Crosstalk of *Escherichia coli* FadR with Global Regulators in Expression of Fatty Acid Transport Genes

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Abstract

*Escherichia coli* FadR plays two regulatory roles in fatty acid metabolism. FadR represses the fatty acid degradation (*fad*) system and activates the unsaturated fatty acid synthetic pathway. Cross-talk between *E. coli* FadR and the ArcA-ArcB oxygen-responsive two-component system was observed that resulted in diverse regulation of certain *fad* regulon β-oxidation genes. We have extended such analyses to the *fadL* and *fadD* genes, the protein products of which are required for long chain fatty acid transport and have also studied the role of a third global regulator, the CRP-cAMP complex. The promoters of both the *fadL* and *fadD* genes contain two experimentally validated FadR-binding sites plus binding sites for ArcA and CRP-cAMP. Despite the presence of dual binding sites FadR only modestly regulates expression of these genes, indicating that the number of binding sites does not determine regulatory strength. We report complementary *in vitro* and *in vivo* studies indicating that the CRP-cAMP complex directly activates expression of *fadL* and *fadD* as well as the β-oxidation gene, *fadH*. The physiological relevance of the *fadL* and *fadD* transcription data was validated by direct assays of long chain fatty acid transport.

Introduction

Much of our current knowledge of bacterial fatty acid metabolism comes from studies with *Escherichia coli* [1]. The fatty acid degradation (*fad*) pathway is primarily responsible for the transport, activation and β-oxidation of fatty acids [2]. The known long-chain fatty acid (LCFA) transport system components are the outer-membrane transport protein, FadL [3,4] and the inner-membrane associated acyl-CoA synthetase, FadD [5,6]. In this system, the FadL transporter delivers exogenous long chain fatty acids across the cell membrane into the periplasmic space [3,4]. From there by an unknown mechanism the LCFA enter the cytosol where they become activated to their acyl-CoA thioesters by FadD which allows entry of the acyl chains into the β-oxidation cycle [5,6]. *E. coli* FadR coordinates the catabolic and anabolic fatty acid pathways (Fig. 1). In this dual role FadR acts as a repressor for the entire set of *fad* regulon genes and also functions as an activator for unsaturated fatty acid biosynthesis pathway by increasing transcription of both *fabA* [7,8] and *fabB* [9] (Fig. 1). The physiological ligands that antagonize FadR binding to its cognate operators are LCFA CoA thioesters synthesized by the cytosolic acyl-CoA synthetase, FadD [7,10]. In strains lacking either FadD or FadL addition of LCFA fails to induce *fad* regulon expression because the regulatory ligand cannot be synthesized. Therefore, extremely stringent regulation of *fadD* and *fadL* genes is precluded because this would block derepression. Similar to most of the other *fad* regulon genes, *fadL* [4,11,12] and *fadD* [12,13] are controlled at the transcriptional level by two different regulatory systems in addition to FadR, the oxygen-sensitive ArcA-ArcB two-component system and the cyclic AMP (cAMP) receptor protein-cyclic AMP (CRP-cAMP) complex. Our interest in *fadL* and *fadD* arose from the presence of two FadR-binding sites in the promoters of both genes whereas the other FadR regulon *fad* genes each have only a single site. This raised the question of regulatory interactions among the multiple regulators, FadR, ArcA and CRP-cAMP when bound to the respective promoter regions. Cho and coworkers [12] initially used quantitative RT-PCR to assay regulation of *fadL* and *fadD* by FadR and ArcA under anaerobic conditions and reported that deletion of either arcA, *fadR* alone, or both arcA and *fadR* resulted in increased *fadL* expression by 34-, 4- or 69-fold, respectively, whereas *fadD* transcription increased 69-, 4.5-, and 84-fold. These workers did not investigate the effects of CRP-cAMP. Classical catabolite repression of the *fad* pathway has long been known. Pauli and coworkers [14] reported that Fad enzyme levels were very low in wild type cells grown in glucose even in the presence of inducing levels of fatty acid (or in constitutive *fad* mutant strains) and that glucose inhibition could be partially relieved by addition of cAMP. CRP mutant strains also had low Fad enzyme activities. In this laboratory Clark [15] using early lacZ fusion technology showed that glucose acted at the transcriptional level in *fadB* and *fadE* expression. Although as discussed below CRP-cAMP binding sites for several *fad* genes have been proposed, none had been directly tested for the ability to bind the complex. Therefore, it remained possible that CRP-cAMP regulation of the *fad* regulon was an indirect effect.
We report experiments defining the interactions of these regulatory proteins and their effects on fatty acid transport.

Materials and Methods

Bacterial strains and growth conditions

All the strains used here are E. coli K-12 derivatives (Table 1). The bacterial media were used for bacterial growth and analyses of β-galactosidase activity. These included LB medium (Luria-Bertani medium containing 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per liter), Rich broth (RB medium containing 10 g of tryptone, 1 g of yeast extract, and 5 g of NaCl per liter) and minimal medium M9 [16] supplemented with 0.4% glucose or other carbon source, 0.1% Vitamin-Free Casamino Acids, 0.1 mM CaCl₂, and 0.001% thiamine. When necessary, antibiotics were used as follows (in mg/liter): sodium ampicillin, 100; sodium chloramphenicol, 50; and kanamycin, 15. To monitor transcriptional regulation by ArcA-ArcB, an oltc promoter lacZ fusion strain JW2341-1 (Table 1) with expression of the FLP recombinase encoded by plasmid pCP20 (Table 1) to give strain FY103, which retained a single FLP recombinase target (FR) site. The FR site was used for site-specific integration of the lacZ fusion plasmid pKG37 (an improved version of pCE71) containing a FRT site upstream of a promoterless lacZ gene, a kanamycin resistance gene, and the R6K origin of replication (Table 1) [19]. The transformants were screened on LB agar plates containing kanamycin and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) at 37°C to obtain the chromosomal fadL-lacZ fusion strain FY104. The fusion plasmid was stably integrated due to its R6K origin and loss of the temperature-sensitive pCP20 plasmid [19]. The fadL promoter-lacZ fusion was validated by a PCR using primers, fadL-P plus lac-Z-R (Table 2).

