Escherichia coli FadR Positively Regulates Transcription of the fabB Fatty Acid Biosynthetic Gene

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In Escherichia coli expression of the genes of fatty acid degradation (fad) is negatively regulated at the transcriptional level by FadR protein. In contrast the unsaturated fatty acid biosynthetic gene, fabA, is positively regulated by FadR. We report that fabB, a second unsaturated fatty acid biosynthetic gene, is also positively regulated by FadR. Genomic array studies that compared global transcriptional differences between wild-type and fadR-null mutant strains, as well as in cultures of each strain grown in the presence of exogenous oleic acid, indicated that expression of fabB was regulated in a manner very similar to that of fabA expression.

A series of genetic and biochemical tests confirmed these observations. Strains containing both fabB and fadR mutant alleles were constructed and shown to exhibit synthetic lethal phenotypes, similar to those observed in fabA fadR mutants. A fadR strain was hypersensitive to cerulenin, an antibiotic that at low concentrations specifically targets the FabB protein. A transcriptional fusion of chloramphenicol acetyltransferase (CAT) to the fabB promoter produces lower levels of CAT protein in a strain lacking functional FadR. The ability of a putative FadR binding site within the fabB promoter to form a complex with purified FadR protein was determined by a gel mobility shift assay. These experiments demonstrate that expression of fabB is positively regulated by FadR.

Bacteria regulate membrane fluidity by manipulating the relative levels of saturated and unsaturated fatty acids within the phospholipids of their membrane bilayers (1, 13). There are eight known genes (fab) involved in fatty acid biosynthesis in Escherichia coli (reviewed in references 8 and 34). Of these, only fabA and fabB are specifically required for the synthesis of unsaturated fatty acids (4, 5, 12, 46). Likewise, there are at least five separate gene products involved in the degradation of long-chain fatty acids to acetyl coenzyme A (for a review, see reference 34). The FadR regulatory protein negatively controls expression of the genes of the fatty acid degradation pathway (33, 40) and also functions as a positive regulator of unsaturated fatty acid synthesis (19, 29, 30, 38).

Only two unique biochemical reactions are required to specifically produce unsaturated fatty acids in the overall course of fatty acid biosynthesis in E. coli (4, 5, 12, 46). When the growing acyl chain coupled to acyl carrier protein (ACP) reaches the 3-hydroxyacyl-ACP stage, either of two enzymes can carry out the dehydration reaction to produce trans-2-decenoyl-ACP. In vitro, the 3-ketoacyl-ACP dehydratases, FabZ and FabA, both have broad and overlapping ranges of substrate chain length specificity (28). In vivo, FabZ is involved in the dehydration of all chain lengths of 3-hydroxyacyl-ACPs and seems especially important in lipid A biosynthesis (36). Genetic data argue that the activity of FabA in vivo is restricted to 10 carbon substrates. The enzyme not only catalyzes the dehydration of 3-hydroxydecanoyl-ACP but also isomerizes the trans-2-enoyl bond of the ACP-bound substrate to the cis-3 isomer (3, 28). This isomerization places the nascent acyl chain in the unsaturated fatty acid synthetic pathway. The 3-ketoacyl-ACP synthase I (FabB) enzyme appears to be required for elongation of the cis-3 decenoyl-ACP produced by FabA and is known to be the primary factor in determining cellular unsaturated fatty acid content (10). The Claissen-type condensation of malonyl-ACP with cis-3 decenoyl-ACP catalyzed by FabB produces cis-5-ene-3-ketododecenoyl-ACP, which is competent to undergo all the subsequent reactions typical of fatty acid biosynthesis. Ultimately this results in production of cis-9 hexadecenoyl and cis-11 octadecenoyl chains, which are incorporated into phospholipid (34).

The regulation of unsaturated fatty acid biosynthesis is complex. The fabA gene is known to have a strong promoter that is positively regulated by FadR (19, 29, 30) as well as a weaker constitutive promoter. The reasons why a regulatory factor for fatty acid degradation is involved in regulating unsaturated fatty acid biosynthesis remain obscure. A model advanced by Cronan and Subrahmanyam (15) addresses the issue of why it seems advantageous to have two promoters for fabA but fails to answer the question of why FadR regulates fabA per se. DiRusso and Nystrom (21) have postulated that FadR interacts with a number of other regulatory activities to coordinate lipid biosynthesis and degradation in response to stress and aging. While this seems an attractive proposal, it still begs the question of why the synthesis of unsaturated acids in particular, as opposed to that of saturated fatty acids, is regulated by FadR. Experimental evidence that both genes involved in unsaturated fatty acid biosynthesis are regulated similarly would discount the possibility that FadR regulation of fabA is merely fortuitous or vestigial in nature. Computer-assisted searches for consensus FadR recognition sites within the E. coli genome identify fabB as a potential target of FadR regulation (45). It
should be noted that although several reviews state that fabB is positively regulated by FadR, neither these reports (2, 18, 21) nor the specific reference cited therein (19) contains data supporting this claim. We report several different lines of evidence showing that FadR positively regulates fabB transcription.

