Rat Long Chain Acyl-CoA Synthetase 5, but Not 1, 2, 3, or 4, Complements *Escherichia coli fadD**

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Long chain fatty acids are converted to acyl-CoAs by acyl-CoA synthetase (fatty acid CoA ligase: AMP forming, E.C. 6.2.1.3; ACS). *Escherichia coli* has a single ACS, FadD, that is essential for growth when fatty acids are the sole carbon and energy source. Rodents have five ACS isoforms that differ in substrate specificity, tissue expression, and subcellular localization and are believed to channel fatty acids toward distinct metabolic pathways. We expressed rat ACS isoforms 1–5 in an *E. coli* strain that lacked FadD. All rat ACS isoforms were expressed in *E. coli fadD* or *fadDfadR* and had ACS specific activities that were 1.6–20-fold higher than the wild type control strain expressing FadD. In the *fadD* background, the rat ACS isoforms 1, 2, 3, 4 and 5 oxidized [^{14}C]oleate at 5 to 25% of the wild type levels, but only ACS5 restored growth on oleate as the sole carbon source. To ensure that enzymes of β-oxidation were not limiting, assays of ACS activity, β-oxidation, fatty acid transport, and phospholipid synthesis were also examined in a *fadD fadR* strain, thereby eliminating FadR repression of the transporter FadL and the enzymes of β-oxidation. In this strain, fatty acid transport levels were low but detectable for ACS1, 2, 3, and 4 and were nearly 50% of wild type levels for ACS5. Despite increases in β-oxidation, only ACS5 transformants were able to grow on oleate. These studies show that although ACS isoforms 1–4 variably supported moderate transport activity, β-oxidation, and phospholipid synthesis and although their *in vitro* specific activities were greater than that of chromosomally encoded FadD, they were unable to substitute functionally for FadD regarding growth. Thus, membrane composition and protein-protein interactions may be critical in reconstituting bacterial ACS function.

Acyl-CoA synthetase (fatty acid CoA ligase: AMP forming, E.C. 6.2.1.3; ACS)[1] catalyzes the conversion of long chain free fatty acids into their CoA esters and plays an important role in fatty acid metabolism in bacteria, yeast, and mammalian cells (1, 2). In yeast, the ACS isoforms Faap1 and Faap4 are required for the utilization of exogenous fatty acids for growth and transport (3), and in NIH 3T3 cells, overexpression of mouse ACS1 increases fatty acid uptake (4).

The *Escherichia coli* ACS enzyme FadD and the fatty acid transport protein FadL are essential components of a fatty acid uptake system (5). Because the outer membrane of *E. coli* contains lipopolysaccharide that constitutes a barrier for hydrophobic molecules, fatty acids are not able to cross it by diffusion but require the transport protein FadL. Protonated fatty acids then cross the bacterial inner membrane in a process favored by the proton gradient and are abstracted from the membrane and concomitantly trapped within the cell as acyl-CoA esters by the membrane-associated ACS, FadD (5). FadL-mediated transport and the subsequent FadD-mediated formation of acyl-CoA esters is defined as vectorial acylation, a process that allows exogenous fatty acids to be used by bacteria as their energy source as well as a substrate for phospholipid biosynthesis. Thus, when no other carbon source is available, exogenous fatty acids are converted to acyl-CoA esters, which bind to the transcription factor FadR and derepress the expression of the *fad* genes that encode proteins responsible for fatty acid transport (FadL), activation (FadD), and β-oxidation (FadA, FadB, FadE, and FadH) (5). When alternative sources of carbon are available, bacteria synthesize fatty acids as acyl-ACPs, which are used for phospholipid synthesis but are not substrates for β-oxidation. Thus, acyl-CoA and acyl-ACP constitute independent pools of fatty acids destined for different fates.

Contrasting with the single ACS present in bacteria, eukaryotes express multiple ACS isoforms that are not functionally equivalent (2). Six yeast isoforms (Faap1–4, Fat1p, and Fat2p) have been cloned and characterized (6). Faap1 and Faap4 are essential for the utilization of exogenous fatty acids, because *faa1*Δfaa4Δ strains cannot grow when endogenous synthesis is inhibited by the fatty acid synthase inhibitor cerulenin unless supplemented with 14:0, 16:0, or 18:1 (3). Faap2 activates endogenous medium chain fatty acids destined for β-oxidation in peroxisomes (7), and Faap3 also uses endogenous fatty acids, but their specific fate is uncertain (8). Fat1p has specificity for very long chain fatty acids (9) and is required for efficient fatty acid uptake (10).

In rats and humans, five long chain ACS isoforms[2] have been cloned (1, 2). These isoforms have different substrate preferences, are expressed in different tissues, and are present in different subcellular locations. The expression of each of the rat

[1] The abbreviations used are: ACS, acyl-CoA synthetase; IPTG, isopropyl-1-thio-β-D-galactopyranoside.

[2] Human FACL1 and FACL2 are the same gene (www.gene.ud.ac. uk/nomenclature/genefamily/acs.html).

isoforms is likely to be regulated independently. For example, ACS1 mRNA in different tissues is variously up-regulated by peroxisomal proliferator-activated receptors α and γ (11, 12) and by insulin (13), ACS2 in brain is up-regulated by peroxisomal proliferator-activated receptor β (14), and ACS4 mRNA is induced by ACTH and by 20:4 and down-regulated by dexamethasone in steroidogenic tissues (15). These and other data suggest that rat ACS isoforms, like those of yeast, are not redundant but rather that they have distinct functions, possibly directing fatty acids to different metabolic pathways. In human skin fibroblasts, triacsin C, which inhibits ACS1 and 4 but not ACS5 (16), blocks *de novo* glycerolipid synthesis but does not affect lysophospholipid reacylation (17), suggesting that different ACS isoforms generate independent acyl-CoA pools that have specific fates. In similar experiments with rat hepatocytes, thiazolidinediones, which are inhibitors of rat ACS4 but not ACS1 or 5 (16), markedly decrease the synthesis of TAG but have less effect on β-oxidation (18). Female mice heterozygous for ACS4 gene inactivation have increased production of eicosanoids in uterine tissue and reduced fertility, suggesting that other isoforms cannot compensate for even a partial lack of ACS4 (19). In humans, mutations in the FACL4 gene (ACS4 homolog) that inactivate the enzyme are linked to mental retardation (20, 21), again suggesting that the unique function of this isoform in brain cannot be replaced.