P1vir phage transduction

P1vir transductions were carried out as described by Miller [20] with minor modifications. Strains FY133, FY134, FY141, FY142, FY169 and FY170 were generated by transduction of strains FY159 (ΔarcA), FY60-2 (Δcrp), FY193 (ΔarcA ΔfadR::Tn10), FY140 (Δcrp ΔfadR::Tn10), FY77 (ΔarcA ΔfadR::Tn10), and FY78 (Δcrp ΔarcA ΔfadR::Tn10), respectively (Table 1) with a P1vir lysate grown on FY104 (fadL-lacZ) with selection for kanamycin resistance. Similarly, a P1vir lysate grown on FY159 (fadD-lacZ) was used for transduction of the following strains FY57, FY59, FY60-2, FY139, FY140, FY177 and FY60 with selection for kanamycin resistance to give strains FY161, FY162, FY163, FY164, FY165, FY166 and FY167, respectively (Table 1). Transduction of strains FY104, FY59 and FY60-2 with a P1vir lysate grown on FY104 (fadD-lacZ) with selection for tetracycline resistance gave strains FY105 (ΔfadR::Tn10, fadL-lacZ), FY139 (ΔarcA ΔfadR::Tn10) and FY140 (Δcrp ΔfadR::Tn10), respectively.

β-Galactosidase assays

Mid-log phase cultures in LB, RB or minimal media (with or without supplementation with various carbon sources), were collected by centrifugation, washed twice with Z Buffer [21] and assayed for β-galactosidase activity after lysis with sodium dodecyl sulfate-chloroform [21]. The data were recorded in triplicate with no less than three independent experiments.

Protein expression and purification

Hexahistidine-tagged E. coli FadR (and/or CRP) proteins were produced in E. coli BL21 (DE3) carrying the expression plasmid pET28-fadRec (and/or pET28-crp) (Table 1) by induction of bacterial cultures at an OD₆₀₀ₐ₅₀ of 0.8–1.0 with 0.5 mM IPTG at 30°C for 3 h [17,22]. The cells were pelleted washed twice with ice cold PBS buffer (101.4 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl, 8% glycerol, pH 7.4), dissolved in the same buffer and lysed using a French pressure cell. The extracts were centrifuged to remove bacterial debris and the supernatants loaded onto a nickel chelate column (Qiagen). Following washing with ten column volumes of with PBS buffer containing 50 mM imidazole, the FadR proteins were eluted with 150 mM imidazole. Appropriate eluted protein fractions were pooled and dialyzed against PBS buffer then concentrated by chemical cross-linking as described previously [18].
Table 1. Strains and plasmids used in this study.