**Materials and Methods**

**Bacterial strains and plasmids.** The bacterial strains and plasmids used in this study are listed in Table 1. Unless otherwise indicated, strains were obtained from local laboratory stocks or from the E. coli Genetic Stock Center (CGSC, Yale University, New Haven, Conn.). Phage transductions and other basic genetic techniques were generally carried out as previously described in reference 53. Strain CAG18497 is from the ordered Tn10 collection of Singer and coworkers (48). Strains JWC264, JWC286, and JWC287 were made by P1-mediated transduction of the fabB15::Tn10 allele of CAG18497 into strains MG1655, MS, and M5, respectively. Strain JWC264 was selected on rich broth plates containing tetracycline at 37°C. Strain JWC276 is a fabB::CAT (chloramphenicol acetyltransferase) transcriptional fusion in the strain MG1655 background containing a wild-type copy of the fabB gene expressed from the araBAD promoter of plasmid pARA14 (47). Strain JWC276 was made by transduction of fabB15::Tn10 from strain CAG18497 into strain JWC264 and selecting for tetracycline resistance at 30°C on rich broth plates supplemented with 0.1% oleate. Plasmid pRC1 carries a 4-kb fragment isolated from chromosomal E. coli DNA that includes intact fabA (20). Plasmid pARAfabB was made by PCR amplification of the fabB gene from MG1655 chromosomal DNA, followed by ligation of the fragment into pARA14 (7). The amplification reactions used a 5’ primer with the sequence 5’-CATTCGGATCCTTACTCTAT-GTGCG-3’ and a 3’ primer with the sequence 5’-GCCTGGATCCCCTTACCCGACC-3’ primer with the sequence 5’-CATTCGGATCCTTACTCTAT-GTGCG-3’ and a 3’ primer with the sequence 5’-GCCTGGATCCCCTTACCCGACC-3’. The unique 1.3-kb product was purified using a Qiagen (Valencia, Calif.) desalting column and digested with BamHI. Approximately 1 µg of plasmid pARA14 DNA was digested with BglII, treated with alkaline phosphatase, and ligated to the fabB PCR product. These reaction mixtures were then digested with BglII and transformed into strain DH10a (51). All enzymes and buffers were from Gibco-BRL (Gaithersburg, Md.) or New England Biolabs (Beverly, Mass.). Restriction enzymes were used at the following concentrations: tetracycline HCL, 12 µg/ml; kanamycin sulfate, 50 µg/ml; and CPTD, 25 µg/ml. CPTD was used for the reasons previously described (51). Chloramphenicol was present at 34 µg/ml, unless otherwise indicated. The detergent, antibiotics, and most bulk chemicals were obtained from Sigma (St. Louis, Mo.). Solid media contained 1.5% (wt/vol) BactoAgar (Difco, Milwaukee, Wis.). The phenotypes of the various fab mutants were verified by testing for a requirement for unsaturated fatty acids (oleate) on rich-broth plates.

**Cultures for genomic expression analyses were grown in minimal M9 medium (35) with 0.4% glycerol as carbon source. Exponentially growing cultures (doubling time of about 2 h) were grown aerobically at 37°C with vigorous rotary shaking. The cultures were repeatedly diluted to ensure exponential growth and were harvested at a cell density of about 10^10 cells/ml (about 1/20 of the maximal cell density attained in this medium). The growth curves of the fadR and wild-type strains were indistinguishable in the exponential phase of growth in all of the media tested, although the wild-type strain reached a slightly higher maximal cell density.**

Cerulein was dissolved in chloroform, and various volumes of this solution were placed into empty sterile culture tubes and allowed to evaporate to dryness at room temperature. One milliliter of rich broth containing kanamycin was added to each tube, and the cultures were incubated for 6 h at 37°C in a roller drum. Cell growth was measured spectrophotometrically at 600 nm.

**Genomic expression profiling analysis.** The Sigma-Genosys (The Woodlands, Tex.) E. coli Panorama array system was used to evaluate the expression of each of the 4,290 open reading frames (ORFs) in the E. coli genome. Experimentally this involved measuring differences in expression in cells grown in M9-glycerol minimal medium with and without supplementation with 0.01% oleic acid. The use of M9-glycerol allowed the expression of the genes to be measured with relative ease. The differences in expression was also measured between the reference strain, MG1655, and the inosine fadR mutant, JWC264, when both strains were grown in M9-glycerol. A third experiment measured the transcriptional differences between the JWC264 (fadR) strain grown with and without oleate supplementation.

