JOURNAL OF BACTERIOLOGY, May 1997, p. 2969–2975 Vol. 179, No. 9
0021-9193/97/$04.00+0

Isolation of Mutants of *Acinetobacter calcoaceticus* Deficient in Wax Ester Synthesis and Complementation of One Mutation with a Gene Encoding a Fatty Acyl Coenzyme A Reductase

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Acinetobacter calcoaceticus **BD413 accumulates wax esters and triacylglycerol under conditions of mineral nutrient limitation. Nitrosoguanidine-induced mutants of strain BD413 were isolated that failed to accumulate wax esters under nitrogen-limited growth conditions. One of the mutants, Wow15 (without wax), accumulated wax when grown in the presence of *cis*-11-hexadecenal and hexadecanol but not hexadecane or hexadecanoic acid. This suggested that the mutation may have inactivated a gene encoding either an acyl-acyl carrier protein or acyl-coenzyme A (CoA) reductase. The Wow15 mutant was complemented with a cosmid genomic library prepared from wild-type *A. calcoaceticus* BD413. The complementary region was localized to a single gene (*acr1*) encoding a protein of 32,468 Da that is 44% identical over a region of 264 amino acids to a product of unknown function encoded by an open reading frame associated with mycolic acid synthesis in *Mycobacterium tuberculosis* H37Ra. Extracts of *Escherichia coli* cells expressing the *acr1* gene catalyzed the reduction of acyl-CoA to the corresponding fatty aldehyde, indicating that the gene encodes a novel fatty acyl-CoA reductase.**

Wax esters are utilized in diverse biological roles. Prominent uses include coating the surfaces of plant leaves as epicuticular waxes to provide desiccation tolerance and protection against ultraviolet light and serving as carbon storage inclusions in some bacterial species such as *Acinetobacter calcoaceticus*. Wax esters also have industrial applications, serving as high-temperature lubricants, surface coatings, and emulsifying agents.

The chemical structures of wax esters produced by *A. calcoaceticus* are similar to those found in other organisms such as jojoba (*Simmondsia chinensis* L.), a plant species native to the southwest region of the United States that is used for commercial wax ester production, and the sperm whale, the commercial source of wax esters prior to the worldwide ban on whaling (7). The proposed pathway for wax ester biosynthesis in *A. calcoaceticus* is illustrated in Fig. 1. In this pathway, three enzymes are directly involved in the conversion of acyl-acyl carrier protein (ACP), or acyl-coenzyme A (CoA), to wax esters. In the first step, acyl-CoA (or acyl-ACP) is reduced by an acyl-CoA (or acyl-ACP) reductase to its corresponding fatty aldehyde. This fatty aldehyde is then further reduced to its corresponding fatty alcohol by a fatty aldehyde reductase. Finally, an acyl-CoA (acyl-ACP) fatty alcohol transferase condenses the fatty alcohol with either acyl-ACP or acyl-CoA to give the final wax ester product. The first two steps of this pathway are indirectly supported by biochemical evidence gathered from studies of the metabolism of alkanes by *Acinetobacter* species and from studying wax ester biosynthesis in other microorganisms (10).

Several previous studies have reported that fatty alcohol and fatty aldehyde dehydrogenases exist in various *Acinetobacter* species (6, 11, 24, 25). These observations have been the result of investigations into the ability of *Acinetobacter* species to metabolize hydrocarbons, particularly alkanes. However, none of these reports directly address the question of whether these enzymes are directly involved in wax ester biosynthesis by assaying for the ability of the enzyme to catalyze the reduction of acyl-CoA (or acyl-ACP) or fatty aldehyde to its corresponding product. There has been no direct biochemical evidence reported for the final step involving the acyl-CoA (or acyl-ACP) fatty alcohol transferase in *Acinetobacter* species.

The purification of enzymes and the cloning of the corresponding genes for enzymes involved in wax ester biosynthesis from jojoba have been described in several United States patents (16, 18). In these patents it is reported that the jojoba acyl-CoA reductase is able to catalyze the conversion of long chain acyl-CoAs (18 to 24 carbons in length) directly to the corresponding fatty alcohol via a fatty aldehyde intermediate. Results presented here indicate that in *A. calcoaceticus* more than one enzyme is required to catalyze the overall conversion of acyl-CoA to the corresponding alcohol. The *A. calcoaceticus* enzyme also exhibits different chain-length specificity from the jojoba enzyme.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1.

Growth and culture conditions. Low nitrogen minimal medium (LNMM) was used in experiments with *A. calcoaceticus* BD413 to induce wax ester formation. LNMM contained the following per liter: 2.0 g of KH_2PO_4, 1.18 g of succinic acid, 0.1 g of NH_4SO_4, and 980 ml of water adjusted to pH 7.0 with KOH; after autoclaving, 20 ml of sterile 2% $MgSO_4$ was added. High nitrogen minimal medium was the same as LNMM except for the addition of 1.0 g of NH_4SO_4 per liter. In some experiments 0.3% hexadecane, hexadecanol, hexadecanoic acid, or *cis*-11-hexadecenal was added to LNMM. Hexadecane, hexadecanoic acid, and hexadecanol were dispersed in growth medium by sonication at full power for approximately 2 min.

A. calcoaceticus cultures were typically grown overnight with shaking at 30°C. For the growth of cultures of 50 ml or more, 3-ml overnight cultures were collected by centrifugation and the cell pellet was washed in medium, suspended in fresh medium, and incubated at 30°C overnight. For other purposes, such as DNA isolation, *A. calcoaceticus* was grown and maintained on Luria Bertani (LB) medium (22). Maintenance and growth of *Escherichia coli* strains was on LB medium with appropriate antibiotics. Antibiotics were commonly used at the following concentrations for both *A. calcoaceticus* and *E. coli*: ampicillin (AP), 100 μg/ml; chloramphenicol (CM), 50 μg/ml; kanamycin (KM), 25 μg/ml; rifampin (RIF), 100 μg/ml; and tetracycline (TC), 15 μg/ml.