| Bacteria or plasmids | Relevant characteristics | References/sources |
|----------------------|--------------------------|--------------------|
| **E. coli strains**   |                          |                    |
| Topo10               | F<sup>−</sup>, ΔlacX74, a cloning host for recombinant plasmids | Invitrogen         |
| BL21(DE3)            | Protein expression host  | Lab collection     |
| MG1655               | Wild type of E. coli K-12| CGSC<sup>c</sup>, Lab collection |
| MC4100               | F<sup>−</sup>, araD139, Λ(lacF-lac)169 | [25] |
| DH5α (λ-pir)         | Δlac host for pAH125 and its derivatives | [25,37] |
| MC1061               | Wild type of E. coli K-12, Δlac | [23] |
| MF8                  | UB1005, fadR::Tn10 | [7] |
| BW25113              | A wild type strain of E. coli K-12 | CGSC<sup>c</sup>, [38] |
| JW2341-1             | F<sup>−</sup> Δ(araD-araB)567 Δlac2478Δ(mnb-3) LAM- ΔfadL752::kan rph-1 Δ (rhaD-rhaB) 568 hsdR514 | CGSC<sup>c</sup>, [38] |
| JW1794-1             | F<sup>−</sup> Δ(araD-araB)567 Δlac2478Δ(mnb-3) LAM- ΔfadD730::kan rph-1 Δ (rhaD-rhaB) 568 hsdR514 | CGSC<sup>c</sup>, [38] |
| FYJ35                | fadH-lacZ transcriptional fusion | [17] |
| FYJ56                | fadH-lacZ transcriptional fusion, ΔfadR::Tn10 | [17] |
| FYJ57                | JW1176-1, ΔfadR | [17] |
| FYJ59                | JW4361-1, ΔarcA | [17] |
| FYJ60-2              | JW5702-4, Δcrp | [17] |
| FYJ65                | fadH-lacZ transcription fusion, Δcrp | [17] |
| FYJ76                | FYJ60-2, Δcrp ΔarcAΔ726Δkan | [17] |
| FYJ77                | FYJ76, ΔarcA Δcrp | [17] |
| FYJ78                | FYJ77, ΔarcA Δcrp ΔfadR::Tn10 | [17] |
| FYJ82                | fadaBA-lacZ transcriptional fusion, ΔarcA | [17] |
| FYJ83                | fadaBA-lacZ transcriptional fusion, Δcrp | [17] |
| SI203                | fadaBA-lacZ transcriptional fusion | [17,39] |
| SI207                | fadaBA-lacZ transcriptional fusion, ΔfadR::Tn10 | [17,39] |
| FYJ103               | JW2341-1 carrying pCP20ts, ΛfadB | This work |
| FYJ104               | FYJ103, fadD-lacZ transcriptional fusion | This work |
| FYJ105               | FYJ104, ΔfadR::Tn10, fadD-lacZ transcription fusion | P<sub>lte</sub>(MFH8)×FYJ104<sup>6</sup> |
| FYJ118               | MC1061, Δfad::kan | This work |
| FYJ119               | FYJ118 carrying pCP20, ΔfadB | This work |
| FYJ122               | JW1116-1 carrying pCP20ts | This work |
| FYJ133               | FYJ59, ΔarcA, fadD-lacZ transcriptional fusion | P<sub>inv</sub>(FYJ104)×FYJ59<sup>6</sup> |
| FYJ134               | FYJ60-2, Δcrp, fadD-lacZ transcriptional fusion | P<sub>inv</sub>(FYJ104)×FYJ60-2<sup>6</sup> |
| FYJ139               | FYJ59, ΔarcA ΔfadR::Tn10 | P<sub>inv</sub>(MFH8)×FYJ59<sup>9</sup> |
| FYJ140               | FYJ60-2, Δcrp ΔfadR::Tn10 | P<sub>inv</sub>(MFH8)×FYJ60-2<sup>9</sup> |
| FYJ141               | FYJ139, ΔarcA ΔfadR::Tn10, fadD-lacZ transcriptional fusion | P<sub>inv</sub>(FYJ104)×FYJ139<sup>5</sup> |
| FYJ142               | FYJ140, Δcrp ΔfadR::Tn10, fadD-lacZ transcriptional fusion | P<sub>inv</sub>(FYJ104)×FYJ140<sup>5</sup> |
| FYJ158               | DH5α (λ-pir) carrying pAH-PfadD | [22] |
| FYJ159               | MC4100 with fadD-lacZ transcriptional fusion at the chromosomal att<sub>B</sub> site. | [22] |
| FYJ161               | FYJ57, ΔfadR, fadD-lacZ transcriptional fusion | P<sub>inv</sub>(FYJ159)×FYJ57<sup>9</sup> |
| FYJ162               | FYJ59, ΔarcA, fadD-lacZ transcriptional fusion | P<sub>inv</sub>(FYJ159)×FYJ59<sup>9</sup> |
| FYJ163               | FYJ60-2, Δcrp, fadD-lacZ transcription fusion | P<sub>inv</sub>(FYJ159)×FYJ60-2<sup>2</sup> |
| FYJ164               | FYJ139, ΔarcA ΔfadR::Tn10, fadD-lacZ transcriptional fusion | P<sub>inv</sub>(FYJ159)×FYJ139<sup>9</sup> |
| FYJ165               | FYJ140, Δcrp ΔfadR::Tn10, fadD-lacZ transcriptional fusion | P<sub>inv</sub>(FYJ159)×FYJ140<sup>5</sup> |
| FYJ166               | FYJ77, Δcrp ΔarcA, fadD-lacZ transcription fusion | P<sub>inv</sub>(FYJ159)×FYJ77<sup>9</sup> |
| FYJ167               | FYJ78, Δcrp ΔarcA ΔfadR::Tn10, fadD-lacZ transcription fusion | P<sub>inv</sub>(FYJ159)×FYJ78<sup>9</sup> |
| FYJ169               | FYJ77, Δcrp ΔarcA, fadD-lacZ transcriptional fusion | P<sub>inv</sub>(FYJ104)×FYJ77<sup>9</sup> |
| FYJ170               | FYJ78, Δcrp ΔarcA ΔfadR::Tn10, fadD-lacZ transcriptional fusion | P<sub>inv</sub>(FYJ104)×FYJ78<sup>9</sup> |
| FYJ187               | MC4100 carrying pINT-ts | [22] |
| FYJ238               | Topo 10 carrying pET28-crp | This work |
| FYJ239               | BL21(DE3) carrying pET28-crp | This work |
| FYJ294               | DH5α (λ-pir) carrying pAH-PfadD | This work |
### Table 1. Cont.

| Bacteria or plasmids | Relevant characteristics | References/sources |
|----------------------|--------------------------|--------------------|
| FYJ295               | MC4100 with fadL-lacZ transcriprional fusion at the chromosomal attB site. | This work |
| **Plasmids**         |                          |                    |
| pET28(a)             | T7 promoter expression KmR vector | Novagen |
| pKD46                | bla P_{Km} gam pSC101, oriTS, AmpR | [40] |
| pKD13                | bla FRT ahp FRT PS1 PS4 oriR6K, KmR | [40] |
| pCP20                | bla cat cI857 s_{P_{f1}} f1 oriTS, AmpR, Cm | [19,41] |
| pKG137               | Transcription fusion plasmid, ahp FRT lacZY+ t_{mp} oriR6K, KmR | [19,40] |
| pINT-ts              | Temperature sensitive helper plasmid expressing Int_{mp}, AmpR | [37] |
| pAH125               | A promoter-less lacZ reporter plasmid in E. coli, KanR | [25,37] |
| pAH-PfadD            | pAH125 carrying fadD promoter region, KanR | [22] |
| pAH-PfadL            | pAH125 carrying fadL promoter region, KanR | This work |
| pET28-fadRec         | pET28 carrying wild type fadR | [23,25] |
| pET28-crp            | pET28 encoding CRP | This work |

*CGSC denotes Coli Genetic Stock Center, Yale University.