Expression of rat ACS1 rescues yeast *faa1Δ faa4Δ* strains and allows growth on a fermentable carbon source when cerulenin and exogenous long chain fatty acids are present (3), indicating that a mammalian ACS can functionally substitute in a lower eukaryote. Because the long chain ACSs may have independent functions, we wondered whether they would vary in their ability to replace the single ACS in *E. coli*, FadD, in fatty acid vectorial transport and the activation of exogenously provided fatty acids. To answer this question, we transformed rat ACS1–5 into *E. coli fadD* and measured transport, β-oxidation, lipid synthesis, and growth in the presence of oleate as the only source of carbon and energy.

EXPERIMENTAL PROCEDURES

Construction of Recombinant pFLAG-CTC-ACS Plasmids—Plasmids pFLAG-CTC-ACS1, -ACS4, and -ACS5 express the ACS isoforms as fusion proteins with a C-terminal FLAG epitope, under the control of a *tac* promoter (16). To obtain cDNA clones of ACS2 and ACS3, rat total brain RNA was isolated using TRIzol reagent (Invitrogen), and cDNAs were synthesized using Moloney murine leukemia virus reverse transcriptase (Invitrogen). Rat ACS3 (GenBank™ accession number D30666) (22) was amplified by PCR using the upper primer 5′-CTAAGCTTCACATGAATAACCACGTATCTTCAACAC-3′ and the lower primer 5′-AAGTCGACTTTTCTTCCGTACATCCGCTCAATG-3′. The product included the open reading frame flanked by the recognition sequences for HindIII and SalI restriction enzymes. Following digestion with HindIII and SalI, the amplified ACS3 cDNA was ligated into the pFLAG-CTC vector to produce pFLAG-CTC-ACS3. Rat ACS2 (GenBank™ accession number D10041) (23) was amplified by PCR using the upper primer 5′-ATAAGCTTAAGATGCAGACCCAGGAGATCCTG-3′ and the lower primer 5′-GTACTCGAGACATGGAGATTGAGTACAGCTCTTCT-3′ and ligated into pCR-BluntII-TOPO vector (Invitrogen) according to the manufacturer's instructions. The ACS2 cDNA was excised with HindIII and XhoI and then ligated into the pFLAG-CTC vector to generate pFLAG-CTC-ACS2. The sequences of ACS2 and 3 were verified by automated DNA sequencing (UNC-CH Automated DNA Sequencing Facility).

Strains, Transformation, and Culture Conditions—*E. coli* strain BL21-CodonPlus(DE3)-RIL was obtained from Stratagene and will be referred to as BL21(DE3)RIL or wild type throughout this paper. A strain containing a *fadD* deletion was generated by P1 transduction of BL21-(DE3)RIL with *fadD*::*kan* as described previously (24) and is referred to as BL21(DE3)RIL*fadD*. A spontaneous *fadR* mutant was obtained by plating BL21(DE3)RIL on minimal medium containing decanoate (25). This mutant, BL21(DE3)RIL*fadR*, constitutively expresses the fatty acid degradative enzymes (Fad) without oleate induction. It was transduced with a P1 bacteriophage grown on

BL21(DE3)RIL*fadD* to generate the strain BL21(DE3)RIL*fadR fadD*. Bacteria were transformed by the Hanahan protocol (26); strains transformed with pFLAG-CTC vector without an insert were used as control. For routine culture, liquid Terrific Broth (TB) medium (Invitrogen) and Luria-Bertani (LB) agar (Fisher) plates were used; 50 μg/ml carbenicillin (Invitrogen) was added to maintain the plasmids. Growth in liquid cultures was monitored by measuring optical density at 600 nm (A_{600}). Frozen stocks were prepared in LB medium, 7% Me$_2$SO and stored at -80 °C.

Transport Assay—BL21(DE3)RIL*fadR fadD* transformed with pFLAG derivatives of rat ACS 1–5 was used to inoculate an overnight culture in TB. The next morning the cells were subcultured at 1:100 dilution in the same medium. When growth reached mid-log phase, protein production was induced with 1 mM IPTG for 1 h. The cells were then harvested by centrifugation (3400 $\times$ g 15 min at room temperature), rinsed once with M9 medium (27) and 0.5% Brij 58, resuspended in one-half volume of the same medium containing 100 μg/ml chloramphenicol, and starved for 15 min at 30 °C. Fatty acid transport was measured by adding 100 μM [³H]potassium oleate (final concentration). At 0, 2, 4, and 6 min aliquots were pipetted onto filter GN-6, washed twice with M9 0.5% Brij 58, air-dried, and counted (28).

Induction of Rat ACSs and fad Genes for β-Oxidation and Lipid Synthesis Experiments—Bacteria were grown overnight in 3 ml of TB medium at 37 °C with shaking. The next morning the bacteria were harvested by centrifugation and resuspended in medium E with 0.01 g/liter thiamine (Med EB$_1$), 25 mM potassium acetate, 1 g/liter casamino acids. The cell density was adjusted to $A_{600} \approx 0.1$, and the bacteria were grown in 250-ml Erlenmeyer flasks at 37 °C with shaking at 240 rpm. When the cultures reached mid-log phase ($A_{600} \approx 0.5$), the expression of the recombinant rat ACS isoforms was induced with 1 mM IPTG. At the same time, in the BL21(DE3)RIL and BL21(DE3)RIL*fadD* strains, the expression from the endogenous *fad* genes responsible for β-oxidation was induced with 5 mM potassium oleate in 0.5% Brij58 (29). No oleate was necessary for *fadR* and *fadR fadD* strains because these *fadR* mutants constitutively express the proteins of the *fad* regulon. The cultures were allowed to grow for 1 h, and aliquots were used to measure lipid incorporation. To measure β-oxidation, the cells were harvested by centrifugation and washed twice with Med EB$_1$, 0.5% Brij58, 1 mM IPTG with no carbon source and resuspended in Med EB$_1$, 1 mM IPTG, 0.1 g/liter chloramphenicol, bringing the cell concentration to $A_{600} \approx 1$. The protein content was determined in separate aliquots. The remaining cultures were further processed to measure ACS activity and to perform immunoblot analyses.

