Characterization of FadR, a Global Transcriptional Regulator of Fatty Acid Metabolism in *Escherichia coli*

INTERACTION WITH THE *fadB* PROMOTER IS PREVENTED BY LONG CHAIN FATTY ACYL COENZYM E AS*

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The *Escherichia coli* *fadR* gene product, FadR, is a multifunctional regulator of fatty acid metabolism. In this work we have purified FadR by a two-step procedure employing two ion-exchange columns. The amino-terminal sequence of the purified protein confirms the sequence of the protein derived from analysis of the DNA sequence (DiRusso, C. C. (1988) Nucleic Acids Res. 16, 7995-8009) and indicates that the initiating methionine is cleaved from the mature protein. Purified FadR binds to a 326-base pair *Hae*III fragment of *fadB* DNA which carries the *fadB* promoter. DNase I footprinting localizes the operator to a sequence, 5′ ATCTGGTACGACCAGAT 3′, at +1 to +17 nucleotides relative to the start of transcription. Using protein-DNA gel retention assays, we estimate the *K*~m~ of FadR binding to the *fadB* operator to be approximately 3 × 10^{-14} M. Binding of FadR is specifically inhibited by long chain fatty acyl-CoA compounds. The apparent *K*~m~ values for oleoyl-CoA, palmitoyl-CoA, and palmitoleoyl-CoA are each 5 nm while that of myristoyl-CoA is 250 nm. Decanoyl-CoA, crotonoyl-CoA, and free fatty acids inhibited binding only at concentrations above 1 μM.

In all organisms fatty acids are essential components of membranes and are important sources of metabolic energy. The metabolism of fatty acids is complex and must necessarily be regulated in such a manner as to allow the cell to adapt quickly to changes in the cell cycle or to environmental stress. Much of our knowledge of the metabolism of fatty acids is derived from studies on *Escherichia coli* (reviewed in Ref. 1).

In *E. coli*, the pathways of fatty acid synthesis and degradation are coordinately controlled by the product of the *fadR* gene, FadR (1). Previous work has shown that FadR exerts negative control over the genes of the *fad* regulon (1) and positive control over at least the *fabA* gene which is required for unsaturated fatty acid biosynthesis (2). The *fad* regulon includes the *fadL, fadD, fadE, fadA, fadB, and fabH* genes which encode proteins required for transport, activation, and β-oxidation of long and medium chain fatty acids. The inducer of the *fad* genes is suggested to be a long chain fatty acid derivative since growth of wild type *E. coli* cells in medium containing fatty acids of greater than 12 carbon atoms in length results in the induction of the *fad* structural genes (3).

FadR was proposed to be a transcriptional regulator based on the following indirect evidence. 1) Spontaneous mutations and transposon insertions in *fadR* cause the constitutive expression of the *fad* structural genes (3, 4). 2) *fadR* supplied in trans on an F′ episome (5) or a plasmid (6) is dominant to mutant *fadR* in restoring the wild type phenotype to the mutant strain. 3) *fadR* strains harboring *fad(L-lacZ)* (7), *fad(B-lacZ)* (8), or *fadA⋅lacZ* (8) have elevated levels of β-galactosidase as compared to *fadR*′ strains whether or not long chain fatty acids are present in the growth medium. 4) *fadR*′ strains harboring the same fusions show inducible β-galactosidase levels (7, 8). 5) *fadA⋅lacZ* strains, in contrast, have reduced levels of β-galactosidase activity in *fadR* strains suggesting that *fadR* is an activator of *fabA* gene expression (2).

In the present study, we have isolated the FadR protein, localized the FadR-binding site on DNA containing the *fadB* promoter, and have shown that long chain acyl-CoAs but not long chain fatty acids prevent DNA binding. This work provides evidence *in vitro* that FadR mediates control of the *fad* genes by binding to DNA and that long chain acyl-CoA compounds prevent DNA binding.

**MATERIALS AND METHODS**

**Strains and Growth Conditions**—The *E. coli* strains used were: C600, F− thf− leuB6 lacY1 tonA21 supE44 lacI5 *Δ*(lac-proB) F′ traD36 proA*Δ* lacI5ΔMIS for M13 growth; and BL21 (DE3)/pLysS F− recA 76 73 mKID RIF for the overexpression of FadR. Bacteria were routinely grown in a New Brunswick gyratory water bath at 37 °C in LB (9). When minimal medium was required, M9 (9) containing 25 mM dextrose was used. Antibiotics were added as required to maintain plasmids at 100 μg of ampicillin/ml and 15 μg of chloramphenicol/ml. Bacterial growth was monitored in a Klett-Summerson Colorimeter equipped with a blue filter.