Mutagenesis. A 25-ml culture of *A. calcoaceticus* BD413 was grown in LB medium at 30°C until the optical density (OD) at 600 nm was approximately 0.6.

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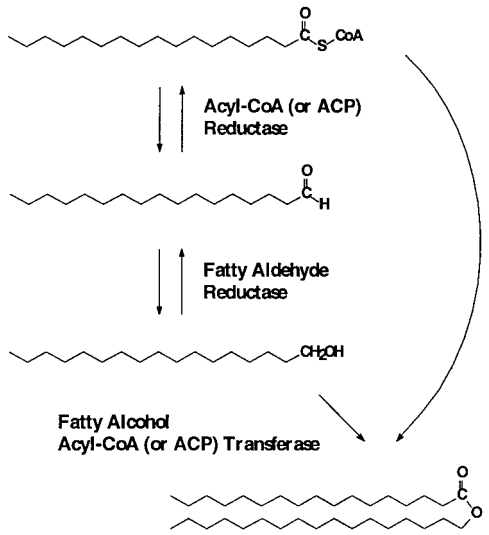

FIG. 1. Proposed pathway for wax ester biosynthesis in *A. calcoaceticus* and other microorganisms (1, 5, 11, 25, 26). Wax biosynthesis could begin with either an acyl-CoA (as shown) or an acyl-ACP substrate.

Nitrosoguanidine was added to the culture at a final concentration of 0.1 mg/ml. The sample was incubated for 50 min at room temperature on an orbital shaker. The cells were collected by centrifugation, washed twice with fresh LB medium, and then incubated overnight at 30°C before being plated onto LB plates. The resulting colonies were transferred onto LB master plates in arrays of 100 colonies per 100-mm-diameter petri plate. The efficiency of mutagenesis was evaluated by scoring for the presence of auxotropic mutations (20).

Mutant screening with Sudan Black B staining. The master plates containing the mutagenized cells were replica plated onto LNMM and incubated overnight at 30°C to induce wax accumulation. The cells were then stained by irrigating the plates with Sudan Black B (0.02% in 50:45:5 dimethyl sulfoxide-ethanol-water) and gently shaking them for approximately 20 min. The stain was aspirated away, and the plates were carefully washed with 70% ethanol by gently shaking them at room temperature for approximately 2 min. Lighter-staining colonies were identified from the stained plates, and the corresponding colony from the master plate was subsequently analyzed by thin-layer chromatography (TLC).

TLC. For mutant screening, *A. calcoaceticus* samples to be analyzed by TLC were typically grown in 3-ml cultures in LNMM. Samples were collected by centrifugation (5 min at 3,000 × *g*), the cells were washed in fresh medium and centrifuged, and the supernatant was discarded. Neutral lipids were isolated by extracting the pellet with 6% of the original culture volume of chloroform-methanol (50:50) followed by phase separation using 0.3 volumes of 1 M potassium chloride in 0.2 M phosphoric acid. Samples were centrifuged at 2,000 × *g* at room temperature for 2 min. The chloroform phase was transferred to a glass tube and dried under nitrogen or in some cases directly spotted onto a TLC plate. Samples were resuspended in a volume of chloroform that was 1% of the original culture volume. Twenty microliters of these samples was spotted onto heat-dried (110°C for 20 min) Si-250 TLC plates with preadsorbant layers (Baker). Standards (0.02 mg) of known lipids were also loaded for comparison during subsequent analysis. The neutral lipids were separated by developing the plates in hexane-ethyl ether-acetic acid (90:10:1). Samples were visualized by spraying the plates with 50% sulfuric acid and charring at 160° for approximately 5 min or by iodine staining.

DNA isolation and library construction. *A. calcoaceticus* genomic DNA for the construction of the cosmid library was prepared in the following manner. A 200-ml culture of *A. calcoaceticus* BD413 was grown overnight in LB medium. Cells were collected by centrifugation and resuspended in 16 ml of buffer-A (8% sucrose, 50 mM Tris-HCl [pH 8.0], 50 mM EDTA [pH 8.0]). Lysozyme (Sigma, St. Louis, Mo.) was added to a final concentration of 2 mg/ml. Cells were incubated at 30°C for 30 min to make spheroplasts. Twenty-four milliliters of lysis buffer (3% sodium dodecyl sulfate [SDS], 0.5 M Tris-HCl [pH 8.0], 0.2 M EDTA) was added, and the sample was incubated at 65°C for 30 min and then cooled on ice. Ten milliliters of the sample was then layered on top of a sucrose step gradient consisting of 5 ml of 50% sucrose, 10 ml of 20% sucrose, and 10 ml of 15% sucrose. The gradient was centrifuged in a Beckman SW22 swing-out rotor at 27,000 rpm for 3 h at 15°C. The DNA was recovered above the 50% sucrose block and dialyzed in Tris-EDTA (TE) buffer overnight. The DNA was gently extracted with 1 volume of phenol, extracted with 1 volume of chloroform-isoamyl alcohol (24:1), and dialyzed against TE buffer. The DNA was partially digested with *Sau*IIIA to a mean size of approximately 25 kb. Fragments of approximately 25 kb and larger were size selected by running the partially digested DNA on a 0.6% agarose gel, excising the band containing the fragments of interest from the gel, and isolating the DNA by electroelution (21). The fragments were ligated to *Bgl*II-digested cosmid pLA2917 and packaged with commercially available packaging extracts (Stratagene, La Jolla, Calif.). Approximately 1,300 *E. coli* HB101 primary transfectants were selected and transferred to 96-well plates. Examination by restriction analysis of 19 randomly chosen cosmids from the library indicated that 68% of the transfectants contained inserts.