*Selection for tetracycline resistance.

*Selection for kanamycin resistance.

**Table 2. Primers used in this study.**

| Primers                | Primer sequences (5’-3’) |
|------------------------|-------------------------|
| fadL-PF                | CGT TGA TTT CCT TGT TAT GTG C |
| lacZ-R                 | GAC CAT GAT TAC GGA TCC ACT G |
| fadL-promoter-F (Sall) | CCG GTGAC GTT TGA TTT CCT TGT TAT GTG |
| fadL-promoter-R (EcoRI) | AACCC GAAATC GCG AGA GCA GAC TTT GTA AAC |
| fadD-promoter-F (Sall) | CCG GTGAC GTT GGG GTA CAA AAC CAG CA |
| fadD-promoter-R (EcoRI) | AACC GAAATC GTC TAA AAT GCG TGG TCG TCG |
| crp-F1 (BamHI)         | CG GAATTC ATG GGG CGG AAA CCG CA |
| crp-R1 (XhoI)          | CCG CGGTAG TTA AGG AGT GCC GTA AAC GAC |
| fadL-FadR-site1-F      | GCA ACA TTC CAG CTG GTC CGA CTT ATA CTC TCG CC |
| fadL-FadR-site1-R      | GGC GAG AGT ATA GGT CGG ACC AGC TGG AAT GTT GC |
| fadL-FadR-site2-F      | CTC TCG CCA CTG GTC TGA TTT CTA AGA TGG ACC TC |
| fadL-FadR-site2-R      | GAG GTA CAT TTT AGA AAT CAG ACC AGG GGC GAG AG |
| fadD-FadR-site1-F      | GTA ATT ATC AAG CTG GTC TAA TGA GGT AAT ATT ATG |
| fadD-FadR-site1-R      | CAT AAT ATT AAC TCA TCA TAC CAG ATT GAT AAT TAC |
| fadD-FadR-site2-F      | GAA ACA GGC GGT GGT CCG CTG TTT CTG CAT TCT |
| fadD-FadR-site2-R      | AGA ATG CAG AAA CAG CGG ACC AGC GGC TGT TTC |
| fadD-CRP-site-F        | GTA AAG ATA AAA ATA AAT AGT GAC GGG CTT CGC AAT CTT TTC GTT GGG |
| fadD-CRP-site-R        | CCC AAC GAA AAG GGT GGG AAG AGG GGC GTC ACT ATT TAT TTT TAT CTT TAC |
| fadL-CRP-site1-F       | CTG CAA AAT CCG ATA AGT GAC GCA AAT CAC ACT TAA AAA TGA TCT |
| fadL-CRP-site1-R       | AGA TCA TTT TTA AGT GTG ATT TGC TGT ACT TAT CGG ATT TGG CAG |
| fadL-CRP-site2-F       | CCC TAC ACT TCG CGG TCT TCG TCT TAC ACG TAA CAT ATG TGG TAT AAA AAT AAA TC |
| fadL-CRP-site2-R       | GAT TTA TTA TAC AAA CTA TGT TAC GTG TTA CAG GAG GCA GAT TAG GG |
| fadH-CRP-site1-F       | GTA ACC TGG ATA AAA CCG GAC AAG CGT CTC ATG CCT GAT CMA CCG |
| fadH-CRP-site1-R       | CGG GTG ATA ACA GCG GAT GCG ACG GTC ATT TGC TGT ACT TAT CCA GAT TAC TAC |
| fadH-CRP-site2-F       | CAC CGG GTC TAT TCT TTA AGA ATC CCA TCA AAA ACC CGGCAC TCC |
| fadH-CRP-site2-R       | GGA GTG CGG GTG TTA TGG TAC TAA AAA AGA ATA CAG CC CG GTG |
| T7-F                   | TAA TAC GAC TCA CTA TAG GG |
| T7-R                   | GCT AGT TAT TGC TCA GCG G |

The sequences underlined are restriction sites, and the bold letters are predicted FadR or the CRP-cAMP sites.