**Enzymes and Chemicals**—Restriction enzymes, Klenow fragment of DNA polymerase, T4 polynucleotide kinase, avian myeloblastosis virus reverse transcriptase, and deoxyribonuclease I were purchased from U. S. Biochemicals, Inc. Fatty acids and fatty acyl-CoAs were obtained from Sigma. [α-32P]ATP (3000 Ci/mmol), [γ-32P]ATP (3000 Ci/mmol), and [35S]methionine (660 Ci/mmol) were from New England Nuclear Corp. Chemicals used in the synthesis of oligonucleotides were purchased from Pharmacia LKB Biotechnology Inc. and American Bionetics, Inc. All other chemicals were from standard suppliers and were of reagent grade.

**Plasmid and Phage Constructions**—For overexpression of FadR, a 4.0-kilobase HindIII restriction fragment was subcloned from pCACfadR3 (6) into pBR322 (10) to generate pCD129. The *fadA* promoter (designated P₃) was subcloned from pCEM (11) on a 326-
bp) FadR fragment into the Small site of pUC19 to generate pCD154. The 377-bp HindIII to EcoRI fragment of pCD154 containing Pb and the FadR-binding site, C, was further subcloned to M13 mp19 (12) to give MD15 and to M13 mp18 (12) to give MD14.

Characterization of Protein FadR—The transcription initiation site was determined by extension of a fadB-specific oligonucleotide hybridized to in situ synthesized RNA using avian myeloblastosis virus reverse transcriptase. Total RNA was extracted as described in Ref. 13 from strain C606 grown in TB (10 g of tryptone and 5 g of NaCl/liter) containing 5 mM cAMP and 0.5 mM Brj 58. The oligonucleotide (CTTCACCAAGGTGACT) complementary to nucleotides 68-88 in Fig. 4 was end-labeled with 32P using T4 polynucleotide kinase and [γ-32P]ATP (14). Annealing mixtures contained: 20 μ g of RNA, 40,000 cpm 32P-labeled oligonucleotide, 50 mM Tris-HCl, pH 8.0, 40 mM NaCl, and 0.5 mM EDTA in a total volume of 10 μ l. The samples were heated at 85 °C for 2 min and allowed to cool slowly to 42 °C. When 42 °C was reached, 75 units of Rnasin (purchased from U. S. Biochemicals) was added, and the samples were incubated at 42 °C. After 1 h, 10 μ l of a mixture containing 2 mM each dATP, dGTP, dCTP, and dTTP, 20 mM MgCl2, 20 mM DTT, 50 mM Tris-HCl, pH 8.0, 150 mM KCl, 0.5 μ g of actinomycin D, and 15 units of avian myeloblastosis virus reverse transcriptase were added. Primer extension was continued at 42 °C for 1 h, then samples were extracted once with 1 volume of phenol saturated with 100 mM Tris-HCl, pH 8.0, 10 mM EDTA and once with 1 volume of CHCl3/isoamyl alcohol (24:1, v/v). Samples were concentrated by precipitation and resuspended in 5 μ l of formamide gel-loading solution (14). Samples were denatured at 85 °C for 3 min. The products of the primer extension analysis were compared to columns which were run at room temperature (21 °C). Cells were lysed by addition of 10 pg of tRNA, 25 μ l of 7.5 M ammonium acetate, and 125 μ l of 70% ethanol, and resuspended in 4 μ l of TE and 3 μ l of formamide gel-loading solution (14). Samples were denatured at 85 °C for 3 min, placed on ice for 3 min, and 3 μ l were loaded onto an 8% acrylamide gel. The gel was run in 4.5 TBE buffer in a 4% acrylamide gel for 4 h (21). The sample buffer contained 5% glycerol and 0.1 mg/ml Bromophenol blue.