**RNA isolation and sample handling procedures were those of Tao et al. (52). Briefly, early-log-phase cultures were removed from the shaking incubator and immediately deproteinized into an equal volume of boiling lysis buffer composed of 2% sodium dodecyl sulfate (SDS), 250 mM sodium acetate (pH 4.5), and 20 mM EDTA. The lysed cells were extracted twice with 60°C phenol equilibrated with 0.1 M sodium chloride at pH 4.5 and then extracted once with phenol-chloroform (1:1) at room temperature. Nucleic acids were precipitated in 0.5 volumes of 2-propanol and rinsed with a small volume of ice-cold 70% ethanol. The pellet was air dried for 10 to 15 min and dissolved in a small volume of sterile RNase-free water. Approximately 20 U of RNase inhibitor (Promega, Madison, Wis.) was added. Residual RNA was removed by incubation with 10 U of RNase-free DNase I (Promega) at 37°C for about 1 h. The RNA samples were applied to RNeasy columns (Qiagen) and recovered in 30 µl of sterile diethyl pyrocarbonate-treated water. The RNA was quantitated by absorbance at 260 nm against a water blank. Sample purity was determined by the A260/A280 ratio. Total yields of 10 to 15 µg of RNA with A260/A280 ratios of 1.8 to 2.1 were routinely obtained.**

**The RNA samples consist of a mixture of the various stable RNAs, including tRNA and rRNA, as well as mRNA. To restrict probe synthesis to mRNA, the Panorama E. coli cDNA labeling and hybridization kit (Sigma-Genosys) was**

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**TABLE 1. Bacterial strains used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG1655</td>
<td>fabB-1 rfp(?)</td>
<td>56</td>
</tr>
<tr>
<td>CAG18497</td>
<td>fabB15::Tn10 rfp-1 fabA18(Ts)</td>
<td>This work</td>
</tr>
<tr>
<td>M5</td>
<td>serUs4 fabB15(Ts)</td>
<td>4</td>
</tr>
<tr>
<td>M8</td>
<td>serUs4 fabA18(Ts)</td>
<td>4</td>
</tr>
<tr>
<td>JWC264</td>
<td>fabB15::Tn10 rfp-1 ftr(?) fabB::CAT</td>
<td>This work</td>
</tr>
<tr>
<td>JWC287</td>
<td>fabB15::Tn10 ftr(?)</td>
<td>This work</td>
</tr>
<tr>
<td>JWC288</td>
<td>pRC1/fabB15::Tn10 ftr(?)</td>
<td>This work</td>
</tr>
<tr>
<td>JWC289</td>
<td>pRC1/fabB15::Tn10 rfp-1 ftr(?)</td>
<td>This work</td>
</tr>
<tr>
<td>JWC276</td>
<td>pARAfabB/fabB-1 ftr(?) fabB-CAT</td>
<td>This work</td>
</tr>
<tr>
<td>JWC277</td>
<td>pARAfabB/fabB-1 rfp-1 ftr(?) fabB-CAT</td>
<td>This work</td>
</tr>
</tbody>
</table>

**FadR regulation of fabB**

5’-GAATTAATCTTCTTCAGCT TGGCGGATCCAGCGTGTCGTT-3’ and 3’-AGGCTTCCACGCGGCCATGTC-5’ were used as the 5’ and 3’ ends, respectively, of the PCR primer to amplify the fabB gene from the MG1655 chromosomal DNA. The resulting fragment was cloned into pMD18-T (Takara) and sequenced. The sequence of this primer was 5’-GAATTAATCTTCTTCAGCT TGGCGGATCCAGCGTGTCGTT-3’ and 3’-AGGCTTCCACGCGGCCATGTC-5’. The sequence of this primer was 5’-GAATTAATCTTCTTCAGCT TGGCGGATCCAGCGTGTCGTT-3’ and 3’-AGGCTTCCACGCGGCCATGTC-5’. The sequence of this primer was 5’-GAATTAATCTTCTTCAGCT TGGCGGATCCAGCGTGTCGTT-3’ and 3’-AGGCTTCCACGCGGCCATGTC-5’. The sequence of this primer was 5’-GAATTAATCTTCTTCAGCT TGGCGGATCCAGCGTGTCGTT-3’ and 3’-AGGCTTCCACGCGGCCATGTC-5’. The sequence of this primer was 5’-GAATTAATCTTCTTCAGCT TGGCGGATCCAGCGTGTCGTT-3’ and 3’-AGGCTTCCACGCGGCCATGTC-5’. This primer was used to amplify the fabB gene from MG1655 chromosomal DNA. The resulting fragment was cloned into pMD18-T (Takara) and sequenced. The sequence of this primer was 5’-GAATTAATCTTCTTCAGCT TGGCGGATCCAGCGTGTCGTT-3’ and 3’-AGGCTTCCACGCGGCCATGTC-5’. The sequence of this primer was 5’-GAATTAATCTTCTTCAGCT TGGCGGATCCAGCGTGTCGTT-3’ and 3’-AGGCTTCCACGCGGCCATGTC-5’. This primer was used to amplify the fabB gene from MG1655 chromosomal DNA. The resulting fragment was cloned into pMD18-T (Takara) and sequenced. The sequence of this primer was 5’-GAATTAATCTTCTTCAGCT TGGCGGATCCAGCGTGTCGTT-3’ and 3’-AGGCTTCCACGCGGCCATGTC-5’.
used in a standard cDNA synthesis reaction. This kit consists of an equimolar mixture containing 4.2 U of super RNase inhibitor. Sample RNA was added to a solution containing 0.33 mM concentration each of dATP, dGTP, dCTP, and dTTP and 4 µl of the Sigma-Genosys primer mix. The reaction mixture was brought to a total volume of 25 µl in first-strand synthesis buffer in a small, thin-walled Eppendorf tube. The reaction mixture was placed into a thermal cycler, heated to 90°C for 2 min, and then linearly cooled to 42°C over a 20-min period. On reaching 42°C, 200 U of Superscript II RNAse H- Reverse Transcriptase ( Gibco-BRL) was added along with 20 U of RNase Inhibitor (Promega) and 20 to 30 µCi of [α-32P]dCTP (3,000 Ci/mmol) (NEN Life Science Products, Inc., Boston, Mass.). The reaction mixture was incubated at 42°C for an additional 3 h. Unincorporated nucleotides were removed by centrifugation through Sephadex G-25 gel filtration columns (Boehringer Mannheim, Indianapolis, Ind.). Incorporation levels of 80 to 90% of the total label were routinely achieved by this procedure.

