ACP dehydratase; FabB, β-ketoacyl-ACP synthase I; FabF, β-ketoacyl-ACP synthase II; SFA, saturated fatty acid; UFA, unsaturated fatty acid; FadR, fatty acid degradation regulator; and FabR, fatty acid biosynthesis regulator; fatty acid abbreviations, 18:1∆11, number of carbon atoms:number of double bonds, ∆ = the location of the double bond.
REFERENCES


### TABLE I

**Bacterial strains used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant Genotype/Plasmid</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>UB1005</td>
<td>metB1 relA1 spoT1 gyrA216 λ− λ F−</td>
<td>(41)</td>
</tr>
<tr>
<td>PDJ1</td>
<td>recD::Tn10</td>
<td>P1(CAG12135) × UB1005</td>
</tr>
<tr>
<td>ANS8</td>
<td>fabR::kan</td>
<td>P1(WMF1) × UB1005</td>
</tr>
<tr>
<td>MWF1</td>
<td>fabR::kan recD::Tn10</td>
<td>“Experimental Procedures”</td>
</tr>
<tr>
<td>JT10</td>
<td>UB1005/pDM4</td>
<td>“Experimental Procedures”</td>
</tr>
<tr>
<td>MWF2</td>
<td>recD::Tn10/pSJ21(fabB)</td>
<td>“Experimental Procedures”</td>
</tr>
<tr>
<td>SJ109</td>
<td>fabF1</td>
<td>P1(CY288)× UB1005</td>
</tr>
<tr>
<td>ANS7</td>
<td>fabR::kan fabF1</td>
<td>P1(MWF1) × SJ109</td>
</tr>
<tr>
<td>PDJ14</td>
<td>fadR::Tn10</td>
<td>P1(RW11) × UB1005</td>
</tr>
<tr>
<td>ANS4</td>
<td>fabR::kan fadR::Tn10</td>
<td>P1(MWF1) × PDJ14</td>
</tr>
</tbody>
</table>

*Strain CY288 is described in Ref. 31.*
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Probe</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>acpP</td>
<td>AGCTGGAATGGCTGCTTGGGA</td>
<td>VIC-AGTTTGAATCGAGATTCCCGAGCACGAAGA</td>
<td>CTGAAGGGATGGGATTTCCTCA</td>
</tr>
<tr>
<td>fabA</td>
<td>CTCTGGTCGCGGCGAATGTG</td>
<td>FAM-TGGCGCTAAAGGCCCGAATTG</td>
<td>GGTCCATCATCGCATGTTTC</td>
</tr>
<tr>
<td>fabB</td>
<td>CTGGCGCGGCGTGCCTGTC</td>
<td>FAM-TGAAATCGTTGCTACGCGAAGA</td>
<td>CAACCATGCACGCCCACAG</td>
</tr>
</tbody>
</table>
### TABLE III

**FabR regulation of fatty acid composition**

Strains were grown on minimal medium until mid-log phase of growth. The lipids were extracted, fatty acid methyl esters prepared and the compositions determined by gas-liquid chromatography as described under “Experimental Procedures.”

<table>
<thead>
<tr>
<th>Strain (Genotype)/Plasmid</th>
<th>C16:0&lt;sup&gt;a&lt;/sup&gt;</th>
<th>C18:0</th>
<th>C16:1&lt;sup&gt;b&lt;/sup&gt;</th>
<th>C18:1&lt;sup&gt;b&lt;/sup&gt;</th>
<th>UFA/SFA</th>
<th>18:1/16:1</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDJ1</td>
<td>48.4</td>
<td>1.9</td>
<td>23.2</td>
<td>20.6</td>
<td>0.87</td>
<td>0.89</td>
</tr>
<tr>
<td>MWF1 (fabR::kan)</td>
<td>32.0</td>
<td>2.2</td>
<td>15.0</td>
<td>45.4</td>
<td>1.77</td>
<td>3.03</td>
</tr>
<tr>
<td>JT10 (UB1005/pDM4(fabB))</td>
<td>31.4</td>
<td>4.9</td>
<td>13.0</td>
<td>45.1</td>
<td>1.60</td>
<td>3.47</td>
</tr>
<tr>
<td>MWF2/pSJ21(fabA)</td>
<td>52.6</td>
<td>3.7</td>
<td>23.0</td>
<td>14.6</td>
<td>0.67</td>
<td>0.63</td>
</tr>
<tr>
<td>SJ109 (fabF1)</td>
<td>50.7</td>
<td>4.5</td>
<td>30.2</td>
<td>7.2</td>
<td>0.68</td>
<td>0.24</td>
</tr>
<tr>
<td>ANS7 (fabF1 fabR::kan)</td>
<td>39.1</td>
<td>6.6</td>
<td>29.0</td>
<td>21.2</td>
<td>1.10</td>
<td>0.73</td>
</tr>
<tr>
<td>PDJ14 (fadR::Tn10)</td>
<td>54.7</td>
<td>3.0</td>
<td>21.1</td>
<td>14.5</td>
<td>0.62</td>
<td>0.69</td>
</tr>
<tr>
<td>ANS4 (fabR::kan fadR::Tn10)</td>
<td>56.6</td>
<td>1.9</td>
<td>22.7</td>
<td>12.6</td>
<td>0.60</td>
<td>0.56</td>
</tr>
</tbody>
</table>