Purification of FadR—BL21(DE3) carrying pLySs and pCD129 cells were grown at 37 °C to a cell density of 3.5 × 108 cells/ml in 1 liter of LB containing 100 μg/ml ampicillin and 15 μg/ml chloramphenicol at which time 4 mM isopropyl-thiol-β-D-galactopyranoside was added to induce T7-RNA polymerase expression (15). Growth was continued for 4 h, and cells were harvested by centrifugation at 7,500 × g, rinsed once with 100 ml of M9 medium, and stored as a pellet at −80 °C until use. When [35S]methionine-labeled FadR was required to trace the protein during purification, cells of the same strain were grown in 100 ml of M9 medium containing 25 mM dextrose, 100 μg/ml ampicillin, and 15 μg/ml chloramphenicol. At a cell density of 3.5 × 108 cells/ml, isopropyl-thiol-β-D-galactopyranoside was added to 0.4 mM, growth continued for 90 min at 37 °C, and then rifampicin was added to 200 μg/ml. Incubation was continued for 30 min, and then 100 μ C of [35S]methionine were added for 15 min. Cells were harvested by centrifugation at 7,500 × g for 15 min, rinsed with M9, and stored as a pellet until use at −80 °C.

For the purification of FadR, the pellet from 1 liter of culture (100-150 mg total protein) was thawed on ice in 50 ml of TNE buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol). All remaining steps were done at 4 °C except the ion-exchange chromatography which was run at room temperature (21 °C). Cells were lysed by sonication with a Virtis ultrasonic cell disintegrator 10 s on, 10 s off seven times at full power. Following sonication, phenylmethylsulfonyl fluoride was added to 1 mM final concentration. Membranes and cellular debris were removed by centrifugation at 70,000 × g in a Beckman SW28 rotor for 30 min. Solid ammonium sulfate was added to the supernatant to 60% saturation at 4 °C. The protein precipitate was identified by SDS-polyacrylamide gel electrophoresis and frozen at −80 °C. Amino-terminal analysis was performed by the method of Edman and coworkers (16) upon elution at 0.16-0.2 M KCl. Appropriate fractions were pooled, concentrated by ammonium sulfate precipitation as above, resuspended in 6 ml of 20 mM MES, pH 6.0, and dialyzed against 2 liters of the same buffer for 3 h. The sample was applied to a 30-ml CM Bio-Gel A (Bio-Rad) column equilibrated with 20 mM MES, pH 6.0. The column was washed with 100 ml of the same buffer and then eluted with a 500 ml of linear gradient of 0-0.4 M KCl in 20 mM MES, pH 6.0. FadR, eluted between 0.18-0.2 M KCl, containing fractions were pooled, concentrated by ammonium sulfate precipitation, and resuspended in 500 μ l of NTED. The sample was dialyzed against 1 liter of the same buffer for 2 h using a Bethesda Research Laboratories model 1200 MA microdialysis unit. For storage at −20 °C, glycerol was added to the purified FadR sample to 30% (v/v).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins was performed using the technique of Laemmli (17) in a Mini-Protein II dual slab cell system (Bio-Rad). Protein bands were stained with the Bradford reagent (17) and scanned using a Bio-Image analyzer (Bio-Rad) system. Protein-DNA gel retention assay was performed using the protein-DNA gel retention assay described below.

Gel Filtration Chromatography—A 2-ml sample containing 75 μ g of final FadR protein and 100,000 cpm specifically labeled as [35S]FadR in TNED and 5% glycerol was applied to a 200 ml, 5 × 40-cm column of P-200 (Bio-Rad) equilibrated in TNE. Elution was carried out with the same buffer at a rate of 14 ml/h. Column fractions (1.75 ml) were monitored for radioactivity by scintillation counting and for DNA binding activity using the protein-DNA gel retention assay described below.

Amino-terminal analysis—A 10-μ g sample of the final FadR preparation after CM Bio-Gel A column chromatography was electrophoresed on a 15% SDS-PAGE and transferred electrophoretically to Immobilon-P transfer membrane (Millipore) as described by Matsusaida (18). FadR was identified after transfer by staining with 1% Ponceau Red, the band was excised, rinsed extensively with cold glass-distilled H2O, and frozen at −20 °C. Amino-terminal analysis was performed using the technique of Laemmli (17) using the Bradford reagent (17) and a Bio-Rad model 1200 microdialysis system. The purified FadR was analyzed using the method of Prescott (19) using a Mini-Protein II dual slab cell system (Bio-Rad). Protein-DNA gel retention assay was performed using the protein-DNA gel retention assay described below.