Triparental filter matings. Identification of complementary cosmids was carried out by triparental filter matings of the cosmid genomic library to the Wow15 mutant in the presence of the helper strain MM294. The cosmid library was contained in *E. coli* HB101, which was Tc^r due to the presence of the cosmid. MM294 transformed with pRK2013 (Km^r) was used as a helper strain that enabled triparental mating through its *mob* genes. A spontaneous Rif^r derivative of Wow15, designated Wow15:Rif^r was the recipient. At the end of the mating, Wow15:Rif^r mutants containing the cosmids were selected by plating the products of the cross onto LB medium containing RIF, to select for Wow15:Rif^r, and TC to select for the presence of the cosmid. MM294 and the HB101 donor fail to grow under these conditions.

The Wow15 strain and MM294 were grown overnight as 3-ml cultures, and the library was grown in 96-well plates as replicates of the original. The day of the mating, 0.5 ml of MM294 was used to inoculate a 50-ml culture, and 3 ml of Wow15 was used to inoculate a 50-ml culture. Cultures were collected at an OD_{600} of 0.6, washed, and then resuspended in 50 ml of LB medium. Twenty-five milliliters of MM294 was combined with 50 ml of the Wow15 culture. Ten milliliters of this mixture was drawn onto a sterilized filter (pore size, 45 μm; diameter, 85 mm) via a vacuum apparatus, creating an even lawn of bacteria. The filter was then removed and placed onto an LB plate. Approximately 3-μl aliquots of the library were transferred onto the lawn of bacteria by using a multipronged device, and matings were allowed to incubate at 30°C overnight. Filters were then transferred to selective medium containing RIF and TC to select for Wow15:Rif^r containing the cosmids. The resulting patches were then transferred onto a master plate containing the selective medium before being replica plated onto LNMM for analysis by Sudan Black staining and TLC.

Transposon mutagenesis of the complementary cosmid. Defective phage λNK1324 (13), which carries a Cm^r gene on a Tn*10* derivative, was used to transfect *E. coli* MG1655 containing the cosmid that complemented the Wow15 mutant. Colonies were selected on TC for the presence of the cosmid and on CM

TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Relevant characteristics	Source or reference
Strains		
A. calcoaceticus		
BD413	ATCC 33305; unencapsulated mutant of BD4	12
Wow1	Wax^- mutant of BD413	20
Wow3	Wax^-Tag^- mutant of BD413	20
Wow15	Wax^- mutant of BD413	This study
Wow15:Rif^r	Rif^r mutant of Wow15	This study
E. coli		
HB101	F^- *proA2 recA13 mcrB*	21
DH5α	F^- *endA1 recA1* ϕ80d *lac* Δ(*lacZM15*)	19
MM294	F^- *endA1 hsdR17 thi-1*	5
BD21(DE3)	F^- *ompT* $hsdS_B$ (r_B^- m_B^-) *gal dcm*	Novagen
MG1655	*rph-1*	13
Phage λNK1324	mini-Tn*10*Cm^r	13
Plasmids		
pRK2013	Km^r Tra^+ RK2-$ColE1_{rep}$	8
pLA2917	Km^r Tc^r cos	1
pET21	*E. coli* expression vector	Novagen
pSER2	*A. calcoaceticus-E. coli* shuttle vector (Tc^r Km^r)	20
pSR2	Bluescript with *Eco*RV fragment from 4A-55	This study
pSR6	Bluescript with *Eco*RV fragment from 4A-55	This study
pSER2:*acr1*	pSER2 derivative containing PCR-amplified *acr1*	This study
pET21:*acr1*	pET21 derivative containing *acr1*	This study
4A-55	pLA2917 derived cosmid clone that complements the Wow15 mutant	This study

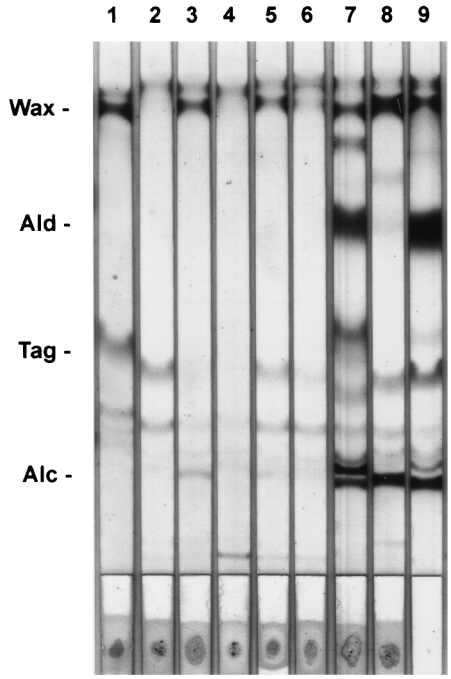

FIG. 2. TLC separation of lipids from mutant and wild-type *A. calcoaceticus* cells grown under low nitrogen conditions. Lane 1, BD413; lane 2, Wow15, a class I (Wax^-) mutant; lane 3, Wow7, a (Tag^-) class II mutant; lane 4, Wow3, a class III (Wax^-Tag^-) mutant; lane 5, Wow15 (pSER2::*acr1*); lanes 6 to 8, lipids from Wow15 grown in LNMM supplemented with 0.3% hexadecane, *cis*-11-hexadecenal, or hexadecanol, respectively (extracted lipids were spotted proportionately based on cellular wet weight); lane 9, standards containing 0.02 mg of palmitoyl-palmitic acid ester (Wax), *cis*-11-hexadecenal (Ald), triacylglycerol (Tag), and hexadecanol (Alc).

for the presence of the transposon. To separate insertion events that were located in the bacterial genome from the desired insertions in the cosmid, approximately 3,000 transfectants were pooled and cosmid DNA was isolated and used to transform *E. coli* DH5α to Tc^r and Cm^r. One hundred ninety-six of the resulting transformants were transferred to 96-well plates and used for triparental matings with Wow15 as previously described. The resulting exconjugates were then screened by TLC for insertions that resulted in the loss of the ability of the cosmid to complement the mutant phenotypes.