<sup>a</sup>Fatty acids that comprised less than 1.5% of the total are not listed.

<sup>b</sup>The contributions of the respective cyclopropane derivatives were included in these percentages.
### TABLE IV

*Fatty acid metabolic genes regulated by FadR versus FabR*

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold Change$^a$</th>
<th>Gene Description</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\text{fadR}$</td>
<td></td>
</tr>
<tr>
<td>fadD</td>
<td>↑3</td>
<td>Fatty acyl-CoA synthetase</td>
</tr>
<tr>
<td>fadL</td>
<td>↑3</td>
<td>Outer membrane long-chain fatty acid transporter</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Fatty acid degradation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fadA</td>
<td>↑16</td>
<td>β-Ketoacyl thiolase I</td>
</tr>
<tr>
<td>fadB</td>
<td>↑48</td>
<td>Fatty acid oxidation complex I$^c$</td>
</tr>
<tr>
<td>fabE (yafH)</td>
<td>↑34</td>
<td>Acyl-CoA dehydrogenase</td>
</tr>
<tr>
<td>fabH (ygjL)</td>
<td>↑15</td>
<td>2,4-dienoyl-CoA reductase</td>
</tr>
<tr>
<td>yfcX (b2341)</td>
<td>↑7</td>
<td>Fatty acid oxidation complex II$^d$</td>
</tr>
<tr>
<td>yfcY (b2342)</td>
<td>↑13</td>
<td>β-Ketoacyl-CoA thiolase II</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Acetate metabolism</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aceA</td>
<td>↑4</td>
<td>Isocitrate lyase</td>
</tr>
<tr>
<td>aceB</td>
<td>↑4</td>
<td>Malate synthase</td>
</tr>
<tr>
<td>aceK</td>
<td>↑3</td>
<td>Isocitrate dehydrogenase kinase/phosphatase</td>
</tr>
<tr>
<td>iclR</td>
<td>↓3</td>
<td>Repressor of aceBAK operon</td>
</tr>
</tbody>
</table>

$^a$The expression levels of metabolic genes were monitored in *E. coli* strain PDJ14 (*fadR*), strain UB1005 (wt), and in the isogenic *fabR* (ANS8) and *fadR fabR* (ANS4) strains. Strains were grown to a density of $3 \times 10^8$ cells/ml in glycerol minimal media supplemented with methionine and thiamine. The fold change corresponds to the fluorescent ratio $\text{fadR/wild-type (PDJ14/UB1005)}, \text{fabR/wild-type (ANS8/UB1005)}$ or $\text{fadR fabR/wild-type (ANS4/UB1005)}$.

$^b$means a fold change less than 2.

$^c$includes enoyl-CoA hydratase; β-hydroxyacyl-CoA dehydrogenase, β-hydroxybutyryl-CoA epimerase and $\Delta^3$-$\Delta^2$-trans-enoyl-CoA isomerase (42).

$^d$predicted to include enoyl-CoA hydratase; β-hydroxyacyl-CoA dehydrogenase and β-hydroxybutyryl-CoA epimerase.
FIGURE LEGENDS

FIG. 1. Location of the FadR and FabR binding sites in the fabB and fabA promoters. The sequence of the promoter regions of the fabA and fabB gene are very similar. The transcriptional start sites are indicated by the arrows for both fabA (18) and fabB (21). The FadR (17,18) and FabR (22) binding sites are indicated by the brackets.