DNAase I footprinting—To detect FadR binding to the top (non-transcribed) strand of the fadB promoter, a 588-bp PvuII-EcoRI restriction fragment from pCD154 containing the fadB promoter which had been end-labeled using [γ-32P]ATP and the Klenow fragment of DNA polymerase I. An assay mixture of 25 μ l contained, approximately 1 × 107 M DNA (30,000 cpm) was incubated in a 50-μ l reaction mixture containing 20 mM Tris-HCl, pH 7.5, 2.5 mM MgCl2, 1 mM CaCl2, 1 mM DTT, 1 mM EDTA, 1 μ g of poly(dI-dC), 1 μ g of fatty acid free BSA (Sigma), and 5% glycerol. FadR was added to the appropriate concentrations and the mixture was incubated 30 min at room temperature, then the samples were transferred to a 37 °C water bath and incubated for 1 min prior to the addition of 2 μ g of DNAse I. The reaction continued for 15 min and was terminated by the addition of 10 μ g of tRNA, 25 μ l of 7.5 M ammonium acetate, and 125 μ l of 100% ethanol. The samples were placed on dry ice for 5 min, and the DNA was pelleted by centrifugation for 10 min in a Beckman microfuge at 13,000 × g. The pellet was rinsed with 70% ethanol, dried, and resuspended in 4 μ l of TE and 3 μ l of formamide gel-loading solution (14). Samples were denatured at 85 °C for 3 min, placed on ice for 3 min, and 3 μ l were loaded onto an 8% acrylamide gel. The gel was run in 4.5 TBE buffer in a 4% acrylamide gel for 4 h (21). The sample buffer contained 5% glycerol, and 1 μ g of poly(dI-dC) (Sigma), and 1 μ g of fatty acid free BSA (Sigma). The gel was stained with ethidium bromide and photographed under UV light.

Quantitation of DNA Binding Activity—Protein-DNA gel retardation assays (20, 21) (gel shifts) were performed using the 377-bp HindIII-EcoRI restriction fragment containing Pb of pCD154 which had been end-labeled using [γ-32P]ATP and the Klenow fragment of DNA polymerase I. An assay mixture of 25 μ l contained, unless otherwise noted, 20 mM Tris-HCl, pH 7.5, 10 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1 μ g of poly(dI-dC) (Sigma), and approximately
1 × 10^{-12} \text{ M} [\text{35S}]\text{DNA} (2,000 \text{ cpm}). The reaction was initiated by the addition of FadR which had been diluted to the appropriate concentration in TNED just prior to assay. The reaction mixture was incubated at room temperature (22°C) for 30 min, then 20 \mu l was loaded onto a 5% polyacrylamide gel (polyacrylamide/bisacrylamide, 60:1, w/w) containing 5% glycerol and 0.5 × TBE (14). Loading was performed using a Hamilton syringe which was rinsed first with acetone and then with 0.5 × TBE between sample loadings. This step was found to limit variability between experiments when fatty acids or fatty acyl-CoA compounds were being tested for ability to inhibit DNA binding. The gels were prerun 60 min at 10 watts constant power, loaded, and run for 75 min. The gels were dried and autoradiographed (16–20 h exposure). FadR binding was estimated as the conversion of the fast mobility complex (unbound DNA) to slow mobility complex (FadR-DNA bound). Quantitation of autoradiographic images was carried out with a Bio Image computer assisted image analysis system (MilliGen/Biosearch).

The concentration of DNA fragments was determined by comparison to φX174 DNA HaeII restriction fragments displayed on a 5% PAGE (polyacrylamide/bisacrylamide, 29:1, w/w) containing 5% glycerol and 0.5 × TBE. The concentration of φX174 DNA was estimated spectrophotometrically at 260 nm using an LKB UltraspecII assuming an extinction coefficient of 20.5 for 1 mg/ml double-stranded DNA. When end-labeled DNA was used, recovery was assumed to be 100% input DNA since the concentration of labeled DNA was too low to be detected by ethidium bromide staining.

**FadR-Fatty Acyl-CoA Interaction—DNA-protein gel retention assays** were performed as described above except that fatty acids or fatty acyl-CoA were added to the reaction mixture prior to the addition of DNA. Fatty acids were prepared as 1 mM stocks in 95% ethanol and stored at −20°C. Fatty acyl-CoA compounds were prepared as 100 \mu M stocks in 100 mM Tris-HCl, pH 8.0, and stored at −20°C. Fatty acids or fatty acyl-CoA were diluted in 100 mM Tris-HCl, pH 8.0, just prior to use in assays. The concentration of CoA esters was determined by absorbance at 257 nm with an extinction coefficient of 15.7 mM cm^{-1} after Tippett and Neet (22). The extent of FadR binding inhibition was estimated as the conversion of the slow mobility complex (FadR-DNA bound) to the fast mobility complex (unbound DNA). K_i was defined as that concentration of fatty acid or fatty acyl-CoA which caused 50% of the DNA to be in the fast or slow complexes.