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| lacZ-R                 | GAC CAT GAT TAC GGA TCC ACT G |
| fadL-promoter-F (Sall) | CCG GTGAC GTT TGA TTT CCT TGT TAT GTG |
| fadL-promoter-R (EcoRI) | AACCC GAAATC GCG AGA GCA GAC TTT GTA AAC |
| fadD-promoter-F (Sall) | CCG GTGAC GTT GGG GTA CAA AAC CAG CA |
| fadD-promoter-R (EcoRI) | AACC GAAATC GTC TAA AAT GCG TGG TCG TCG |
| crp-F1 (BamHI)         | CG GAATTC ATG GGG CGG AAA CCG CA |
| crp-R1 (XhoI)          | CCG CGGTAG TTA AGG AGT GCC GTA AAC GAC |
| fadL-FadR-site1-F      | GCA ACA TTC CAG CTG GTC CGA CTT ATA CTC TCG CC |
| fadL-FadR-site1-R      | GGC GAG AGT ATA GGT CGG ACC AGC TGG AAT GTT GC |
| fadL-FadR-site2-F      | CTC TCG CCA CTG GTC TGA TTT CTA AGA TGG ACC TC |
| fadL-FadR-site2-R      | GAG GTA CAT TTT AGA AAT CAG ACC AGG GGC GAG AG |
| fadD-FadR-site1-F      | GTA ATT ATC AAG CTG GTC TAA TGA GGT AAT ATT ATG |
| fadD-FadR-site1-R      | CAT AAT ATT AAC TCA TCA TAC CAG ATT GAT AAT TAC |
| fadD-FadR-site2-F      | GAA ACA GGC GGT GGT CCG CTG TTT CTG CAT TCT |
| fadD-FadR-site2-R      | AGA ATG CAG AAA CAG CGG ACC AGC GGC TGT TTC |
| fadD-CRP-site-F        | GTA AAG ATA AAA ATA AAT AGT GAC GGG CTT CGC AAT CTT TTC GTT GGG |
| fadD-CRP-site-R        | CCC AAC GAA AAG GGT GGG AAG AGG GGC GTC ACT ATT TAT TTT TAT CTT TAC |
| fadL-CRP-site1-F       | CTG CAA AAT CCG ATA AGT GAC GCA AAT CAC ACT TAA AAA TGA TCT |
| fadL-CRP-site1-R       | AGA TCA TTT TTA AGT GTG ATT TGC TGT ACT TAT CGG ATT TGG CAG |
| fadL-CRP-site2-F       | CCC TAC ACT TCG CGG TCT TCG TCT TAC ACG TAA CAT ATG TGG TAT AAA AAT AAA TC |
| fadL-CRP-site2-R       | GAT TTA TTA TAC AAA CTA TGT TAC GTG TTA CAG GAG GCA GAT TAG GG |
| fadH-CRP-site1-F       | GTA ACC TGG ATA AAA CCG GAC AAG CGT CTC ATG CCT GAT CMA CCG |
| fadH-CRP-site1-R       | CGG GTG ATA ACA GCG GAT GCG ACG GTC ATT TGC TGT ACT TAT CCA GAT TAC TAC |
| fadH-CRP-site2-F       | CAC CGG GTC TAT TCT TTA AGA ATC CCA TCA AAA ACC CGGCAC TCC |
| fadH-CRP-site2-R       | GGA GTG CGG GTG TTA TGG TAC TAA AAA AGA ATA CAG CC CG GTG |
| T7-F                   | TAA TAC GAC TCA CTA TAG GG |
| T7-R                   | GCT AGT TAT TGC TCA GCG G |

The sequences underlined are restriction sites, and the bold letters are predicted FadR or the CRP-cAMP sites.

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Electrophoretic mobility shift assays

These assays of the interaction between the \( \text{fadD} \) and \( \text{fadL} \) promoters, FadR and the cAMP-CRP complex were done essentially as previously reported [18,23]. All of the FadR (and/or CRP) probes were prepared by annealing two complementary primers (Table 2) by incubation in TEN buffer (10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, pH 8.0) at 95°C for 5 min followed by slow cooling to 25°C and then DIG labeling by terminal transferase with DIG-ddUTP (Roche). DNA probes (Table 2) for assay of DNA binding by the CRP-cAMP complex were similarly synthesized. The digoxigenin-labeled DNA probes (either 0.1 or 0.2 pmol) were incubated with either DNA binding protein in binding buffer (Roche) for 15 min at room temperature and then analyzed by native PAGE (6.5% PAGE for the CRP probes and 7% PAGE for all other probes). The separations were then visualized as previously described [18,23].

Fatty acid transport assays

Fatty acid transport was assayed as described by Klein et al. [24] with minor modifications. To avoid complications by \( \beta \)-oxidation strains that carried a \( \text{fadB}1 \) disruption (strains FYJ82, FYJ83 and SI203) were used (Table 1). To test ArcA-P regulation of fatty acid transport, FYJ82 strain (\( \Delta \text{arcA} \)) was compared with the wild type strain SI203 (Table 1). Overnight cultures were inoculated into 10 ml of RB liquid media supplemented with potassium nitrate (5 mM) as electron acceptor and kept in a fully anaerobic chamber at 37°C for 10 hrs [17]. The anaerobic environment (5% H\(_2\), 75% N\(_2\), and 20% CO\(_2\)) was generated by an anaerobic environmental chamber (Bio-Bag type A, Becton Dickinson) [17]. Cultures in exponential phase were treated with 100 mM chloramphenicol for 10 min prior to assay. 1-\(^{14}\)C-Oleic acid (American Radio-labeled Chemicals) was injected into the anaerobic bacterial cultures using a syringe fitted with a fine
Figure 3. E. coli fadL and fadD both carry two functional FadR-binding sites. A. sequence alignments of several known FadR binding sites from E. coli fad regulon. White letters with red background denote strictly conserved bases whereas yellow background letters denote highly conserved residues. The dual FadR sites of fadL and fadD are highlighted in bold italics. B and D. Gel shift assays of FadR binding to both fadL promoter sites. F and H. Gel shift assays of FadR binding to both fadD promoter sites. Long chain fatty acyl-CoA species block binding of FadR to the two fadL sites (C and E), as well as to the two fadD sites (G and I). FadR was used at concentrations of 0 (denoted by a minus sign), 1, 2, 5, or 10 pmol. FadR was incubated with 0.1 pmol of DIG-labelled probe in a total volume of 10 µl. For the acyl-CoA experiments the components were: probe, 0.1 pmol; FadR, 1 pmol and acyl-CoA; 50 pmol. Designations: C9:0, nonanoyl-CoA; C10:0, decanoyl-CoA; C16:0, palmitoyl-CoA; C16:1, palmitoleoyl-CoA; C18:0, stearoyl-CoA; C18:1, oleoyl-CoA.