β-Oxidation Assay—To measure β-oxidation, 2-ml aliquots of the bacterial cultures described above were incubated for 1 h at 37 °C with shaking at 240 rpm with 500 μM [¹⁴C]oleic acid in 0.5% Brij58 in rubber-stoppered vials with center wells. Then 0.5 ml of 5 N H$_2$SO$_4$ was added to the medium to promote the evolution of [¹⁴C]CO$_2$, which was trapped in center wells filled with 0.2 ml of ethanolamine:ethanol 1:1 (v/v). After an additional hour of shaking, the contents of the center wells were transferred to vials, and the [¹⁴C]CO$_2$ produced was measured by scintillation counting (30). The experiments were performed in triplicate, and the results are presented as the means $\pm$ S.E. of a representative experiment, which was repeated two to six times for BL21(DE3)RIL wild type and *fadD* strains and twice with *fadR* and *fadR fadD* strains. The differences were analyzed by Student's *t* test.

Lipid Synthesis—Bacteria were grown to mid-log phase, and the expression of rat ACS isoforms was induced for 1 h as described above. Then 1 ml of culture was incubated with 20 μM of [1-¹⁴C]oleate (1 μCi) (final concentration) for 30 min at 37 °C. The cultures were then chilled on ice and washed three times with phosphate-buffered saline buffer containing 0.5% Brij58. The lipids were extracted (31), and [¹⁴C]oleate incorporation was measured by scintillation counting. Incorporation into phospholipid species was determined by thin layer chromatography in CHCl$_3$/ethanol/H$_2$O/triethylamine (30/35/7/35; v/v) using authentic standards (32).

Preparation of Bacterial Lysate—Bacterial cultures described above were harvested by centrifugation for 10 min at 5000 rpm in a Sorval SA-600 rotor at 4 °C and resuspended in 10 mM HEPES, pH 7.8, 0.5 mM EDTA buffer. The bacteria were lysed by sonication on ice, with six 10-s bursts and 10-s rest intervals using a heat system ultrasonic cell disruptor sonicator (Heat Systems, Ultrasonics) at setting 4. Lysate aliquots were stored at -80 °C until used for enzyme assay.

ACS Activity Assay—ACS activity was measured using 17–80 μg of bacterial lysate. The assays contained 175 mM Tris-HCl, pH 7.4, 8 mM MgCl$_2$, 5 mM dithiothreitol, 10 mM ATP, 0.25 mM CoA, and 50 μM [¹⁴C]palmitic acid (New England Nuclear) in 0.5 mM Triton X-100, 0.01 mM EDTA in a total volume of 200 μl. The assays were performed at

TABLE I

Only rat ACS5 complements absence of FadD

BL21 (DE3)RIL and BL21(DE3)*RILfadR* containing the empty vector or BL21 (DE3)RIL*fadD* and *fadR fadD* containing vectors expressing ACS1, 2, 3, 4, or 5 were streaked on medium EB$_1$ agar plates supplemented with 5 mM oleate in 0.5% Brij58 as the sole energy and carbon source. EB$_1$ agar plates containing 25 mM glucose were used as controls. The plates were incubated at 37 °C and checked daily. This experiment was repeated 6–11 times with both carbon sources and IPTG concentrations of 0.005 to 0.5 mM. +++, WT growth; ++, intermediate growth; −, no growth.

| Strain | Vector | EB$_1$ media | |
		25 mM glucose	5 mM oleate
BL21 (DE3)RIL	Empty	+++	+++
fadD	ACS1	+++	−
	ACS2	+++	−
	ACS3	+++	−
	ACS4	+++	−
	ACS5	+++	++
fadR	Empty	+++	+++
fadD fadR	ACS1	+++	−
	ACS2	+++	−
	ACS3	+++	−
	ACS4	+++	−
	ACS5	+++	++

37 °C for 5 min with shaking. The reaction was started by adding lysate protein and terminated by adding 1 ml of Dole's reagent (isopropanol, heptane, 1 M H$_2$SO$_4$ (80:20:2)). The unreacted free fatty acid was removed with two 2-ml heptane washes, and the labeled acyl-CoA produced by the reaction was measured by scintillation counting (33). The assays measured initial rates.

Immunoblot Analysis—Proteins from the bacterial lysate (1 or 2 μg) were resolved on a 10% polyacrylamide gel containing 0.1% SDS and transferred onto polyvinylidene difluoride membranes (Bio-Rad). Immunoreactive bands were detected by incubating the membranes with anti-FLAG M2 monoclonal antibody (Sigma), horseradish peroxidase-conjugated goat anti-mouse IgG (Pierce), and SuperSignal West Pico Chemiluminescent Reagent (Pierce).

Growth Complementation of fadD Deletion—Bacteria were transformed with plasmids containing different isoforms of rat ACS or no insert. These were examined for their ability to grow on oleate as the sole energy and carbon source. Transformants were streaked on Med EB$_1$ agar plates supplemented with 50 μg/ml carbenicillin, 5 mM potassium oleate solubilized in 0.5% Brij 58 (final concentration), and different concentrations of IPTG (0.005, 0.01, 0.02, 0.05, 0.1, 0.2, and 0.5 mM). Control plates were supplemented with 25 mM glucose and the appropriate concentration of IPTG. The plates were incubated at 37 °C, and growth was checked daily for 7 days.

Other Methods—The protein content was measured using bovine serum albumin as the standard (34).

RESULTS

Rat ACS Isoforms 1–5 Are Expressed and Active in BL21(DE3)RILfadD—To determine whether rat ACS isoforms can functionally replace FadD, the sole ACS enzyme in bacteria, the coding region of *fadD* was deleted in BL21(DE3)RIL to generate strain BL21(DE3)RIL*fadD*. The *fadD* strain had ACS activity levels indistinguishable from background and was unable to grow when oleate was the sole energy and carbon source (see Fig. 2 and Table I). BL21(DE3)RIL*fadD* was transformed with the expression vector pFLAG-CTC-ACS1, 2, 3, 4, or 5 or with the empty vector as a control. The rat ACS isoforms were each expressed as a fusion protein with a C-terminal FLAG epitope. The FLAG epitope does not interfere with ACS activity or kinetic properties (16).