FIG. 2. The fabR gene targeting strategy and characteristics of fabR::kan deletion strain. Panel A, a diagram of the fabR replacement in the genome showing the locations of the kanamycin gene insert, the PCR primers and the diagnostic restriction enzymes used in constructing and analyzing the gene replacement. Abbreviations for the restriction enzymes are: Nd, Ndel; E, EcoRI; S, Scal; Nc, Ncol; C, ClaI. Kanamycin-resistant strains were isolated following linear transformation of strain PDJ1 (recD::Tn10) as described under “Experimental Procedures” and homologous recombination of the targeting construct was verified by PCR (Panel B) and Southern blotting (Panel C). Panel B, PCR analysis of wild type and fabR::kan strains with primers P1 and P2. The PCR product from a wild type cell was 2.4-kb (lane 1), compared to the longer 3.0-kb product obtained from the fabR::kan strain MWF1 (lane 2). The PCR products of strain PDJ1 (wild type; lanes 3, 4 and 5), and strain MWF1 (fabR::kan; lanes 6, 7 and 8) were digested with three different restriction enzymes, Ncol (lanes 3 and 6), BamHI (lanes 4 and 7) and HindIII (lanes 5 and 8). The digested products showed patterns expected from the location of the restriction sites shown in Panel A. Panel C, Southern blot analysis was performed using a probe prepared from a 230-bp Clal-Scal fragment downstream of fabR gene as shown in Panel A. The 32P-labelled probe was hybridized with genomic DNA fragments of the expected sizes from either strain MWF1 (fabR::kan) (lanes 2, 4 and 6) or strain PDJ1 (wild type) (lanes 1, 3 and 5) digested with Scal (lanes 1 and 2), EcoRI (lanes 3 and 4) or Ndel (lanes 5 and 6) restriction enzymes.

FIG. 3. Increased expression of both fabB and fabA in strain MWF1 (fabR::kan). Northern blot analysis showed that the level of fabA mRNA in strain MWF1 (fabR::kan) was 3 times higher than control (strain PDJ1). The level of fabB mRNA was 7-fold higher in FabR negative
cells. The bands were localized and the intensities quantitated using a PhosphorImager. The size of the fabA mRNA was 550 bp and the fabB was 1280 bp.

**FIG. 4. Increased condensing enzyme activity in strain ANS7 (fabR::kan).** Cell-free extracts of strains SJ109 (fabF1) and ANS7 (fabR::kan fabF1) were prepared, dialyzed and assayed for condensing enzyme activity using myristoyl-ACP as the substrate as described under “Experimental Procedures.” The condensing enzyme specific activity in strain ANS7 extracts was 25.2 µmoles/min/µg, which was 2.5 times higher than in strain SJ109 (10.5 µmoles/min/µg).

**FIG. 5. Levels of fabA and fabB mRNA in fabR and fadR strains determined by microarray analysis and real-time PCR.** RNA preparations from strains UB1005 (wild type), PDJ14 (fadR::Tn10), ANS8 (fabR::kan) and ANS4 (fabR::kan fadR::Tn10) were used for the Affymetrix microarray analysis and real-time PCR. Panel A, two independent fabR::kan RNA microarrays were compared to two control RNA microarrays to obtain 4 analyses of the amount of fabB and fabA mRNA in the two strains. The fabB and fabA fluorescence was normalized to the acpP mRNA value and the results were averaged from 4 independent chip comparisons. In the FabR-negative cells, the fabA level was increased by 2-fold and the fabB level in the fabR mutant was 4 times higher than in wild type. The analysis of the double mutant fabR fadR showed a decrease in the fabA expression level, as observed in the case of the fadR mutant. The fabB mRNA levels in the fabR fadR mutant were 2-fold higher than in the wild type. Panel B, TaqMan real-time PCR was performed with primers and probe sets specific for acpP, fabB or fabA as described under “Experimental Procedures.” The amounts of fabB and fabA cDNA were determined and normalized to the amount of acpP cDNA in the same samples. The data are means of four determinations on two independent RNA/cDNA preparations. The expression level of fabA in FadR-negative cells was 8-fold lower than in wild type cells, and no significant change was observed in fabB levels. In the FabR-negative cells, the fabA level was elevated 3-fold and the fabB level was elevated 5-fold. Analysis of the fadR fabR double mutant showed a decrease in fabA expression, whereas the fabB levels were 1.5-fold higher than in the fadR strain.
Figure 1
Figure 2
Figure 3

Relative Expression Level

WT

fabR

fabA

fabB

Figure 3
Figure 5
The FabR (YijC) transcription factor regulates unsaturated fatty acid biosynthesis in Escherichia coli
Yong-Mei Zhang, Hedia Marrakchi and Charles O. Rock

J. Biol. Chem. published online February 21, 2002

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