Typically, about 10 to 15 ng of cDNA was recovered from the reverse transcriptase reactions. Estimates of the mRNA content of E. coli range from 3% of total RNA based on pulse-labeling studies to 1.4% based on hybridization experiments (6, 31). These estimates place the total amount of mRNA available in the reverse transcriptase reaction in the neighborhood of 14 to 30 ng. This closely matches the amount of cDNA recovered from the reaction. A crude estimate of the molar amount of mRNA, based on an average mRNA length of 1 to 2 kb, places the total number of transcripts at about 100 pmol or approximately 1010 molecules. The available nucleoside triphosphates in the reaction mixture could theoretically support the synthesis of about 1015 molecules of 1-kb single-stranded DNA. Under these conditions, with limiting template concentration, excess nucleotides and primer, and a single annealing cycle, the cDNA products approximate both the complexity and the relative abundance of the individual ORFs within the mRNA population.

The hybridization and washing steps were carried out as recommended by the manufacturer of the array. Hybridization buffer consists of 5x SSPE (1× SSPE is 0.18 M NaCl, 10 mM Na2HPO4, and 1 mM EDTA [pH 7.7]), 2% SDS, and 1× Denhardt’s reagent supplemented with 100 µg of sheared and sonicated herring sperm DNA per ml. The membranes were prehybridized overnight at 65°C in a roller oven. After prehybridization, the buffer was exchanged for 6 ml of fresh, presaturated buffer. The cDNA probe sample was heated to 94°C for a few minutes and then added to the hybridization bottle. Samples were hybridized for at least 18 h at 65°C in the roller oven.

When hybridization was complete the membranes were washed twice in 50 ml of a wash solution consisting of 0.5x SSPE and 0.2% SDS at room temperature. This was followed by two washes at 65°C with the same wash solution for 20 min each in the hybridization oven. After the final wash step, the membranes were placed onto blotting paper, wrapped in plastic food wrap, and placed in a PhosphorImager cassette (Molecular Dynamics, Sunnyvale, Calif.). The phosphor screen was exposed for 2 to 3 days prior to quantitation.

The TIFF images of each blot were analyzed for pixel depth at each spot position on the membrane using Molecular Dynamics ImageQuant software. The phosphoryl screen was exposed for 2 to 3 days prior to quantitation.

PhosphorImager cassette (Molecular Dynamics, Sunnyvale, Calif.). The phosphor screen was exposed for 2 to 3 days prior to quantitation.

A CAT assay kit, produced by Roche, Inc., was used to measure the mass of CAT protein in cell extracts. The method is based on a sandwich enzyme-linked immunosorbent assay involving antibodies to CAT bound to the surface of microtiter wells. The cells were lysed, and the cell extracts containing CAT enzyme were added to the wells. Following a 1-h incubation at 37°C the sample extract was discarded. The wells were then washed five times with 250 µl of a washing buffer composed of phosphate-buffered saline and tween 20. A digitonin-membrane was added to the wells.