All five ACS isoforms were expressed in BL21(DE3)RIL*fadD*, with ACS1 and ACS4 exhibiting the greatest expression and ACS5 exhibiting the least (Fig. 1*A*). All of the isoforms exhibited ACS activity (Fig. 2*A*). In each case, the specific activity was at least 1.6-fold higher (ACS5) and as much as 20-fold higher (ACS4) than the specific activity of the bacterial enzyme FadD in the wild type control strain containing the empty
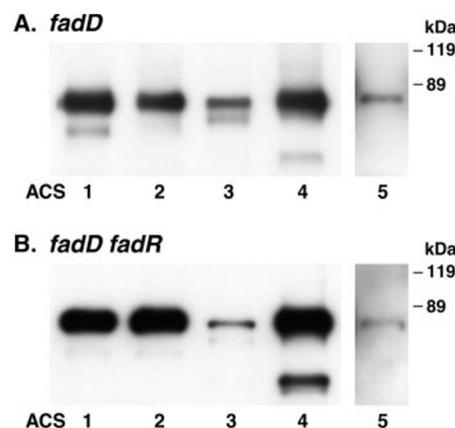

FIG. 1. **Rat ACS isoforms 1–5 are expressed in *E. coli fadD*.** BL21(DE3)RIL*fadD* (*A*) or BL21(DE3)RIL*fadR fadD* (*B*) transformed with different ACS isoforms was grown as described under "Experimental Procedures." Expression was induced for 1 h with 1 mM IPTG. The lysates were prepared, and 1 μg (*A*) or 2 μg (*B*) of protein was resolved by 10% SDS-PAGE and subjected to immunoblot analysis using M2 anti-FLAG antibody. Exposure time was 2 s except for ACS5, which was exposed for 20 s (*A*) or 30 min (*B*). The experiment was repeated twice. Representative results are shown.

vector. Further, the correlation was poor between expressed protein and enzyme activity. Although the expression of ACS4 protein was not substantially different from that of ACS1, ACS4 specific activity was 8-fold higher than that of ACS1, perhaps because ACS4 is a peripheral membrane protein like FadD. In contrast, ACS1 and ACS5 (and probably ACS2 and ACS3) are integral membrane proteins whose activity might be negatively affected by the composition of the bacterial membrane, which differs considerably from the phospholipid composition of mammalian membranes (35).

Rat ACS Isoforms 1–5 Activate Exogenous Fatty Acids for β-Oxidation—When carbohydrate is not available, *E. coli* can import and activate exogenous fatty acids to acyl-CoA esters that are then oxidized to provide energy. BL21(DE3)RIL*fadD* expressing each of the rat ACS isoforms was studied to determine whether these rat ACS isoforms would be able to supply fatty acyl-CoA for β-oxidation in the absence of FadD (Fig. 3*A*). As expected, BL21(DE3)RIL*fadD* transformed with the empty vector was unable to oxidize exogenous oleate. However, transformation with rat ACS isoform 1, 2, 3, 4, or 5 allowed some β-oxidation to occur, indicating that each of the rat ACS isoforms partially replaced FadD for this function. There was no correlation between *in vitro* specific activities of the ACS isoform and the rates of *in vivo* β-oxidation. Despite ACS activities that were 1.6–20-fold higher than that of the control BL21(DE3)RIL, which contains active FadD (Fig. 2), the relative rates of β-oxidation ([^{14}C]CO$_2$ produced/ACS activity) were 0.2–7.5% of those observed in the wild type strain (Fig. 3*B*). Thus, although the ACS isoforms were able to catalyze the formation of acyl-CoAs *in vitro*, either they did not form acyl-CoAs *in vivo*, the acyl-CoAs formed were not accessible to the β-oxidation enzymes, or the activities of the β-oxidation enzymes were limiting because of the presence of FadR.