Overexpression of *acr1* in *E. coli*, protein purification, and separation. To construct pSER2:*acr1*, the *acr1* gene was amplified from cosmid 4A-55 by PCR with oligonucleotide primers (GCAGGATCCTTGGGATTGAACATATTG and GCAGGATCCGGTGCGATTTATGATGTA), which created *Bam*HI sites at the ends. The PCR product was digested with *Bam*HI and ligated into the *Bam*HI site of an *A. calcoaceticus-E. coli* shuttle vector, pSER2 (20).

To construct pET21:*acr1* the *acr1* gene was amplified by PCR from the complementary cosmid with oligonucleotide primers containing *Bam*HI (GCAGGA TCCAAAACATTGGTAATTTCAGATACT) and *Eco*RI (GCAGAATTCGG TGCGATTTATGATGTA) linkers for directional cloning. The PCR product was gel purified, digested, and subcloned into pET21 (Novagen, Madison, Wis.) to produce plasmid pET21:*acr1*, which was used to transform *E. coli* BL21(DE3). For protein expression studies, *E. coli* BD21(DE3) was grown in 50 ml of LB broth with AP to an OD_{600} of 0.6. Expression of the *acr1* gene was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Cells were grown for 2.5 h and collected by centrifugation at 5,000 × *g* for 10 min. Cells were resuspended in 125 mM sodium phosphate buffer (pH 7.4) and incubated at 30°C for 15 min in the presence of 100 μg of lysozyme/ml. Cells were then sonicated for two 40-s bursts at maximum power. Soluble proteins were separated from cell walls and insoluble materials by centrifugation at 35,000 × *g* for 30 min at 4°C. The soluble fraction was collected as fraction I. The insoluble fraction was resuspended in 0.5 ml of phosphate buffer by a 10-s burst at maximum power with a sonicator equipped with a microtip. SDS-polyacrylamide gel electrophoresis (PAGE) analysis of proteins was carried out using Pharmacia's Phast Gel system with 12.5% homogeneous gels and silver staining.

Enzymatic assays for reductase activity. Acyl-CoA reductase activity was measured at 30°C for 12 min in 30-μl assays containing 125 mM sodium phosphate buffer (pH 7.4), 100 μM NADPH, 30 μM palmitoyl-1-[^{14}C]CoA (44.4 mCi/mmol; Dupont/NEN, Wilmington, Del.), and 13.2 μg of protein. The assays were then extracted with 75 μl of chloroform-methanol (50:50), vortexed for 10 s, and centrifuged for 20 s in a microcentrifuge. The chloroform phase was removed and spotted onto a TLC plate, and the lipids were separated with hexane-ethyl ether-acetic acid (90:10:1). After drying, the TLC plate was exposed to a phosphorimaging cassette. Assays for Acyl-ACP reductase (9 μM final concentration), palmitic acid reductase (65 μM final concentration), or hexadecanal reductase (1 μM final concentration) were performed by substituting the indicated substrate for acyl-CoA in the standard assay conditions described above.

^{14}C-radiolabelled 1-hexadecanal was prepared enzymatically by incubating protein extracts of *E. coli* cells transformed with pET21:*acr1* with palmitoyl-1-[^{14}C]CoA and resolving the reaction products as described above. The aldehyde was recovered from the TLC plates and solubilized in 0.1% of either Triton X-100, Tween 40, or Nonidet P-40. This substrate was then used in place of acyl-CoA in the assay described above. The final concentration of detergent in these assays was 0.01%.

Spectroscopic assays were carried out by measuring the decrease in absorbance at 340 nm from the oxidation of NADPH to $NADP^+$. Activity was measured at room temperature in 100-μl assays containing 125 mM sodium phosphate buffer (pH 7.4), 250 μM NADPH, 100 μM acyl-CoA, and 13.2 μg of protein. Assays were initiated by the addition of NADPH.

DNA hybridization, cloning, and sequencing. Southern blot hybridization and colony hybridization were performed with Hybond-N membranes (Amersham, Arlington Heights, Ill.) by standard techniques (21). The nucleotide sequence of cloned fragments was determined by the dideoxy chain-termination method on an Applied Biosystems (Foster City, Calif.) AB1310 Sequenator according to the manufacturer's instructions. Nested deletions of fragments to be sequenced were made by using Erase-a-Base (Promega). Nucleotide sequences and deduced amino acid sequences were compared to sequence information in GenBank release 92.0 by using the BLAST programs (2) via an electronic mail server.

Nucleotide sequence accession number. The nucleotide sequence reported here has been deposited at GenBank with accession number U77680.