RESULTS

Genomic array experiments indicate that fabB is positively regulated by FadR. Genomic array analysis of global differential transcription patterns in bacteria is useful in defining the extents of metabolic regulons in bacteria (44, 52, 56). A transcriptional regulator such as FadR, having both positive and negative regulatory roles, provides a good test of this experimental approach. Data sets of differential transcription analy-
sis from three separate experimental conditions involving various combinations of FadR and long-chain fatty acid supplementation have been assembled and are available online at http://www.life.uiuc.edu/~jwcampbe. Analysis of these data confirms a number of features of the current model of FadR regulation.

The FadR protein is known to bind long-chain fatty acyl coenzyme A (acyl-CoA) at nanomolar concentrations (22, 30). When FadR binds acyl-CoA, the ability of the protein to bind DNA is greatly decreased and it no longer functions as an effective transcriptional repressor or activator (11, 19, 30). Transcription of fabBA (encoding the two subunits of the fatty acid degradation complex) is higher in wild-type strains grown in the presence of oleate than in the same strains grown without fatty acids (9) (Table 2). Conversely, transcription of fabA decreased in the absence of FadR or in the presence of long-chain fatty acids (29, 30) (Table 2).

Three separate genomic differential transcriptional analyses were used to measure the effects of various fatty acid and FadR conditions on global gene expression patterns. The first experiment involved examining the differences in gene expression between a fadR null mutant, strain JWC264, with the otherwise isogenic wild-type reference strain, strain MG1655. It should be noted that this fadR null allele, fadR613::Tn10 (also called fadR13::Tn10), has been widely used (19, 20, 26, 29, 30, 47) and that recently the site of transposon insertion into the gene was determined (37). It seems likely that the truncated protein is degraded in vivo since the point of insertion is downstream of the regions important for DNA binding (54). If a protein having functional DNA binding activity was present in cells carrying this insertion, the insertion mutation should act as a dominant negative allele (43), which it does not (20). A second experiment examined transcriptional activity in strain MG1655 grown in the presence and absence of 0.01% oleic acid. This concentration of oleic acid is sufficient for maximal growth of unsaturated fatty acid auxotrophs (5, 12, 46), and similar concentrations result in full induction of the β-oxidation regulon (23, 40). The third data set was produced by comparing gene expression patterns in strain JWC264 grown in the presence and absence of 0.01% oleic acid. Not surprisingly, the magnitude of the differential transcriptional response of FadR regulated genes was greatest in the data set that compared the global transcriptional patterns of the FadR null mutant with the wild-type strain. The data set produced by comparing the global transcriptional activities in a wild-type FadR strain in the presence and absence of oleic acid was similar to that observed in the FadR mutant versus wild type experiment. However, the overall magnitude of the differential transcription of FadR regulated genes was generally lower. This was probably due to residual FadR DNA binding activity in the presence of long-chain acyl-CoAs, versus the complete absence of FadR in strain JWC264.

The fabB4 β-oxidation operon is transcribed from a FadR-regulated promoter (9), and thus, the parallel behavior of these two genes within the global transcriptional arrays was expected. However, fabA and fabB are well-separated transcriptional units (one-fifth of the genetic map). The major fabA promoter is regulated by FadR and is downstream of a second weak constitutive promoter. The promoter of fabB is not as well characterized (32) but is likely to be complex. The similarity of the differential transcriptional responses of fabB to fabA under the various experimental conditions suggests that fabB expression is modulated by a mechanism similar to that of fabA.

**Temperature-sensitive fabB mutations are synthetically lethal with fadR.** The first indications that fabA was positively regulated by FadR were the discoveries that fabR strains contained abnormally low levels of cellular unsaturated fatty acid and, more definitively, that fabA(Ts) fadR double mutants were unable to grow even at low temperature unless unsaturated fatty acids were provided (38). The explanation for this finding is that in the presence of functional FadR, sufficient mutant FabA is produced to provide a level of isomerase activity that satisfies cellular unsaturated fatty acid requirements. Indeed, fabA(Ts) strains have been shown to contain abnormally low levels of cellular unsaturated fatty acid at permissive temperatures (38). In contrast, even at the permissive temperature (30°C), fabA(Ts) fadR strains are unable to synthesize enough mutant FabA to provide sufficient unsaturated fatty acid for functional cell membranes (15). Our interpretation of these data is that E. coli can tolerate normal levels of expression of a FabA enzyme of compromised catalytic efficiency or low levels of expression of the wild-type enzyme but cannot survive low levels of expression of a catalytically compromised mutant enzyme.