**RESULTS**

**Isolation of FadR**—FadR was isolated from cells over-expressing the protein using the T7 expression system of Studier and Moffat (15). The conditions for purification were established by monitoring FadR specifically labeled by the incorporation of [35S]methionine as described under “Materials and Methods.” These conditions allowed monitoring of FadR purification both by scintillation counting and by monitoring the appearance of the protein on 15% SDS-PAGE stained with Coomassie Blue or as detected by autoradiography. Using the T7 expression system, we estimated that 2–10% of the total cellular protein was FadR. FadR remained in the soluble fraction after a 30-min centrifugation at 75,000 × g and did not appear to be associated with inclusion bodies. There was no apparent degradation of the protein during the 2-h induction period as evaluated using a 1-min pulse of [35S]methionine followed by a 2-h chase with 0.01% methionine (data not shown).

FadR was purified to greater than 97% homogeneity using two ion-exchange columns. A typical protein profile is given in Fig. 1. A 15-fold enrichment for FadR was achieved by passage over DEAE-cellulose using 20 mM Tris-HCl, pH 8.0, and a linear gradient of 0–0.5 M KCl. As shown in Fig. 1, FadR eluted under these conditions at 0.16–0.2 M KCl. There were a substantial number of proteins which coeluted with FadR from this column which were eliminated by passage of the partially pure preparation over a CM-Bio-Gel A column using 20 mM MES, pH 6.0, and linear gradient of 0–0.4 M KCl. FadR eluted from the CM-Bio-Gel A column between 0.18–0.21 M KCl. A few minor bands in addition to FadR were visible in some preparations on staining an SDS-PAGE with Coomassie Blue. Only FadR preparations which contained less than 3% contaminating proteins were used for the experiments reported here. The purified protein was stored in TNED containing 50% glycerol at −20°C.

**Protein Analysis**—The amino-terminal sequence was determined using an ABI 470A Protein Sequencer to be Val-Ile-Lys-Ala-Gln-Ser-Pro-Ala-Gly-Phe. It appears that the initiator methionine was cleaved from the mature protein. The subunit composition of FadR was estimated by gel filtration chromatography using 35S-labeled FadR. The calculated molecular weight of the protein predicted from DNA sequence analysis and estimated by SDS-PAGE is 26,972 (6, 13). The protein eluted from a P200 column as a monomer with an apparent native molecular weight of 27,000. A typical elution profile appears in Fig. 2.

**Localization of the FadR-binding Site on fadB DNA**—The E. coli fadBA operon encodes two proteins of 78,000 and...
42,000 Da, respectively, which form a complex in an αβ2 conformation. The operon is inducible by growth of wild type cells in minimal medium containing long chain fatty acids and is repressed by fadR. The fadBA operon has been sequenced (23). In this work we have localized the transcription initiation site by primer extension analysis to be 42 base pairs from the start of translation (Fig. 3). The same initiation site was identified whether the RNA used for the primer extension was isolated from cells grown in TB (noninducing medium) or TB containing oleate (inducing medium), and only the intensity of the band differed (data not shown). The promoter region of fadBA was cloned on a 326-bp HaeIII restriction fragment into pUC19 (Fig. 4) for use in DNase I footprinting and in protein-DNA gel retention assays (also known as gel shifts). By enzymatic cleavage of the subclones with the restriction endonuclease EcoRI or HindIII within the polylinker of pUC19 followed by end-labeling, we were able to detect FadR binding to the top (non-transcribed) or bottom (transcribed) strands, respectively, as shown in Fig. 5, A and B. FadR binds to a single site on this fragment of fadb DNA which was localized on the top strand from +1 to +16 base pairs relative to the start of transcription. The binding of FadR at this position would be expected to repress fadBA transcription. The sequence of the FadR-binding site is 5′ ATCTGGTGACAGGACGAT 3′ which has perfect dyad symmetry about the central ACG residues as underlined. Binding of FadR to the bottom strand gave a broader footprint which included two additional nucleotides on either end of the region covered on the top strand (Figs. 4 and 5, A and B). This extends the symmetry noted above by one pair: 5′ TCTCTGGTAGCAGGACGATCA 3′.

Binding Affinity of FadR for FadB DNA—FadR binding to the 377-bp HindIII-EcoRI DNA fragment containing the fadb promoter (designated Pe) was easily detected by gel shift assays using crude extracts or highly purified protein preparations (see activity and purification values in Table I). A single shift was detected suggesting there was a single binding site for FadR within Pe as confirmed by DNase I footprinting described above.