β-Oxidation by E. coli requires expression of the *fad* genes encoding proteins that mediate transport (FadL), activation (FadD), and β-oxidation of exogenous fatty acids (FadA, FadB, FadE, and FadH) (29, 36). When carbohydrate is present, the transcription factor FadR represses the expression of these proteins. If long chain fatty acids are provided as the sole carbon and energy source in the culture medium, however, the resulting acyl-CoAs produced by the activity of FadD bind to FadR and regulate FadR activity (25) by promoting the dissociation of FadR from the promoter region of the genes of the *fad*
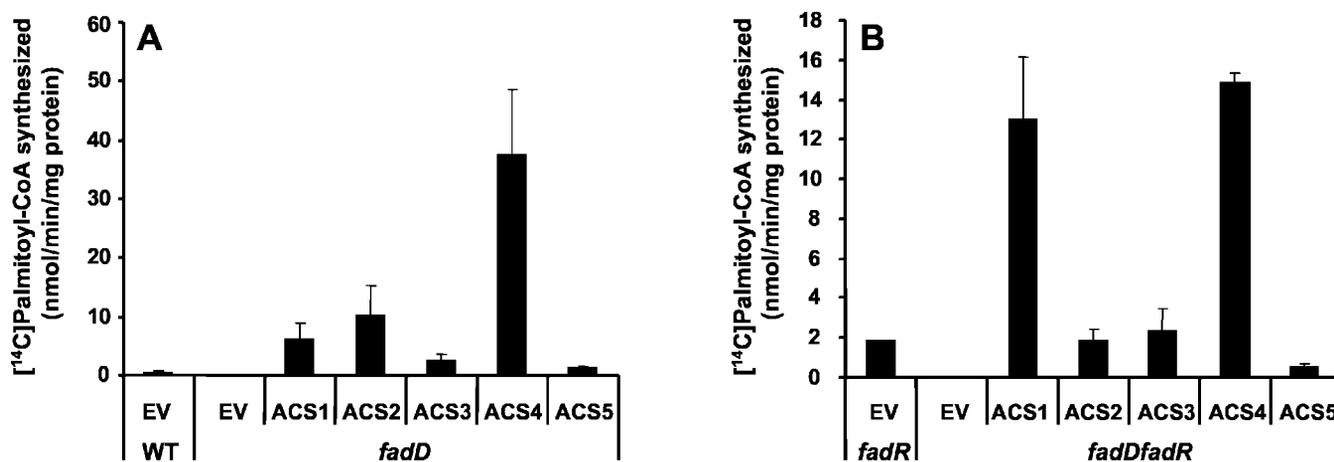
FIG. 2. **Rat ACS isoforms 1–5 expressed in *E. coli fadD* are active.** BL21(DE3)RIL*fadD* (*A*) or BL21(DE3)RIL*fadR fadD* (*B*) transformed with the empty vector (*EV*) or in which rat ACS expression was induced for 1 h with 1 mM IPTG was used to prepare lysates as described under "Experimental Procedures." ACS specific activity was assayed with 14–70 μg of protein. BL21(DE3)RIL (*WT*) (*A*) and BL21(DE3)RIL*fadR* (*B*) strains transformed with the empty vector were used as controls. The data are presented as the means ± S.E. ($n \geq 3$).
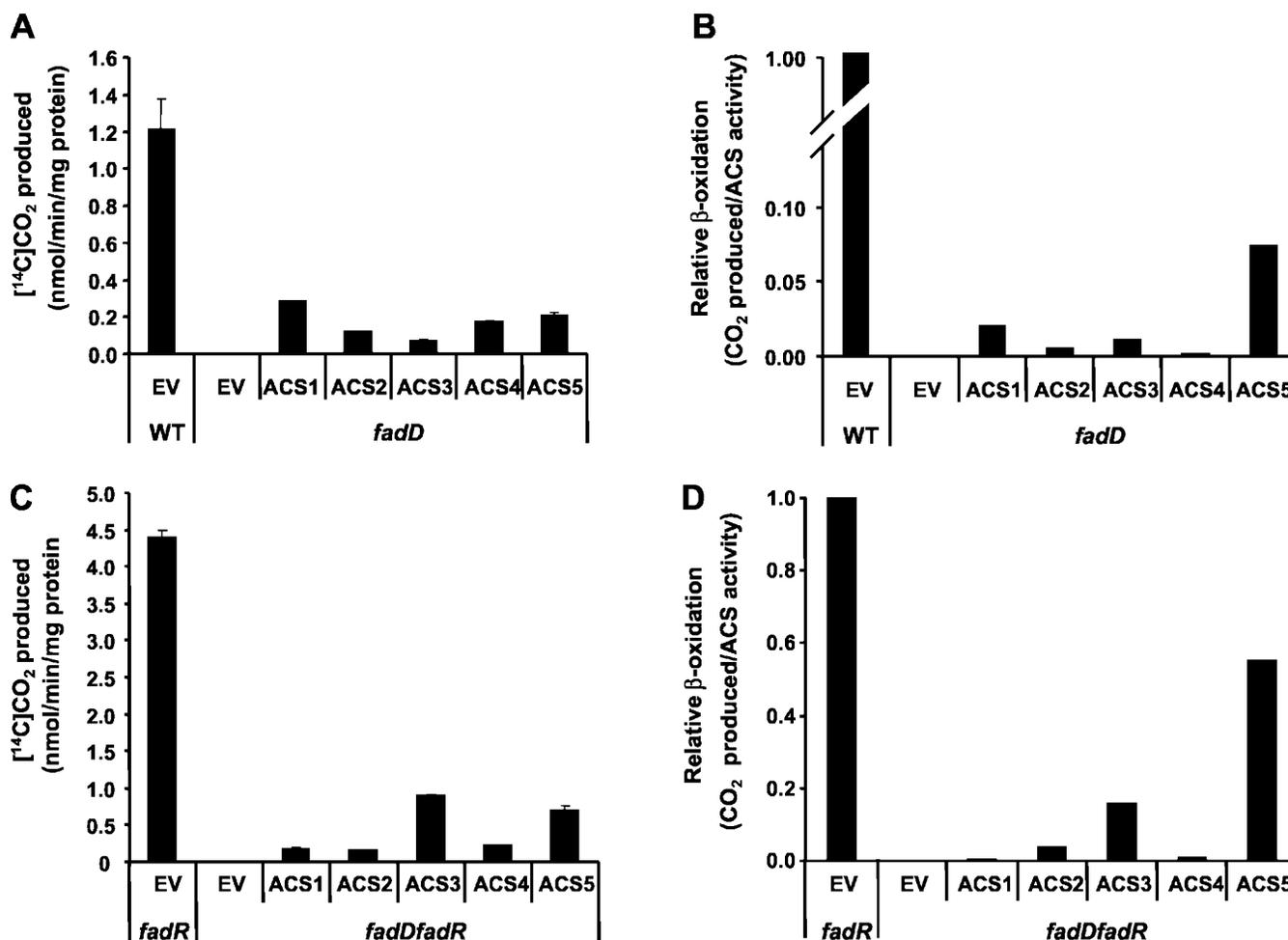

FIG. 3. **Rat ACS isoforms 1–5 partially restore β-oxidation in *E. coli fadD*.** BL21(DE3)RIL*fadD* (*A* and *B*) or BL21(DE3)RIL*fadR fadD* (*C* and *D*) were transformed with the empty vector (*EV*) or with the ACS isoforms. BL21(DE3)RIL (*WT*) (*A* and *B*) and BL21(DE3)RIL*fadR* (*C* and *D*) strains transformed with the empty vector were used as controls. ACS expression was induced for 1 h with 1 mM IPTG. *A* and *B*, β-oxidation was measured using 0.5 mM [¹⁴C]oleic acid for 1 h at 37 °C as described under "Experimental Procedures." The experiments were performed in triplicate. The means ± S.E. of a representative experiment, repeated two to six times, are plotted. *C* and *D*, relative β-oxidation was calculated ([¹⁴C]CO₂ produced/ACS activity) and normalized (WT and *fadR* controls set at 1).