RESULTS

Isolation of wax-deficient mutants. Approximately 6,400 mutagenized colonies of *A. calcoaceticus* BD413 were screened for reduced wax ester content on LNMM by staining with Sudan Black B. This resulted in the identification of 25 mutants which were divided into three phenotypic classes based

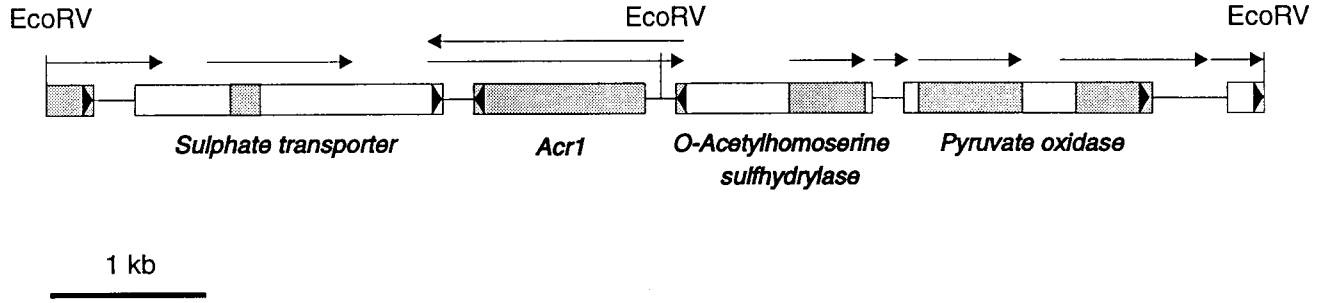

FIG. 3. Putative ORFs flanking the *acr1* gene. The arrows depict the regions for which nucleotide sequences were obtained (20). Boxed regions indicate the approximate extent of ORFs with regions of significant deduced amino acid sequence homology to entries in GenBank release 92.0. Regions of significant homology between the *A. calcoaceticus* sequence and the GenBank entries are represented by the shaded areas. The arrowheads indicate the direction of transcription.

on the abundance of wax esters and triacylglycerol in cells grown under nitrogen-limited conditions (Fig. 2). Class I mutants failed to accumulate wax esters. Class II mutants lacked triacylglycerol, and class III mutants did not accumulate either wax esters or triacylglycerol. A class I mutant designated Wow15, which exhibited a normal growth rate in LNMM and lacked detectable wax, was chosen for detailed analysis.

It has previously been shown that *A. calcoaceticus* is able to metabolize various hydrocarbons and fatty alcohols as carbon sources (7, 15, 23). Additionally, under low nitrogen conditions, these compounds are directly incorporated into wax esters (6, 22, 23). The ability of the Wow15 mutant to incorporate exogenous precursors into wax was tested by growing the mutant on nitrogen-limited minimal medium in the presence of hexadecane, hexadecanoic acid, *cis*-11-hexadecenal, and 1-hexadecanol. Growth of Wow15 in hexadecane did not result in wax accumulation (Fig. 2). By contrast, growth of the Wow15 mutant in the presence of *cis*-11-hexadecenal or hexadecanol restored the ability of the mutant to accumulate wax esters when grown under low nitrogen conditions (Fig. 2). These observations implied that the mutant was able to reduce a fatty aldehyde to the corresponding alcohol and to condense the fatty alcohol with acyl-CoA or acyl-ACP to produce wax esters. Thus, this result suggested that the Wow15 mutant may be the result of a mutation in a gene encoding an acyl-CoA or acyl-ACP reductase.

Complementation of the Wow15 mutant. The Wow15 mutant was genetically complemented by two cosmids from a genomic library of the wild type, constructed in the broad-host-range vector pLA2917. In order to identify the relevant gene, one of these cosmids, 4A-55, was mutagenized with mini-Tn*10*Cm^r carried on λNK1324 (13). Of 196 mini-Tn*10*Cm^r insertions into 4A-55, 8 inactivated the ability of the cosmid to complement the Wow15 mutant. The eight mutagenized cosmids were digested with several restriction enzymes, and the resulting restriction patterns were compared to that of 4A-55. It was found that all of the transposon insertions were in two adjacent *Eco*RV fragments of approximately 4 kb in size (Fig. 3).

Because the region of interest was known to span the two *Eco*RV fragments, a preliminary nucleotide sequence for most of the 8-kb region was determined on one strand, and the coding capacity of the region was assessed by comparing the hypothetical translation products to the polypeptide sequences in GenBank release 92. An 834-nucleotide open reading frame, designated *acr1*, was identified 17 nucleotides downstream of the central *Eco*RV site (Fig. 4). Inspection of the *acr1* open reading frame indicated that a protein of 32,468 Da, would be encoded with GTG as a translational initiation codon compared to a 24,655 Da protein when the first in-frame ATG is used. The use of GTG as an initiation codon was suggested by the presence of a relatively conserved Shine-Dalgarno sequence upstream of the predicted start site (346 to 349 bp), while there is no such conserved sequence upstream of the first ATG codon (Fig. 4).

In order to test the function of the *acr1* gene, the open reading frame along with 156 nucleotides of putative 5′-noncoding sequence and 110 nucleotides of putative 3′-noncoding sequence was amplified by PCR with cosmid 4A-55 as the template, and the product was cloned into pSER2, an *A. calcoaceticus-E. coli* shuttle vector constructed for this purpose (20). The resulting plasmid, pSER2:*acr1*, was used to transform the Wow15 mutant. Comparison of the lipid content of the mutant and the transformed mutant grown under low nitrogen, wax-inducing conditions revealed that pSER2:*acr1* complemented the Wow15 mutant phenotype (Fig. 2).