The final preparation of FadR was at least 50% active in DNA binding as estimated by titrating FadR against 1 × 10^{-6} M Pe (specific activity 2,000 cpm/1 × 10^{-8} M Pe). Activity was stable for at least 3 months at −20℃. Binding of FadR to Pe was at equilibrium under our standard reaction conditions. There was no detectable difference in reactions incubated for 1 min prior to gel loading from those incubated for

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**Fig. 3. Transcript mapping of fadb by primer extension.** An fadb-specific oligonucleotide was 5′ end-labeled, hybridized to E. coli in vivo synthesized mRNA, and extended using reverse transcriptase. The product of the extension reaction is in lane 1. The product of dideoxy sequencing reactions using the same oligonucleotide as primer and an M13 fadb derivative are labeled G, A, T, and C. The sequence for the DNA region around the start site is given to the left.

**Fig. 4. Nucleotide sequence of the HaeIII restriction fragment carrying the fadB promoter used in this study.** The 326-base pair HaeIII fragment was cloned into the Smal site of pUC19 as shown to generate pCD154. Also noted are the PvuII sites within pUC19 used to excise the fragment for end-labeling. The EcoRI to HindIII fragment from pCD154 labeled at the EcoRI end. Lane 2, no FadR; lane 3, 0.1 nM; lane 4, 0.5 nM; lane 5, 5 nM; lane 6, 50 nM FadR. The sequence of MD 16 from the same end is to the right and labeled G, A, T, C, B, Digestion pattern of the coding strand of the EcoRI to HindIII fragment from pCD154 labeled at the EcoRI end. Lane 1, no FadR; lane 2, 0.1 nM; lane 3, 0.5 nM; lane 4, 5 nM; lane 5, 50 nM FadR. The sequence of MD 16 from the same end is to the right and labeled G, A, T, C, B, Digestion pattern of the coding strand of the EcoRI to HindIII fragment from pCD154 labeled at the HindIII end. Lane 1, 50 nM FadR; lane 2, 5 nM; lane 3, 2.5 nM; lane 4, 1 nM; and, lane 5, no FadR. The position of the bands relative to the start of fadb transcription are as shown to the right of the autoradiogram. The region of dyad symmetry noted within the footprints are marked by lines next to the sequence.
Characterization of FadR

### TABLE I

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<th>Purification of FadR</th>
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<tr>
<td>Fraction</td>
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<tr>
<td>Sonicate</td>
</tr>
<tr>
<td>(NH4)2SO4, 0–60% dialyzed</td>
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<tr>
<td>DEAE-cellulose eluate</td>
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* One unit is defined as amount of protein in sample required for a 50% shift of the fast mobility complex to the slow mobility complex.

90 min. The reaction was insensitive to changes in pH in the range of 5.5 to 8.5 and to the addition of salt (NaCl or KCl) up to 200 mM (data not shown). Inclusion of 1 μg of BSA in the reaction mixture had a slightly positive affect on DNA binding; however, we chose not to include it in our standard gel shift assays because BSA is a fatty acid-binding protein and might interfere with our inducer studies. Under our standard assay conditions, binding of FadR to 1 × 10^{-12} M P_B was linear from approximately 1 × 10^{-10} M to 8 × 10^{-10} M with an apparent K_m of 3 × 10^{-10} M (Fig. 6). Binding of 1 × 10^{-7} M FadR to [P]EcoRI DNA was specifically inhibited by the addition of unlabeled DNA fragment containing P_f, above 1 × 10^{-11} M while the addition of poly(dI-dC) or yeast tRNA did not affect binding.