To test whether fabB mutants might also be synthetically lethal with fadR mutations, the behaviors of fabB(Ts) and fabB(Ts) fadR strains grown under various conditions were examined. Each strain was grown overnight on medium supplemented with 0.01% oleate prior to inoculating experimental cultures. The cultures were incubated at 30 or 42°C overnight and then examined for growth (Table 3). The fabA(Ts) strain M8 grew at 30°C in the presence or absence of oleate, but at 42°C the strain grew only when supplemented with oleate (4, 5). In contrast, at either temperature a fabA(Ts) fadR double mutant grew only in the presence of exogenous oleate (38). A phenotypic behavior that paralleled the fabA case was shown

### TABLE 2. Results of differential transcriptional array analysis of fabBA, fabA, and fabB

<table>
<thead>
<tr>
<th>Gene</th>
<th>JWC264 fadR/MG1655</th>
<th>MG1655 + olate/ MG1655 − olate</th>
<th>JWC264 fadR + olate/JWC264 fadR − olate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rank</td>
<td>Ratio</td>
<td>Rank</td>
<td>Rank</td>
</tr>
<tr>
<td>fabA</td>
<td>44</td>
<td>2.39</td>
<td>112</td>
</tr>
<tr>
<td>fabB</td>
<td>2</td>
<td>6.41</td>
<td>101</td>
</tr>
<tr>
<td>fabA</td>
<td>4,090</td>
<td>0.61</td>
<td>4,227</td>
</tr>
<tr>
<td>fabB</td>
<td>3,954</td>
<td>0.70</td>
<td>4,279</td>
</tr>
</tbody>
</table>

| All                           | 1.04 ± 0.32c             | 1.03 ± 0.27                   | 1.18 ± 0.29                            |

a Strain JWC264 is isogenic to the wild-type strain MG1655 except for introduction of fadR613::Tn10. Wei et al. (56) have established the precedence of using relative ranking to describe genomic array data. In this system, the differential expression ratios of each gene represented on the array are sorted by magnitude and the rank of a given gene is reported relative to the remaining 4,290 genes. The expression ratios of each gene represented on the array are sorted by magnitude relative ranking to describe genomic array data. In this system, the differential transcriptional ratio derived from the raw phosphorimager data.

b Values are means and standard deviations for all genes, providing an estimate of the quality of the data and the statistical significance of the ratio values. Complete data sets are available online (see text).
by fabB(Ts) and fabB(Ts) fadR strains. The fabB(Ts) strain M5 required oleate for growth only at 42°C, whereas the fabB(Ts) fadR strain, JWC287, failed to grow at either temperature unless oleate was provided. Thus, the synthetic lethal behavior towards fadR reported for fabA(Ts) mutants was also found for a fabB(Ts) mutant. This result strongly suggests that FadR positively activates expression of fabB, as is the case with fabA. Two other observations support this hypothesis. The first is that the fabB(Ts) fadR strain grew at the permissive temperature on solid media, but the same strain failed to grow in liquid cultures shaken in flasks under standard conditions. However, the fabB(Ts) fadR strain grew in liquid medium when mechanical stress was minimized (in a thin film of liquid with minimal agitation as in Table 3). It therefore seems that the structural integrity of the membranes of these strains was compromised to the point that the cells became very sensitive to mechanical stress (5).

The second observation is based on the previously noted synthetic lethality seen in fabB(Ts) fabF strains (24). Such strains failed to grow at the nonpermissive temperature when supplemented with oleic acid, although the fabB(Ts) strain grew well under these conditions and fabF mutants lack a growth phenotype. The fabB(Ts) fabF strains grow well at permissive temperature with or without oleic acid supplementation, and the failure to grow at nonpermissive temperature is due to the lack of sufficient condensing enzyme activity for saturated fatty acid synthesis (17, 24, 53). We attempted to obtain fadR613::Tn10 transductants of a fabB(Ts) fabF strain at permissive temperature and failed even when the medium contained oleic acid. The triple mutant fabB(Ts) fabF fadR613::Tn10 strain could be constructed only when the recipient strain carried a plasmid expressing fabB (the pARA fabB plasmid was used). Apparently, in the absence of FabF and FadR, even at the permissive temperature the synthetic demand placed on the decreased level of the FabB(Ts) enzyme exceeded its capacity to produce sufficient fatty acids to support growth.

A fadR strain is hypersensitive to cerulenin. The behavior of the fabB(Ts) strains with respect to FadR suggested that fadR mutants might be more susceptible to the synthetic 3-ketoacyl-ACP synthase inhibitor cerulenin (39). Although both 3-ketoacyl-ACP synthases I and II are inhibited by this antibiotic, synthase I (FabB) is about sevenfold more sensitive than synthase II (FabF) in vitro (41). In vivo results indicate similar differential effects on the two enzymes (6, 53). Therefore we expected that, due to decreased FabB levels, fadR strains should be more sensitive to cerulenin than were wild-type strains. This was found to be the case (Fig. 1). The cerulenin concentration that gave one-half the maximal growth of the wild-type strain was between 8 and 16 μg/ml, which agrees well with the value of 12.5 μg/ml reported by Omura (39). In contrast, the fadR mutant strain failed to show detectable growth even at low cerulenin concentrations and almost complete growth inhibition was seen at 1 μg of cerulenin per mg. Therefore, the fadR strain was at least 10-fold more sensitive to cerulenin than was the wild-type strain, indicating lower levels of FabB, the primary target of the antibiotic. Both strains used in this experiment contained plasmid pRC1 which produces sufficient FabA enzyme to allow growth of fabA(Ts) mutants at the nonpermissive temperature (14). This plasmid was introduced to ensure that the effect of cerulenin was due to changes in the level of FabB and not to possible secondary effects of the fadR mutation on fabA expression.