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   1 CAGAAGATATGGTTCGGTTATCGGTTGGGATTGAACATATTGATG
  46 ATTTGATTGCAGATCTGGAACAAGCATTGGCCACAGTTTGAGCGT
  91 AAATTTTATAAAAAACCTCTGCAATTTCAGAGGTTTTTTTATATT
 136 TGCTTTATTATCGTATGATGTTCATAATTGATCTAGCAAATAATA
 181 AAAATTAGAGCAATTACTCTAAAAACATTTGTAATTTCAGATACT
 226 TAACACTAGATTTTTTAACCAAATCACTTTAGATTAACTTTAGTT
 271 CTGGAAATTTTATTTCCCTTTAACCGTCTTCAATCCAAATACAAT
   1                  EcoRV                      M
 316 AATGACAGCCTTTACAGTTTGATATCAATCAGGGAAAAACGCGTG
   2  N  K  K  L  E  A  L  F  R  E  N  V  K  G  K
 361 AACAAAAAACTTGAAGCTCTCTTCCGAGAGAATGTAAAAGGTAAA
  17  V  A  L  I  T  G  A  S  S  G  I  G  L  T  I
 406 GTGGCTTTGATCACTGGTGCATCTAGTGGAATCGGTTTGACGATT
  32  A  K  R  I  A  A  A  G  A  H  V  L  L  V  A
 451 GCAAAAAGAATTGCTGCGGCAGGTGCTCATGTATTATTGGTTGCC
  47  R  T  Q  E  T  L  E  E  V  K  A  A  I  E  Q
 496 CGAACCCAAGAAACACTGGAAGAAGTGAAAGCTGCAATTGAACAG
  62  Q  G  G  Q  A  S  I  F  P  C  D  L  T  D  M
 541 CAAGGGGGACAGGCCTCTATTTTTCCTTGTGACCTGACTGACATG
  77  N  A  I  D  Q  L  S  Q  Q  I  M  A  S  V  D
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  92  H  V  D  F  L  I  N  N  A  G  R  S  I  R  R
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 137  L  P  H  M  I  K  R  K  N  G  Q  I  I  N  I
 766 CTGCCACATATGATTAAGCGTAAAAATGGCCAGATCATCAATATC
 152  S  S  I  G  V  L  A  N  A  T  R  F  S  A  Y
 811 AGCTCTATTGGTGTATTGGCCAATGCGACCCGTTTTTCTGCTTAT
 167  V  A  S  K  A  A  L  D  A  F  S  R  C  L  S
 856 GTCGCGTCTAAAGCTGCGCTGGATGCCTTCAGTCGCTGTCTTTCA
 182  A  E  V  L  K  H  K  I  S  I  T  S  I  Y  M
 901 GCCGAGGTACTCAAGCATAAAATCTCAATTACCTCGATTTATATG
 197  P  L  V  R  T  P  M  I  A  P  T  K  I  Y  K
 946 CCATTGGTGCGTACCCCAATGATCGCACCCACCAAAATTTATAAA
 212  Y  V  P  T  L  S  P  E  E  A  A  D  L  I  V
 991 TACGTGCCCACGCTTTCCCCAGAAGAAGCCGCAGATCTCATTGTC
 227  Y  A  I  V  K  R  P  T  R  I  A  T  H  L  G
1036 TACGCCATTGTGAAACGTCCAACACGTATTGCGACGCACTTGGGT
 242  R  L  A  S  I  T  Y  A  I  A  P  D  I  N  N
1081 CGTCTGGCGTCAATTACCTATGCCATCGCACCAGACATCAATAAT
 257  I  L  M  S  I  G  F  N  L  F  P  S  S  T  A
1126 ATTCTGATGTCGATTGGATTTAACCTATTCCCAAGCTCAACGGCT
 272  A  L  G  E  Q  E  K  L  N  L  L  Q  R  A  Y
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1396 GAACTATGCTGAATAATGTATTGAATATTATAGTGAACATTCATA
1441 GCAGTCACCTGATCAATAAATGCTTTGCTTAATTCACGCGGTTGC
1486 ATAATATCAAATACACTGCCAACCGAATGCATGAGTGGCCCAAGC
1531 ACGTATTGGATGTGTGGCATTTCCTGAATATCGGAAATCTGCTTA
1576 TGTTGCAATCTTAACTGGCATGCGCTGGTGACCGCACCACAGTCG
1621 GTATGTCCCAAAACCAGAATCACTTTGGAACCTTTGGCTTGACAG
1666 GCAAA
```

FIG. 4. DNA and protein sequence of the region containing *acr1*. A possible Shine-Dalgarno sequence is indicated in boldface type (346 to 349 bp). The *Eco*RV site which divides the two *Eco*RV fragments which were subcloned to give pSR2 and pSR6 is indicated at 336 to 341 bp. Priming sites used for PCR to generate pSER2:*acr1* and pET21:*acr1* are double underlined.

Analysis of the open reading frames on either side of the *acr1* gene indicated that the nearest downstream open reading frame encodes a product which is homologous to a 667-amino-acid high-affinity sulfate transporter from *Stylosanthese hamata* (BLASTX score, 140; probability, 5.5×10^{-10}). This putative gene is transcribed from the opposite strand from that of the *acr1* gene. The nearest upstream open reading frame encodes a product which had significant homology to a 443-amino-acid *O*-acetylserine sulfhydrylase from yeast (BLASTX score, 359; probability, 4.8×10^{-52}) which is encoded by a gene transcribed in the same direction as the *acr1* gene. Upstream of this was a gene product that was highly homologous to pyruvate oxidase from *E. coli* (BLASTX score, 330; probability, 4.9×10^{-51}) and encoded by a gene that was transcribed from the

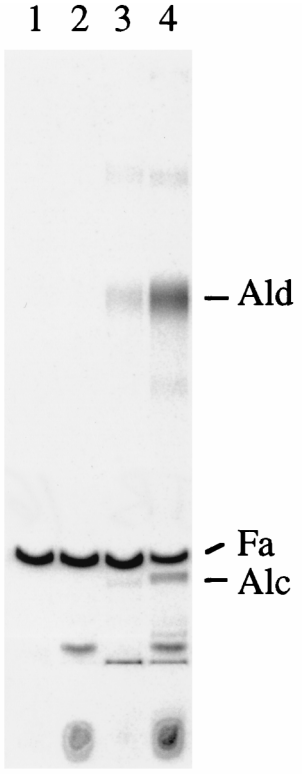

FIG. 5. Measurement of acyl-CoA reductase activity. Soluble (lane 1) and insoluble (lane 2) fractions from extracts of *E. coli* containing pET21 were compared to soluble (lane 3) and insoluble (lane 4) protein preparations from *E. coli* transformed with pET21:*acr1*. Ald, fatty aldehyde; Alc, fatty alcohol; FA, fatty acid.

other strand relative to the *acr1* gene. These putative assignments suggest that the *acr1* gene is not contained in an operon of functionally related genes.