regulon, thereby inducing their expression (37). If the rat ACSs were not as efficient as FadD in providing the long chain acyl-CoA ligand for FadR, the *fad* genes might be expressed at low levels and limit β-oxidation. To rule out this possibility, we generated BL21(DE3)RIL*fadR* and *fadR fadD* strains. These strains have a mutation in the *fadR* gene that leads to constitutive expression of FadL and the β-oxidation enzymes, independent of the presence of acyl-CoA. Expression of the ACS isoforms and their activities in the *fadD fadR* strain were similar to that shown for BL21(DE3)RIL*fadD* (Figs. 1*B* and
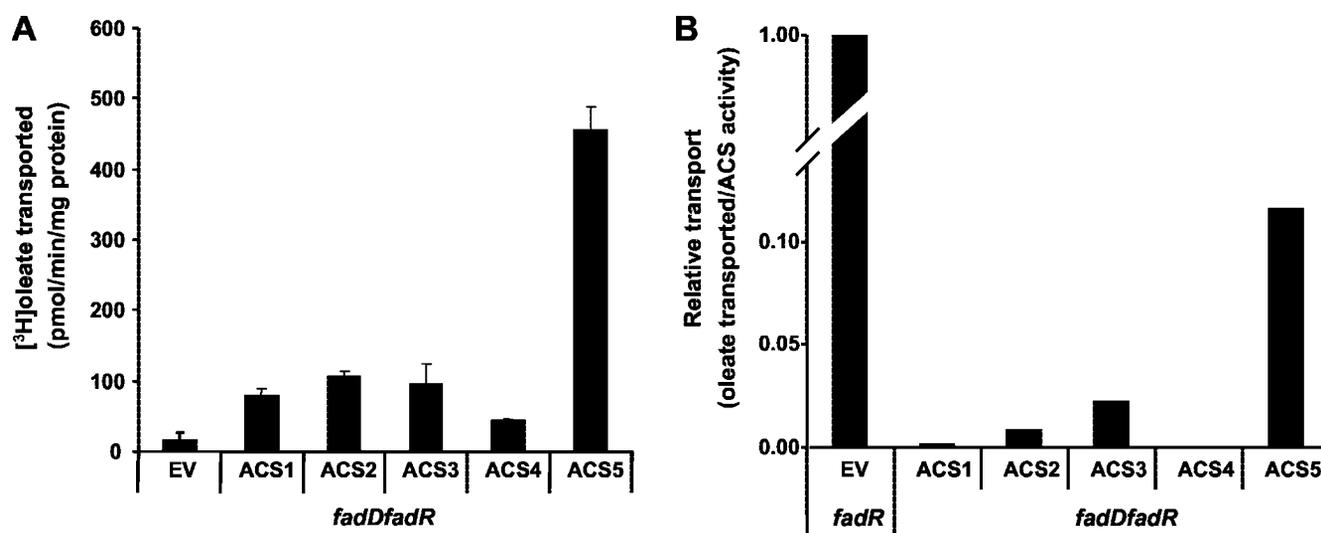
FIG. 4. **Rat ACS isoforms 1–5 partially restore fatty acid transport in *E. coli fadR fadD*.** *A*, BL21(DE3)RIL*fadR fadD* transformed with rat ACS isoforms 1–5 or empty vector (*EV*) were grown to mid-log phase in LB medium. Expression of rat ACSs was induced with 1 mM IPTG for 1 h, and fatty acid transport was measured by adding 100 μM [^{3}H]oleate as described under "Experimental Procedures." The results represent the averages of two independent experiments, each performed in triplicate and plotted as the means $\pm$ S.E. *B*, relative transport was calculated ([^{3}H]oleate transported/ACS activity) and normalized (*fadR* control set at 1).

2*B*). As expected, β-oxidation in BL21(DE3)RIL*fadR* was 3.7-fold higher than observed in the wild type strain (compare the *first bars* in Fig. 3, *A* and *C*). However, the rescue of *fadR fadD* by the five rat ACS isoforms was still only partial (Fig. 3*C*); the values for relative β-oxidation remained low for ACS 1–4 at 0.6–16% of the positive control BL21(DE3)RIL*fadR* (Fig. 3*D*). In contrast, relative β-oxidation for *fadDfadR* bacteria expressing ACS5 was 55% of the *fadR* control. The lower efficiency of rat ACS isoforms 1–4 was not due to low expression of FadL or limiting enzymes of β-oxidation. In both the *fadD* (Fig. 3*B*) and *fadD fadR* (Fig. 3*D*) strains, ACS5 had the highest rate of β-oxidation relative to expression of activity.

Rat ACS Isoforms 1–5 Partially Restore Fatty Acid Transport in BL21(DE3)RILfadR fadD—FadD and FadL comprise the *E. coli* fatty acid import system that pairs fatty acid transport and acylation (5). To determine whether rat ACS isoforms can replace FadD functionally in this import system, oleate transport was measured in BL21(DE3)RIL*fadR fadD* expressing each of the five rat ACS isoforms. Transport was defined as ^{3}[H]oleate incorporation into cells in a traditional filtration assay (see "Experimental Procedures"). Although transport cannot be distinguished from metabolic use in this assay, the experiments were performed during a 4-min period in which the data points are linear and dependent upon both the transporter FadL and an acyl-CoA synthetase, generally bacterial FadD. In the absence of FadD, [^{3}H]oleate import into cells transport was minimal, reflecting the importance of ACS for this process (Fig. 4*A*). Expression of rat ACS isoforms increased fatty acid transport with respect to the negative control harboring the empty vector (Fig. 4*A*). Compared with the transport activity of wild type *E. coli* (1000 pmol/min/mg protein) (28), the relative transport ranged from 0.02% (ACS4) to 12% (ACS5) (Fig. 4*B*). Thus, relative to specific activity, the amount of transport was greatest for ACS5. Although ACS3 protein expression was also low (Fig. 1*B*), its ability to promote fatty acid transport was equivalent to that of ACS1 and 2; thus, toxicity from protein overexpression itself cannot explain these results.

Rat ACS Isoforms 1–5 Activate Fatty Acids for Lipid Synthesis—Vectorial acylation by FadL and FadD allows *E. coli* to use exogenous fatty acids for phospholipid synthesis (36). To study the ability of the rat ACS isoforms to substitute for FadD in

this process, we incubated BL21(DE3)RIL*fadR* and BL21(DE3)RIL*fadDfadR* with [^{14}C]oleate and measured label incorporation into lipid. When FadD was absent, the incorporation of exogenous fatty acid into phospholipid was negligible (Fig. 5*A*). The residual incorporation probably reflects [^{14}C]oleate incorporation into phosphatidyl ethanolamine by the acyl-ACP synthetase/2-acyl-glycerophosphoethanolamine acyltransferase system (38). Expression of the rat ACS isoforms significantly increased the incorporation of [^{14}C]oleate into phospholipid compared with the vector control (Fig. 5*A*), reaching 10–41% of the values for the control strain BL21(DE3)RIL*fadR* during the 30-min incubation. When expressed relative to activity, ACS5 substituted most effectively (85%), and ACS4 substituted least effectively (1%) (Fig. 5*B*). The percentage of [^{14}C]oleate incorporated into different phospholipid species was similar for each of the ACS isoforms (data not shown). Thus, all five rat ACS isoforms could partially replace FadD in providing acyl-CoA for phospholipid synthesis, although each was less efficient than the endogenous bacterial enzyme.