Decreased expression of fabB in fadR strains. Our genomic array experiments indicated that fabB was positively regulated by FadR and that the level of expression was depressed in cultures of the fadR mutant strain, JWC264. Measuring fabB expression by assays of 3-ketoacyl-ACP synthase activity is complicated by the presence of a second 3-ketoacyl-ACP synthase (FabF), which has a similar level of activity and overlapping substrate specificity. We therefore constructed a chromosomal transcriptional fusion in which the fabB promoter was used to drive expression of a promoterless CAT gene. Since FabB provides at least one essential cellular function, we provided a functional copy of fabB in trans to allow growth in the absence of unsaturated fatty acid supplementation. The plasmid used, pARA fabB, expresses fabB from the Salmonella enterica serovar Typhimurium araBAD promoter. Although

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**TABLE 3. Growth of strains M5, M8, JWC286, and JWC287**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>OD&lt;sub&gt;600&lt;/sub&gt; at:&lt;sup&gt;b&lt;/sup&gt;</th>
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<tbody>
<tr>
<td></td>
<td>30°C</td>
<td>42°C</td>
</tr>
<tr>
<td></td>
<td>With oleate</td>
<td>Without oleate</td>
</tr>
<tr>
<td>M5</td>
<td>fabB(Ts)</td>
<td>+</td>
</tr>
<tr>
<td>JWC287</td>
<td>fabB(Ts) fadR</td>
<td>+</td>
</tr>
<tr>
<td>M8</td>
<td>fabA(Ts)</td>
<td>+</td>
</tr>
<tr>
<td>JWC286</td>
<td>fabA(Ts) fadR</td>
<td>+</td>
</tr>
</tbody>
</table>

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<sup>a</sup> Growth was assayed after overnight incubation at the indicated temperatures in liquid cultures grown with maximal surface area and minimal agitation.

<sup>b</sup> OD<sub>600</sub> optical density at 600 nm; −, OD<sub>600</sub> < 0.073; +, OD<sub>600</sub> between 0.750 and 1.10; ++, OD<sub>600</sub> > 1.10.

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![FIG. 1. Growth inhibition by cerulenin. Cells were grown overnight and inoculated (1:200) into rich broth. One-milliliter samples were placed into culture tubes containing the indicated amounts of cerulenin. The cultures were incubated for 6 h at 37°C prior to measuring growth. Open circles, parental fadR wild-type strain; solid circles, strain fadR613::Tn10; OD, optical density.](http://jb.asm.org/Downloaded-from)
this promoter is generally considered to have low residual activity in the absence of inducer (7, 27), the basal level of this promoter is generally considered to have low residual activity from the uninduced promoter provided sufficient activity in the absence of inducer (7, 27), the basal level of this promoter is generally considered to have low residual activity. OD, optical density.

FIG. 2. Growth curves of fabB::CAT strains. Cells grown overnight in rich broth supplemented with 0.01% oleate were inoculated (1:200) into 200 μl of medium lacking oleate. The experimental cultures were placed in 96-well microtiter dishes at 37°C, and growth was monitored for 18 h. Solid circles, parental fabB wild-type strain; solid triangles, fabB::CAT strain; open circles, fabB::CAT strain carrying the pARA/fabB plasmid. Similar results were found in the presence or absence of arabinose. OD, optical density.

The entire promoter regions of fabA and fabB are shown. The transcriptional start sites, indicated as +1, are from the FadR-dependent fabA promoter (30) and the fabB promoter (32).
FIG. 4. Gel shift assays of FadR and the putative fabB-associated binding site. The assay tests the ability of FadR to specifically bind and retard the electrophoretic mobility of the fabB-associated putative FadR binding site. The binding conditions are given in the text, and the ratios of FadR dimer to target DNA are essentially equimolar. The presence or absence of FadR in the binding assay is indicated by + or − at the top of each lane. Lanes 1 and 2 are the products of an FspI restriction digest of the labeled fragment. In the presence of FadR, the 605-bp fragment was shifted, indicating that FadR specifically binds to that DNA fragment. Likewise, when the 950-bp fragment was digested with SphI only one of two resulting fragments was bound by FadR, as shown in lanes 3 and 4. The relative positions of the FspI and SphI sites and the fabB start codon are given in the lower left of the figure.