Expression of the *acr1* gene in *E. coli*. In order to assess the enzymatic properties of the *acr1* gene product, the *acr1* open reading frame was subcloned into pET21, an *E. coli* expression vector. Induction of transcription of the *acr1* gene carried on pET21:*acr1* in *E. coli* resulted in the accumulation of a protein with an apparent molecular mass of 32 kDa when whole-cell extracts were analyzed by SDS-PAGE (results not presented). Sonicated extracts of lysozyme-treated IPTG-induced cells were fractionated into buffer-soluble and -insoluble fractions by centrifugation (35,000 × *g* for 30 min). The 32-kDa polypeptide was found almost exclusively in the insoluble fraction. The size of the induced protein supports the notion that translational initiation takes place at the GTG initiation codon rather than at the first ATG, which would give rise to a 25-kDa product.

Assays for acyl-CoA, acyl-ACP, and palmitic acid reductase activity were carried out using both the soluble and insoluble fractions from protein extracts of *E. coli* cells carrying pET21:*acr1*. NADPH-dependent acyl-CoA reductase activity was observed to be associated primarily with the insoluble fraction (Fig. 5). No activity was observed in extracts prepared from *E. coli* transformed with pET21. No activity was observed when NADH was used as a cofactor, nor was the enzyme able to reduce free fatty acids or acyl-ACP to their corresponding fatty aldehydes (21).

When [^{14}C]palmitoyl-CoA was used as the substrate in the acyl-CoA reductase assay, the principal product of the reaction was the corresponding aldehyde, 1-hexadecanal (Fig. 5). However, significant amounts of 1-hexadecanol were also observed, which presumably resulted from the reduction of the fatty aldehyde. This observation raised the possibility that the *acr1* gene product might also catalyze the conversion of fatty aldehydes to fatty alcohols. Such a bifunctional acyl-CoA reductase has been reported in jojoba (18). Fatty aldehyde reductase activity was assayed by the same method used to measure fatty acyl-CoA reductase except that the substrate was hexadecanal solubilized in a final concentration of 0.01% nonionic detergent (Triton X-100, Tween 40, or Nonidet P-40). Extracts of *E. coli* lacking the *acr1* gene product catalyzed the reduction of hexadecanal to hexadecanol at a rate that was indistinguishable from that of extracts from IPTG-induced cells of *E. coli* containing pET21:*acr1* (20). Because of the insolubility of the *acr1* gene product in *E. coli*, it was not feasible to purify the Acr1 enzyme from the endogenous aldehyde reductase activity in order to assay it for aldehyde reductase activity. Indeed, spectroscopic assays for acyl-CoA reductase activity carried out with the level of detergent required to solubilize hexadecanal indicated that acyl-CoA reductase activity was inactive (results not presented). Thus, we concluded that the conversion of fatty aldehyde to fatty alcohol observed in the assays could be attributed to an endogenous activity in *E. coli*, and there was no evidence that the Acr1 acyl-CoA reductase has any aldehyde reductase activity. The band corresponding to free fatty acid is most likely the result of contaminating thioesterase activity present in the crude extracts.

TABLE 2. Assays of Acr1 enzyme activity in extracts of *E. coli* (pET21:*acr1*) with various acyl-CoA substrates

Substrate	Oxidation of NADPH (pmol μg^{-1} min^{-1})	Relative activity	Relative activity observed in competition assay[b]
C12	N.D.[a]	N.D.	70.2
C14	6.97×10^{-2}	65.1	33.4
C16	1.07×10^{-1}	100	19.5
C18	8.26×10^{-2}	77.2	16.6
C20	8.33×10^{-3}	7.8	17.1
C22	1.36×10^{-2}	12.7	9.9
C24	N.D.	N.D.	13.2

[a] N.D., not detected; no activity above background was detected.
[b] Relative rate of reduction of [^{14}C]palmitoyl-CoA in the presence of 100 μM unlabelled substrate compared to the rate in the absence of unlabelled substrate.

The substrate specificity of the Acr1 protein for acyl-CoA substrates of various chain lengths was assayed spectroscopically by measuring the acyl-CoA-dependent oxidation of NADPH (Table 2). The results of these experiments indicated that the Acr1 protein was able to act on acyl-CoAs with chain lengths of 14 to 22 carbons. The acyl-CoAs tested can be ranked in the following order of substrate preference based on the rates of NADPH oxidation observed: $C_{16} > C_{18} > C_{14} > C_{20} > C_{22}$. No activity was observed to be associated with C_{12} and C_{24} acyl-CoAs by this assay.

The chain length specificity of the Acr1 protein was also examined by examining the ability of equimolar amounts of unlabelled acyl-CoAs of various chain lengths to compete with [^{14}C]palmitoyl-CoA as a substrate (Table 2). The results of these experiments showed that the long-chain acyl-CoAs were almost as effective inhibitors of activity as oleoyl-CoA, even though they did not appear to be effective substrates by the spectrophotometric assays. Because of the different solubility properties of the various acyl-CoAs, we believe that this observation is not readily interpretable.