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needle (30G1 PrecisionGlide) to a final concentration of 45.5 µM. The cultures were mixed well by vigorous vortex mixing and incubated at room temperature for about 15 min with anaerobiosis monitored by an anaerobic indicator (0.001% resazurin). Finally, the bacteria were collected by centrifugation (4,200 x g, 16 min), and washed for five times with iced RB medium. One ml of the cultures were subjected to membrane phospholipid extraction [18,25] after measuring culture absorbance (A600) and adjusted to an absorbance of 1.5. The phospholipids were then separated from any residual fatty acid by thin layer chromatography [18,25]. Assay of the effects of the CRP-cAMP system on

logarithmic phase cultures aerobically grown in LB liquid proceeded in a similar manner.

Bioinformatic analyses

The known or predicted DNA binding sites recognized by either FadR or ArcA (or CRP) were all from the E. coli literature. Multiple alignments were done using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index html), and the resultant output was processed by program ESPript 2.2 (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi), generating the final BLAST version.

Results and Discussion

Regulatory complexity in the LCFA transport promoters, fadL and fadD

The fadL and fadD genes encode the proteins known to be required for LCFA transport (Fig. 2A). The fadL and fadD transcriptional start sites are located 95 bp and 60 bp upstream of the translation start sites, respectively [6,56] (Fig. 2B). Despite a seemingly straightforward role in metabolism, both promoters contain demonstrated or annotated binding sites for three different transcription factors, FadR, ArcA and CRP (http://www.ecocyc.org). Moreover, fadL and fadD promoters are the only fad regulon promoters that contain two distinct FadR binding sites [6,26]. In the fadL promoter the FadR sites are separated by only 8 bp whereas in the fadD promoter the two FadR sites are separated by 68 bp (Fig. 2B and C). The locations of the ArcA binding sites determined by Cho and coworkers [12] also differ. The fadL ArcA site is 5 bp from FadR site 1 whereas the fadD ArcA site overlaps FadR site 2 by one bp, raising the possibility of crosstalk between the two repressor proteins (Fig. 2). The fadL CRP site was reported to lie downstream of the transcription start site [4] (Fig. 2B), a position incompatible with the usual activator function of CRP. Searches using the CRP consensus sequence of Zheng et al. [27] produced another fadL candidate binding site upstream of the transcription start site. Although this seemed a much more plausible position for activation of transcription, experimental verification of the CRP binding site was required. Indeed, although CRP-cAMP regulation of the fad regulon genes at the physiological level has been known for many years [14], none of the proposed sites have been experimentally validated.

The two FadR-binding sites of fadL and fadD result in only modest repression

To obtain a parallel comparison of the FadR binding sites of the two promoters we used electrophoretic mobility shift analyses (EMSA) to assay the binding abilities of the individual sites over a range of FadR concentrations (Fig. 3). The two fadL FadR sites bound FadR with equivalent affinities (essentially complete binding of the probes by 50 nM FadR, Fig. 3B, D). In contrast, although fadD site 1 showed a binding affinity comparable to that of the fadL sites (Fig. 3F), FadR binding by fadD site 2 (Fig. 3H) was >10-fold weaker than the other three sites (Fig. 3B, D and F). Addition of 25–50 µM oleoyl-CoA resulted in loss of FadR binding by all four sites (Fig. 3C, E, G and I). Therefore, our data are in good agreement with the reported DNase I footprinting results [6,11] and provide data on the effects of the acyl-CoA regulatory ligand that is lacking in the foot-printing experiments. To monitor expression of fadL and fadD, each promoter was fused to a LacZ reporter gene to allow expression to be assayed by β-galactosidase activity (Fig. 4). Deletion of FadR resulted in only modest derepression of the two LCFA transport system genes (2 to 2.5 fold for fadL and 2 to 3-fold for fadD) in
Figure 4. FadR repression and oleate induction of fatty acid transport system. A and B. Repression of fadL and fadD by FadR in cultures grown on various carbon sources. C and D. Parallel experiments with two β-oxidation genes fadBA and fadH are given for comparison. Strains FYJ104 (wild type) FYJ105 (ΔfadR::Tn10) were used for fadL whereas strains FYJ159 (wild type) and FYJ161 (ΔfadR) were used for fadD. Strains SI203 (wild type) and SI207 (ΔfadR) were used for fadBA whereas strains FYJ55 (wild type) and FYJ56 (ΔfadR) were used for fadH. E Induction of fadL and fadD expression by oleate. Oleic acid (5 mM) was added as the sole carbon source and compared to 5 mM acetate. Four strains expressing wild type FadR (FYJ295 (fadL-lacZ)), FYJ159 (fadD-lacZ), SI203 (fadBA-lacZ), and FYJ55 (fadH-lacZ), were used. All strains were grown under aerobic condition (10 ml of culture in a 50 ml flask shaken at 200 rpm at 37°C). β-Galactosidase (β-gal) assays were conducted in triplicate and the error bars indicate standard deviations. doi:10.1371/journal.pone.0046275.g004
medium with acetate as sole carbon source (Fig. 4A and B) whereas expression of fadBA and fadH increased by 5 to 10-fold (Fig. 4C and D). Increased expression of genes fadL and fadD in the ΔfadR strain was also seen when the carbon source was either glucose or glycerol (Fig. 4A and B). These observations are generally consistent with those obtained upon oleate induction of glucose or glycerol (Fig. 4A and B). These observations are generally consistent with those obtained upon oleate induction of glucose or glycerol (Fig. 4A and B).