Only ACS5 Supports the Growth of E. coli fadD Mutants on Fatty Acid—Growth of *E. coli* when long chain fatty acids are its sole energy and carbon source requires FadD to activate fatty acids used for both β-oxidation and for the synthesis of membrane phospholipids. To determine whether the low rates of transport, β-oxidation, and phospholipid synthesis observed with the rat ACSs were sufficient for growth, bacteria were streaked on medium EB$_1$ agar plates supplemented with 5 mM oleate in 0.5% Brij58. Plates containing 25 mM glucose were used as controls. As expected, BL21(DE3)RIL*fadD* transformed with the empty vector was unable to grow on oleate (Table I). BL21(DE3)RIL*fadD* transformed with rat ACS5 grew on oleate, although growth was slower than for the wild type strain BL21(DE3)RIL. Bacteria lacking fadD but expressing ACS1, 2, 3, or 4, however, did not grow on EB$_1$ plus oleate. BL21(DE3)RIL*fadD* transformed with either empty vector or the rat ACS isoforms grew in medium that contained glucose, indicating that lack of growth on oleate was due to the unavailability of a usable carbon and energy source. These experiments were repeated using BL21(DE3)RIL*fadR* and BL21(DE3)RIL*fadD fadR* with comparable results (Table I).
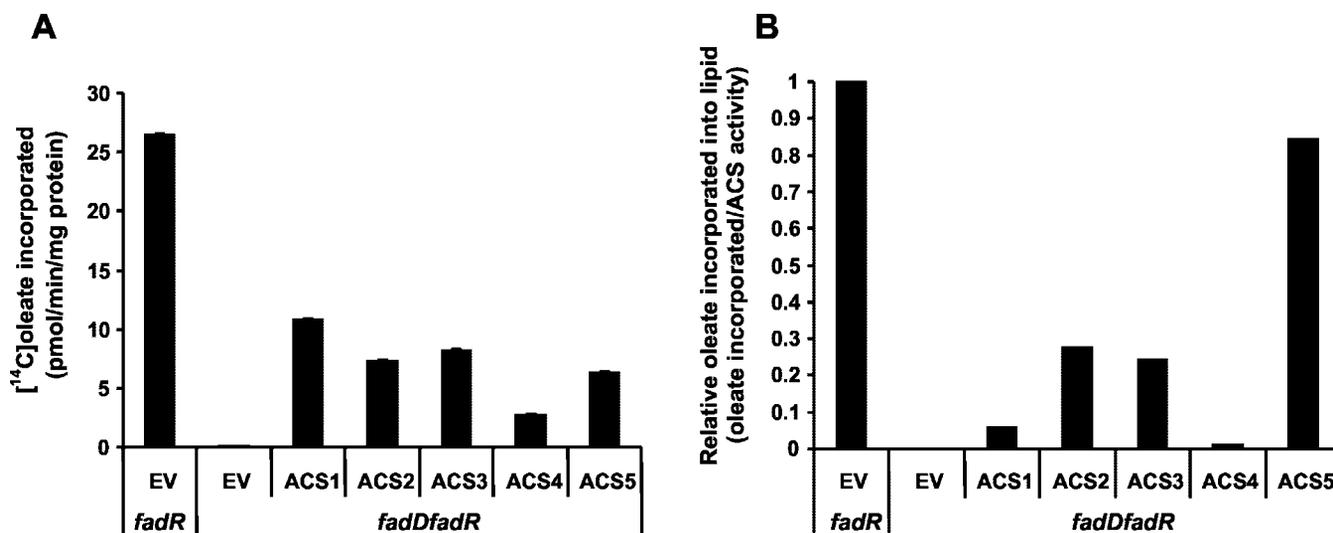
FIG. 5. **Lipid synthesis is partially restored by rat ACS isoforms 1–5.** *A*, BL21(DE3)RIL*fadR* was transformed with empty vector (*EV*). BL21(DE3)RIL*fadR fadD* was transformed with EV or with ACS isoforms. ACS expression was induced for 1 h with 1 mM IPTG. Incorporation of fatty acid into phospholipid was then measured with 20 μM [^{14}C]oleic acid for 30 min at 37 °C as described under "Experimental Procedures." The experiments were performed in triplicate. The means $\pm$ S.E. of a representative experiment, repeated two times, are plotted. *B*, relative oleate incorporation into phospholipids was calculated ([^{14}C]oleate incorporated/ACS activity) and normalized (*fadR* control set at 1).

DISCUSSION

It has become increasingly apparent that the presence of multiple homologs of an enzyme may permit them to perform nonoverlapping functions and that the ability of a specific mammalian enzyme involved in lipid biosynthesis to complement a defective bacterial or yeast gene may provide critical clues about the function or active site of the mammalian ortholog. For example, complementation of the yeast *faa1Δfaa4Δ* strain by rat ACS1 showed the ability of this ACS isoform to activate exogenously provided 14:0 and 16:0 for incorporation into neutral lipid and phospholipid (3). Further, the triacsin C sensitivity of both Faa4p and ACS1 suggested functional analogy. Therefore, we attempted to determine whether any or all of the rat ACS isoforms could complement *E. coli* FadD. FadD contains the AMP/ATP consensus binding site and the putative fatty acid-binding site (39) present in the luciferase/ACS superfamily, and its amino acids show considerable similarity to those of the rat ACSs. Clustal W (www.ebi.ac.uk/clustalw/) analysis shows that the rat ACS isoforms most similar to FadD are isoforms 1, 2, and 5 with 143, 132, and 131 identical amino acids, respectively, and that ACS3 and ACS4 are the least similar, with only 115 and 119 identical amino acids, respectively. LALIGN analysis (40) showed no regions of similarity that were uniquely specific to ACS5 and FadD; nor did ACS3, which was functionally the least active, have unique differences with FadD.