DISCUSSION

The evidence presented indicates that FadR positively regulates fabB transcription. The fact that both fabA and fabB, alone among the fatty acid biosynthetic genes, are directly regulated by FadR indicates that E. coli has a vested interest in regulating unsaturated fatty acid biosynthesis even when exogenous fatty acids are available. In retrospect, coordinated regulation of fabA and fabB might have been anticipated since in Pseudomonas aeruginosa it was recently shown that the two genes are cotranscribed in a two-gene fabA-fabB operon (31), although there is no evidence of transcriptional regulation of the operon. The available partial genomic sequences of Pseudomonas putida and Pseudomonas syringae that fabA and fabB are also adjacent genes in these organisms (data not shown).

The two E. coli long-chain acyl-ACP elongation enzymes, FabB and FabF, play different roles in unsaturated fatty acid synthesis. Both enzymes catalyze the Claissen-like condensation of malonyl-ACP with the growing acyl chain. Although the two enzymes are structurally and functionally very similar, they play different roles in cellular metabolism, as shown by the very different phenotypes of fabB and fabF mutants and of strains carrying recombinant plasmids encoding either of the two enzymes. Null fabF mutants have no growth phenotype, whereas fabB mutants require an exogenous source of unsaturated fatty acids. Likewise, strains carrying recombinant fabB plasmids are well behaved, whereas similar plasmids carrying fabF are prone to deletion and rearrangement (34, 51). Recently, it has been shown that FabF effectively titrates FabD (malonyl coenzyme A:ACP transacylase) and that overproduction of FabD offsets the toxicity of fabF plasmids (51). The exact role of FabB in unsaturated fatty acid biosynthesis has not been directly demonstrated in vitro and currently seems to be a puzzle (41, 55). FabB is generally thought to catalyze elongation of the cis-3-decanoyl-ACP produced by FabA to 3-ketododecanoyl-ACP. This is inferred from the findings that fabB strains have no apparent growth phenotype and that fabB mutants require only unsaturated fatty acids for growth. In vitro tests with purified FabF and FabB proteins show that both enzymes are capable of catalyzing the condensation of malonyl-ACP and cis-3-decanoyl-ACP (24, 55). The discrepancy between the genetic and biochemical observations indicates that the biochemical characterization of these enzymes does not accurately reflect their behavior in vivo. Unfortunately, resolution of these issues is problematic, since double knockouts of fabB and fabF are nonviolate and fabB(Ts) strains are not available. Furthermore, double mutant fabF fabB(Ts) strains are nonviolate at high temperature despite supplementation with both saturated and unsaturated fatty acids probably due to the inability to provide precursors for lipid A biosynthesis (24).

The levels of FabA activity normally present do not limit the synthetic capacity for unsaturated fatty acid biosynthesis in E. coli. This has been shown by examining the effects of FabA overproduction on cellular fatty acid composition (10). Unexpectedly, the cellular content of unsaturated fatty acids did not change, but the content of saturated fatty acids increased markedly. This effect was reversed by introduction of a second plasmid encoding FabB. These results were interpreted as indicating that the level of FabB governs the overall rate of unsaturated fatty acid biosynthesis. This is supported by studies that showed that overproduction of FabB about 10-fold (without manipulating FabA) results in increased unsaturated fatty acid production (17). Therefore, fabB may be the most effective point at which to regulate flux of this pathway. However, in order to regulate the level of unsaturated fatty acid biosynthesis without the complication of altering the level of saturated fatty acids produced, E. coli may need to simultaneously coordinate changes in expression of both fabA and fabB.

Why does FadR regulate only the synthesis of unsaturated fatty acids in response to exogenous supplementation? One clue may be that although exogenous supplementation can satisfy the entire unsaturated fatty acid requirement of E. coli, supplementation with saturated fatty acids cannot completely replace the function of that branch of the pathway. The reason for the differing efficiencies of supplementation is the fatty acid chains of the essential outer membrane component lipid A, which are derived from the saturated pathway. These fatty acids (mainly 3-hydroxymyristic acid) cannot be effectively sup-
plied in the medium. Although when added to the culture medium 3-hydroxymyristic acid enters E. coli (and can serve as a carbon source), the supplied acid is not incorporated into lipid A (34). This is probably due to lack of conversion of the acid to the required ACP thioester (42). Therefore, if E. coli were to shut down both the saturated and unsaturated fatty acid synthetic pathways in response to exogenous supplementation, the organism would perish from a lack of the acyl chains needed to synthesize lipid A.

In general, the amplitudes of transcriptional regulation which we observed in our microarray analyses are less than those observed in enzyme assays, Northern blot analyses, and gene fusion experiments. To an unknown extent this may be due to the use of ratio values as the output of the current array techniques. If the expression signal of a gene in the control over. Bacterial mRNA turnover is not yet well understood and, for logical reasons), these factors can have very large effects on the calculated ratios. Another possibility is that the differences which we observed in our microarray analyses are less than needed to synthesize lipid A.

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REFERENCES
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