**Fatty Acyl-CoA Inhibition of FadR-DNA Binding**—The induction of FadR-responsive genes and operons occurs upon growth of *E. coli* in minimal medium containing long chain (C_{12-18}) but not medium chain (C_{6-10}) fatty acids. This has led to the suggestion that long chain fatty acids or a long chain fatty acid derivative interacts with FadR to prevent binding to DNA. We used the gel shift assays to survey a set of compounds which are intermediates in fatty acid and lipid metabolism for their ability to inhibit binding of FadR to P_f. These included oleic acid, palmitic acid, myristic acid, decanoic acid, oleoyl-CoA, palmitoyl-CoA, myristoyl-CoA, decanoyl-CoA, crotonoyl-CoA, acetyl-CoA, and coenzyme A. In these experiments the reaction mixture contained, in addition to the test compound, 1 × 10^{-12} M [P]EcoRI DNA and 1 × 10^{-9} M FadR (to give 95–100% of the labeled DNA in the slow mobility complex). Care was taken to use concentrations below the critical micellar concentration for each compound (24). An autoradiogram of a gel shift in which each of the compounds was tested at a final concentration of 1 μM is given in Fig. 7. The long chain fatty-acyl CoA compounds oleoyl-CoA, palmitoyl-CoA, and palmitoleoyl-CoA clearly inhibited FadR-DNA binding while long chain fatty acids, crotonoyl-CoA, acetyl-CoA, and free coenzyme A had no effect at a final concentration of 1 μM. The apparent affect of oleic acid in preventing FadR-DNA binding at 1 μM seen in Fig. 7 was not reproducible. Note for comparison Fig. 8 in which palmitic acid was added in a similar experiment at 0.1–5 μM. By performing titrations of each long chain fatty acyl-CoA using the gel shift assay, we were able to estimate a K_i for each 5 nm oleoyl-CoA (C_{18:1}), 5 nm palmitoyl-CoA (C_{16:0}), 5 nm palmitoleoyl-CoA (C_{16:1}), 250 nm myristoyl-CoA (C_{14:0}), and 2 μM decanoyl-CoA. The inhibition of FadR-P_f binding by the fatty acyl-CoA compounds was chain length dependent with C_{18} and C_{16} > C_{14} > C_{10}. There was no detectable difference between the saturated CoA compound palmitoyl-CoA (C_{16:0}) and the unsaturated CoA compound with the same chain length, palmitoleoyl-CoA (C_{16:1}). At high concentrations (2–5 μM) of fatty acids, we did detect some inhibition of formation of the slow mobility complex. We suspect this was due to nonspecific detergent affects since the inhibition of binding was not chain length specific, and the detergents Nonidet P-40 and Brij 58 also inhibited formation of the FadR-DNA complex by at least 50% at 5 μM. In contrast, acetyl-CoA and coenzyme A had no affect up to a final concentration of 50 μM. Our results are consistent with earlier suggestions that long chain acyl-CoA molecules are the inducer molecules of the fad genes and operons (3, 25).

**DISCUSSION**

FadR is an important mediator in the maintenance of balanced levels of fatty acid synthesis and degradation in *E. coli*. In this study we have initiated a molecular analysis of FadR control of the fadBA promoter. The fadBA operon...
encodes two subunits of a multifunctional protein with at least five enzyme activities required for the β-oxidation of fatty acids (26). We chose to study FadR interaction with the promoter of this operon because it is well characterized biochemically and genetically. The proteins of the multienzyme complex were initially suggested to compose an operon due in part to their coordinate induction after growth of wild type *E. coli* on minimal medium containing long chain fatty acids (3). Previously, we have reported that the coding sequences of the *fadB* and *fadA* genes are separated by only 10 base pairs supporting the conclusion that *fadBA* forms an operon (23). In this work, we have mapped the transcription start site of *fadBA*, identified the FadR operator, and preliminarily characterized FadR binding to this site.

We report here the purification of the *fadR* gene product to near homogeneity and the *in vitro* characterization of this important regulatory protein. Our results confirm earlier work predicting that FadR mediates the control of gene expression by directly binding to DNA. We have shown that FadR binds to a single site, 5’-ATCTGGTACGACCAGAT-3’, +1 to +17 relative to the start of transcription of the *fadBA* operon. Binding of FadR at this position would be expected to cause repression of *fadB* and *fadA* gene expression. We have estimated the native molecular weight of purified FadR to be 29,000 using gel filtration chromatography. This apparent molecular weight is equivalent to the monomeric form of the protein. This result was unexpected since the FadR-binding site has nearly perfect dyad symmetry as is common for regulatory proteins which bind to DNA as dimers or tetramers. A similar result was obtained, however, for purified OmpR (27). We have obtained negative trans-dominant mutations in FadR which would argue that the protein functions in multimeric form. At this time we cannot reconcile our biochemical and genetic data. It is possible the dimer form is the active form and yet was undetected in our gel filtration experiments.

The standard protein-DNA gel retention procedure was used to estimate the affinity of FadR binding to DNA restriction fragments containing O1 (20, 21). Under our standard assay conditions, in the absence of BSA, the apparent binding affinity of FadR to DNA containing O1 is tolerant to changes in pH but decreases with salt concentrations above 200 mM indicating protein-DNA binding involves ionic interactions. Similar sensitivity to ion concentrations has previously been noted for other DNA-binding proteins (28). Our standard binding reactions were performed at low salt concentrations (10 mM) primarily because the critical micellar concentrations of the fatty acyl-CoA compounds decreases substantially in 200 mM salt (24). This was an important consideration for our inducer studies discussed below.