Expression of fadL and fadD is directly activated by the CRP-cAMP complex

The global regulator cAMP-CRP complex [28] can act as either an activator [29] or a repressor [30] in expression of genes involved in many E. coli metabolic pathways. Although putative class I cAMP-CRP binding sites had been proposed upstream of some fad regulon genes and activation of fadH transcription was observed in vivo [17], direct physical evidence for DNA binding of these promoters by the cAMP-CRP complex was lacking. We therefore used EMSAs with purified CRP in the presence or absence of cAMP to test the proposed sites. SDS-PAGE analyses indicated that the apo-form of CRP of molecular weight ~23 kDa was homogenous (Fig. 5A). Chemical cross-linking showed the protein was dimeric as previously reported [28] (Fig. 5B). Liquid chromatography mass spectrometry analysis of tryptic peptides demonstrated that our recombinate protein matched E. coli CRP with 66% coverage (Fig. 5C).

Higashitani and coworkers [4] predicted a fadL CRP binding site (Fig. 2B) centered at 57 bp downstream of the fadL transcription start site (termed fadL2) that is covered by our fadL probe 2, Fig. 6C). In contrast, we favored a site centered 157 bp upstream (Fig. 2C and 6A) as also seen in fadH [17] where in the fadH promoter the ArcA and FadR site 1 sequences are separated by 5 bp (Fig. 2B). Thus, cross-talk between FadR and ArcA in fadL and/or fadD expression seemed likely.

Expression of fadL and fadD are additively repressed by FadR and ArcA under anaerobic conditions

The weak effects of FadR inactivation on expression of fadL and fadD suggested that other regulatory proteins may be involved and thus we tested the effects of deletion of the genes encoding the other proteins proposed to bind these promoters. The oxygen-sensitive two-component system ArcA-ArcB was reported to negatively regulate transcription of several fad regulon genes, including fadL, fadD, fadBA and fadH [12]. That report [12] also contained in vitro and in vivo evidence that the phosphorylated ArcA regulator (ArcA-P) bound the promoters of the target genes (Fig. 8A). We recently reported cross-talk between FadR and ArcA in expression of other fad regulon genes and found that the interaction could be either additive (fadH) (Fig. 8E) or synergistic (fadBA) (Fig. 8D) [17]. The fadD promoter FadR site 2 and the ArcA site overlap by a single bp (Fig. 2C) as also seen in fadH [17] whereas in the fadL promoter the ArcA and FadR site 1 sequences are separated by 5 bp (Fig. 2B). Thus, cross-talk between FadR and ArcA in fadL and/or fadD expression seemed likely.
To test for cross-talk strains carrying arcA and/or fadR null mutations plus the chromosomal fadL-lacZ (or fadD-lacZ) transcriptional fusions were constructed. In general agreement with the report of Cho et al. [12], we found that the absence of arcA under anaerobic conditions resulted in increased transcription of fadL and fadD by 2 to 3-fold and 20-fold, respectively, (Fig. 8B and C). Our levels of derepression are somewhat less than those reported by Cho and coworkers [12] which we attribute to the gene used by these workers as their internal reference [17,33]. However, our data differed from those of Cho and coworkers in the effects reported on FadR regulation of fadL transcription under anaerobic conditions when ArcA is functional (we see no effect versus their reported 4-fold increase). Although our statistical analysis argued that these two regulatory proteins control transcription of fadL and fadD in an additive manner, the wide variations in the data and the overlapping error bars provide caveats to this interpretation.

In the absence of the cAMP receptor protein (CRP) complex (cAMP-CRP), FadR and ArcA-ArcB repression of fadL and fadD expression was relatively weak (Fig. 8) and similar to that seen with fadBA and fadH [17]. Thus it seems that regulation by both FadR and ArcA-ArcB rely on the cAMP-CRP complex to activate transcription such that it can be further modulated.

Physiological relevance of ArcA and the CRP-cAMP complex to fatty acid transport

Uptake of [1-14C]oleic acid was assayed to evaluate the physiological consequences of ArcA-ArcB and the CRP-cAMP complex on fatty acid transport (Fig. 9). To rule out potential interference by β-oxidation, the strains studied lacked fadBA (Table 1). Incorporation of [1-14C]oleic acid into the membrane phospholipids of the ΔarcA strain (FY82) was consistently 2-fold greater than that of its parental strain under anaerobic conditions (Fig. 9A). Further analyses by thin layer chromatography showed that incorporation into the three major membrane phospholipids phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and cardiolipin (CL) (Fig. 9B), consistent with anaerobic repression of fadL (and/or fadD) by ArcA-P (Fig. 8B and C).

Figure 6. The cAMP-CRP complex binds the fadL, fadD and fadH promoters. A. Sequence analyses of the CRP binding sites of fadL, fadD and fadH. White letters shaded in black denote strictly conserved bases, bold letters in grey represent highly similar residues, and dots mean gaps. The newly deduced CRP-binding site (fadL1 of A) binds the cAMP-CRP complex (panel B) whereas the CRP-binding site predicted earlier [17] (abbreviated fadL2) does not (panel C). D Binding of the fadD promoter region by the cAMP-CRP complex. E and F. The fadH promoter region contains two functional CRP-binding sites, fadH1 and fadH2, respectively. The DIG-labeled probe shifted by the cAMP-CRP complex is indicated by an arrow. All EMSA experiments were carried out using 6.5% native PAGE and representative results are shown. The protein samples were incubated with 0.6 pmol of DIG-labeled probe in a total volume of 20 μl that contained 200 pmol cAMP (when added). The right hand four lanes of each contained (left to right) 10, 5, 10 and 20 pmol of CRP, respectively.