Except for ACS4 (41), the rat ACSs are, or are predicted to be, integral membrane proteins that have active sites facing the cytosol (42). Like FadD, the ACSs use a broad range of fatty acid substrates from 10 to 22 carbons, but they are differentially inhibited by triacsin C and thiazolidinediones and are believed to link acyl-CoAs to different and distinct fates within mammalian cells (2). For example, rat liver ACS1 and 4 are present on endoplasmic reticulum and the related mitochondrial associated membrane (43), and in NIH 3T3 cells ACS1 is located at the plasma membrane (4). In addition, the fungal metabolite triacsin C inhibits recombinant ACS1 and 4 (16), and in human fibroblasts and HepG2 human hepatoma cells, triacsin inhibits triacylglycerol synthesis and *de novo* synthesis of phospholipids from glycerol-3-phosphate (17, 44). Taken together, these data suggest that ACS1 and 4 provide acyl-CoAs for glycerolipid biosynthesis. Conversely, ACS5 is present on

mitochondrial membranes and is not inhibited by triacsin C (16, 43), suggesting that ACS5 might play a role in β-oxidation. ACS2 and 3 have not been extensively studied.

Bacteria synthesize fatty acids as acyl-ACPs that are used primarily for the biogenesis of phospholipids but cannot be used as substrates for β-oxidation. In contrast, exogenous fatty acids are converted by FadD to acyl-CoAs, which are primarily substrates for β-oxidation and, to a minor extent, can be used for phospholipid synthesis (2% activity of acyl-ACP pathway). Because mammalian ACS isoforms appear to be linked to specific pathways that use acyl-CoAs, we wondered whether they would vary in their ability to substitute for FadD in providing acyl-CoAs for β-oxidation and phospholipid synthesis.

We found that rat ACS5, but not ACS1, 2, 3, or 4, was able to complement *E. coli fadD* functionally to allow growth on oleate as the sole carbon source. Whereas all the rat ACS isoforms exhibited some ability to allow fatty acid transport, β-oxidation, and phospholipid synthesis, each of these actions was minimal for ACS1, 2, 3, or 4. It should be noted that fatty acid transport, phospholipid synthesis, and β-oxidation were measured in assays of 2–6, 30, and 60 min, respectively. In contrast, complementation for growth requires sustained energy production and phospholipid synthesis from exogenous fatty acids sufficient for bacterial doubling over about 70–140 generations (24–48 h). The differences we observed are particularly striking when viewed relative to ACS activity. The most divergent pair, ACS4 and ACS5, showed large differences in β-oxidation, oleate transport, and oleate incorporation into phospholipids relative to activity.

Our data are limited by differences in ACS protein expression and relative differences in enzyme activity. It is unclear why expression was excellent for ACS1, 2, 3, and 4 and poor for ACS5 in both bacterial strains and why specific activity relative to expressed protein was excellent for ACS5, moderate for ACS4, and poor for ACS1, 2, and 3.

Why then were four of the rat ACS isoforms functionally ineffective in *E. coli*? It may be that FadD normally interacts with other proteins. These could be related to fatty acid transport across the bacterial membrane or in pathways of β-oxidation or glycerolipid synthesis. Such protein-protein interactions have not been reported in *E. coli* lipid metabolic pathways, although it has been proposed that mammalian cells channel

acyl-CoAs toward distinct fates (2). Although FadD could interact directly with bacterial enzymes of β-oxidation, such interactions would not be possible for the mammalian ACSs, whose active sites face the cytosol (42) rather than the site of fatty acid oxidation within peroxisomes or the mitochondrial matrix.

On the other hand, FadD is thought to "abstract" fatty acids from the inner membrane after their transport through the cell wall by FadL. Unlike most of the rat ACSs, FadD is a soluble protein that is activated upon movement to the bacterial membrane (45). The mammalian ACSs, which would be present in the bacterial membrane (16), should have been able to function similarly in a transport assay. Except for ACS4, the ACSs are integral membrane proteins, and ACS1, 2, and 3 would interact in *E. coli* in a phospholipid milieu very different from the one they would encounter in a mammalian cell. ACS5, however, is positioned on the outer mitochondrial membrane, which, like bacterial membranes, contains cardiolipin (46). No studies have been performed on the possible cardiolipin dependence of ACS5. If, however, the *E. coli* membrane composition altered the transmembrane or activating domains of the ACSs such that catalysis was poor, a false estimate of activity may have been obtained during an *in vitro* measurement in the presence of detergent. Alternatively, the mammalian ACSs might require post-translational modifications that are critical for their *in vivo* function.

Another possibility is that overexpression of the rat ACSs was toxic to the bacteria and that ACS5 supported growth only because less ACS protein was expressed. Certainly the rates of growth on oleate were markedly decreased in the bacteria expressing the rat enzymes. However, if the amount of protein expressed was the problem, growth should also have been poor on minimal glucose plates containing the inducer IPTG. The high expression of exogenous proteins in bacteria growing in a minimal medium might also have diverted limited substrates like amino acids and ATP, thereby compromising the synthesis of other proteins that decrease the growth rate, but growth remained poor, even when low IPTG concentrations were used to decrease the amount of protein expressed. Further, when we repeated the studies of the ACSs in a FadR mutant to ensure that FadL and enzymes of β-oxidation were not limiting and that ATP and amino acids were not being diverted, growth did not improve despite the expected 3.7-fold increase in β-oxidation.

In complementation experiments with both the BL21-(DE3)RIL*fadD* and *fadD fadR* strains, ACS5 consistently supported growth in oleate, whereas ACS isoforms 1–4 were unable to substitute for a lack of FadD. These studies show that although ACS isoforms 1–4 variably exhibited moderate transport activity, β-oxidation, and phospholipid synthesis and although their *in vivo* specific activities were greater than that of FadD, they were unable to substitute functionally for FadD regarding growth. ACS5 consistently showed the highest relative rates of β-oxidation, fatty acid transport, and oleate incorporation into phospholipids. Thus, the membrane composition and protein-protein interactions may be critical in reconstituting bacterial ACS function.

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