Binding of FadR to DNA containing the promoter of *fadB* is prevented by the addition of long chain fatty acyl-CoA compounds. The *K* for palmitoyl-CoA is approximately 5 mM which is a reasonable range for a physiological affect. The *K* for decanoyl-CoA is estimated to be above 1 μM and is not expected to be physiologically relevant. This work is the first direct evidence that long chain fatty acyl-CoAs mediate induction by interacting directly with FadR. Previous work had suggested that long chain fatty acyl-CoAs were the inducer molecules based on the observation that *fadD* mutants which are defective in acyl-CoA synthetase are noninducible (25). However, this could be an indirect result since *fadD* strains are deficient in long chain fatty acid transport (1). Overath also argued that the chain length specificity of induction was due to the substrate length specificity of the acyl-CoA synthetase (25). However, Kameda and Nunn (29) purified the *E. coli* acyl-CoA synthetase and showed that a single enzyme had broad chain length specificity. Our evidence supports the conclusion that induction of *fad* gene expression results from the binding of long chain acyl-CoAs to FadR to prevent DNA binding and is not simply due to substrate specificity of the transport system and acyl-CoA synthetase. Inhibition of O1-FadR binding by acyl-CoA compounds using the gel shift assays has prompted us to suggest that these compounds bind to FadR. However, the inhibition of DNA binding using the gel shift assay is an indirect test of FadR-acyl-CoA interaction. Therefore, we plan to evaluate direct binding of fatty acyl-CoAs to the purified protein in future work.

The binding site for FadR within the *fadB* promoter can be used to predict putative FadR-binding sites within other FadR-responsive genes. Three which have been sequenced include: *fadL*, which encodes an outer membrane protein required for long chain fatty acid binding and transport (30); aceBAK which encodes enzymes of the glyoxylate shunt (31); and *fabA* which encodes β-OH-decanoyl thioester dehydrase (32). *fadL* and *aceBAK* are predicted to be negatively controlled by FadR (1) while *fabA* is predicted to be activated by FadR (2). Table II lists putative binding sites, identified by sequence comparison to O1, within *fadL* and *fabA*. We were not able to identify by sequence comparison to the *fadB* operator a FadR-binding site within the aceBAK promoter or aceB amino terminus, operator positions which are typical of a repressor (33). For the *fadL* promoter (30) two operators can be predicted, *fadL* O1 and *fadL* O2, as listed in Table II. *fadL* O1 has 11, *fadL* O2 7, and *fabA* 10 out of 17 bases in common with the O1. The most highly conserved positions are 3 through 14 relative to the central base pair of the O1.

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**Table II**

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<tr>
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<td>2</td>
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</table>

*Position relative to the start of transcription.

*A base was included in the consensus if it appeared at least three out of four times in the sequences.

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C. C. DiRusso, unpublished observations.
(Table III). The location of these putative operators relative to the transcription start site for these genes is consistent with the role of FadR as a repressor of fadL gene expression and activator of fabA expression (33). Currently, in vitro studies are being conducted to determine the similarities and differences in FadR-mediated control of fadL, fabA, and fadBA, genes which function at several critical steps in fatty metabolism.

Recently, Haydon and Guest (34) placed FadR in a distinct family of bacterial regulatory proteins which includes Bacillus subtilis GntR and Pseudomonas putida HutC. Each of the proteins possesses a predicted helix-turn-helix motif and 69-residue amino terminus which is more homologous within the group of proteins compared than with the αCro-like proteins (34). The operators for GntR and HutC share identical nucleotides at 11 of 14 positions: 5’ CTTGTATANANTTA 3’ (where N is nonidentity between the sequences) (34). The FadR operator in fadB shares 6 of 14 of these sequence identities: 5’ CTNNTANNNNNNA 3’. Much more information regarding the specific amino acid base pair contacts between these regulators and their operator sites will be required to evaluate what the significance of these sequence alignments is (if any) in operator recognition and binding.

REFERENCES
Characterization of FadR, a global transcriptional regulator of fatty acid metabolism in Escherichia coli. Interaction with the fadB promoter is prevented by long chain fatty acyl coenzyme A.

C C DiRusso, T L Heimert and A K Metzger