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In agreement with the observed CRP-cAMP activation of \( \text{fadL} \) and \( \text{fadD} \) transcription (Fig. 7A and B), quantitative determination of total \( ^{14} \text{C} \)-labeled bacterial membrane phospholipids showed that the level of oleic acid incorporation in the strain lacking \( \text{crp} \) is only about one third that of the parental strain (Fig. 9C and D). To our knowledge these are the first direct physiological data that directly show that these two global regulators modulate LCFA transport pathway in \( \text{E. coli} \).

**Conclusions**

Transcriptional regulation of fatty acid transport in \( \text{E. coli} \) involves three distinct regulatory systems, the specialized FadR system and the two global systems, ArcA and CRP-cAMP. FadR action is straightforward, it acts as a classical LacI-type repressor and only weakly represses \( \text{fadD} \) and \( \text{fadL} \) expression. CRP-cAMP regulation is also straightforward, fatty acids are a low status carbon source and \( \text{E. coli} \) prefers to use the highest status carbon source, glucose. In the presence of glucose (or in the absence of CRP), the other two regulators have little transcription to regulate. The apparent surprise is the stringent repression by ArcA seen under anaerobic conditions even in the absence of FadR because \( \text{E. coli} \) has a pathway to degrade fatty acids under anaerobic conditions [34] and low expression of FadD and FadL would seem likely to compromise function of the anaerobic pathway. However, under anaerobic conditions a new acyl-CoA synthetase, FadK, is induced that replaces FadD [35]. Unlike FadD which is inactive with short chain fatty acids [24,35], FadK strongly prefers short chain length acids and such acids are the preferred growth substrates of the anaerobic \( \beta \)-oxidation pathway [34] although LCFA can also be utilized. Short chain acids readily enter \( \text{fadL} \) strains [36] and thus decreased expression of FadL is irrelevant for these growth substrates. However, degradation of LCFA such as oleate via the anaerobic pathway should require FadL. These apparent contradictions can be reconciled by our finding that ArcA represses \( \text{fadL} \) expression less than \( \text{fadD} \) expression (only about 4-fold) (Fig. 8B and C) and that FadK activates oleate poorly [35]. Hence, the significant level of FadL expressed under ArcA repression probably provides sufficient oleate transport to satisfy the poor catalytic activity of FadK with this substrate and allow the observed slow anaerobic growth on oleate [35].

Figure 7. Activation of fatty acid transport system gene expression by the CRP-cAMP complex. A and B. Decreased expression levels of \( \text{fadL} \) and \( \text{fadD} \), respectively, were seen in the absence of CRP-cAMP. Strains FYJ104 (wild type) and FYJ134 (\( \Delta \text{crp} \)) carry the \( \text{fadL-lacZ} \) transcriptional fusion whereas FYJ159 (wild type) and FYJ165 (\( \Delta \text{crp} \)) contain the \( \text{fadD-lacZ} \) transcriptional fusion. C and D. Expression of \( \text{fadBA} \) and \( \text{fadH} \) are positively regulated by the functional CRP-cAMP complex. Strains SI203 (wild type) and FYJ83 (\( \Delta \text{crp} \)) carry the \( \text{fadBA-lacZ} \) transcriptional fusion whereas FYJ55 (wild type) and FYJ65 (\( \Delta \text{crp} \)) contain the \( \text{fadH-lacZ} \) transcriptional fusion. The cultures were grown in either LB or RB media. beta-Galactosidase activities were from at least three independent experiments, and the error bars indicate standard deviations. doi:10.1371/journal.pone.0046275.g007
Figure 8. Negative regulation of fadL and fadD by ArcA-P under anaerobic conditions. A. Alignments of the ArcA-P binding sites of the fadBA-lacZ transcriptional fusion by different regulatory proteins under anaerobic conditions. The fadBA-lacZ strains were SI203 (WT), SI207 (ΔfadR), FYJ82 (ΔarcA), FYJ83 (Δcrp), FYJ84 (ΔarcA ΔfadR), FYJ85 (Δcrp ΔfadR), FYJ86 (Δcrp ΔarcA), and FYJ87 (Δcrp ΔarcA ΔfadR), respectively. The strains were grown on LB agar plates supplemented with 5 mM nitrate (KNO₃) as the electron receptor. Anaerobic environments were generated using Bio-Bag environmental chamber type A as described [17]. β-Galactosidase activities were recorded from at least six independent assays and are expressed as means ± standard deviations. *, P<0.005; **, P<0.001.

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Figure 9. Effects of ArcA and CRP on incorporation of [1-14C]oleic acid into membrane phospholipid. A. Incorporation in the ΔarcA strain, FYJ82, and the wild type strain, SI203. At least three independent experiments were carried out and the data are expressed as mean ± standard deviation. B. A representative autoradiogram of a TLC separation of the 14C-labeled phospholipids of a panel A experiment. The phospholipid species are phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and cardiolipin (CL). A representative autoradiogram is given. C. Incorporation in the Δcrp strain, FYJ83 and the wild type strain, SI203. At least five independent experiments were performed and the data are expressed in means ± SD. D. A representative autoradiogram of a TLC separation of the 14C-labeled phospholipids of a panel C experiment. Bacterial strains used in panel A and B were kept under anaerobic condition (details seen in Materials and Methods), whereas experiments in panel C and D were routinely conducted under aerobic condition.

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Author Contributions
Conceived and designed the experiments: YF JC. Performed the experiments: YF. Analyzed the data: YF JC. Contributed reagents/materials/analysis tools: YF JC. Wrote the paper: YF JC.

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