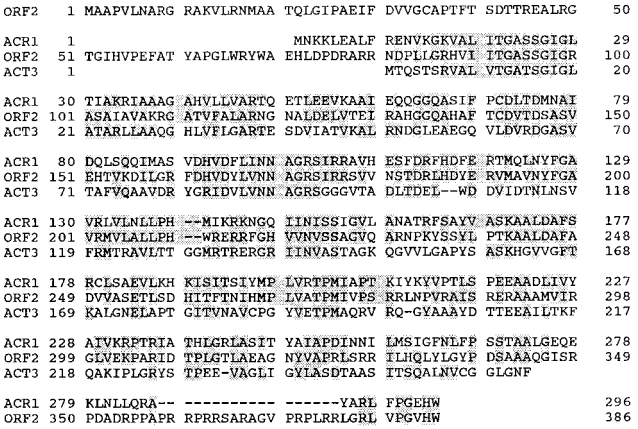

FIG. 6. Alignment of similar regions (shaded areas) of Acr1, the ORF2 product from *M. tuberculosis* and ActIII from *S. cinnamonensis*.

Sequence comparisons with known proteins. Comparison of the deduced amino acid sequence of the *acr1* gene with the sequences in GenBank release 92.0 indicated that the *acr1* gene product was very similar to a product encoded by an open reading frame designated ORF2 identified in *Mycobacterium tuberculosis* H37Ra (accession number U27357). A direct comparison of the two protein sequences showed them to be 44.7% identical over 264 amino acids (Fig. 6). ORF2 is an open reading frame that resides between *cma1*, which encodes cyclopropane mycolic acid synthase, and ORF3, an open reading frame which encodes a product with 35% identity (over 278 amino acids) to a trifunctional hydratase/dehydrogenase/epimerase from *Candida tropicalis* which is involved in peroxisomal degradation of fatty acids (27). The ORF2 gene product is also homologous to ActIII (30% identity over 188 amino acids), a β-ketoacyl reductase involved in chain elongation during polyketide biosynthesis in *Streptomyces cinnamonensis* (3, 27). Based on this similarity and its location between *cma1* and ORF3, Yuan et al. concluded that the probable role of ORF2 was in mycolic acid metabolism (27).

A three-way alignment of Acr1, the ORF2 product, and ActIII is shown in Fig. 6. It can be observed from this alignment that Acr1 is more similar to the ORF2 product (47% identity over 258 amino acids) than ActIII (29% identity over 258 amino acids). Thus, it appears that the ORF2 product probably catalyzes reduction of a fatty acyl substrate.

Hydropathy analysis of the Acr1 protein sequence via the Kyte and Doolittle prediction (14) as implemented in the DNASIS for Windows program (Hitachi, San Francisco, Calif.) shows many potential membrane-spanning domains when amino acid window sizes of both 10 and 21 are used. This is in keeping with ultrastructural observations by Scott and Finnerty that wax ester inclusion bodies in *A. calcoaceticus* HO1-N are surrounded by a single phospholipid membrane (22). It is therefore possible that this protein is associated with the membrane of inclusion bodies. Analysis of the primary protein sequence using the program MotifFinder (A. Ogiwara, Institute for Chemical Research, Kyoto University, Kyoto, Japan) indicated that the Acr1 protein possessed all the amino acids in the predicted spatial orientation associated with the short-chain alcohol dehydrogenases motif ($TGX_3GIGX_{10}GX_2VX_{28}DX_{21}DXLX_2NAGX_{23}NX_{20}GXIX_4SXGX_{10}YXAXKX_9L$) (17). Additionally, based on this motif and secondary structure predictions, it is possible that amino acids 21 to 28 represent a nucleotide binding site for either NAD^+ or $NADP^+$.

DISCUSSION

The *acr1* gene from *A. calcoaceticus* encodes a novel 32.5-kDa enzyme capable of reducing a broad range of acyl-CoA substrates to their corresponding fatty aldehydes. The gene was isolated by its ability to complement a mutant (Wow15) of *A. calcoaceticus* that is deficient in accumulation of wax esters under conditions that induce accumulation in the wild type. The ability of the Wow15 mutant to utilize exogenously provided fatty aldehyde for wax synthesis suggests that aldehyde reductase activity is encoded by a different gene. Direct evidence for the enzymatic activity of the *acr1* gene product was obtained from enzyme assays of extracts of *E. coli* that express the gene. Fractionation of these crude extracts by centrifugation indicated that the enzyme is associated with the membrane fraction. This is consistent with hydropathy analysis of the amino acid sequence which suggests the presence of several potential membrane-spanning domains.

A similar enzymatic activity has been reported in seeds of jojoba (18). However, unlike the *A. calcoaceticus* enzyme, which does not appear to catalyze conversion of the aldehyde to the alcohol, the jojoba enzyme was reported to catalyze both the reduction of the acyl-CoA to the aldehyde and the reduction of the aldehyde to the alcohol. Also, whereas the Acr1 protein was most active with acyl-CoAs of 14 to 18 carbons, the jojoba enzyme had a preference for C_{24} fatty acids.

A. calcoaceticus HO1-N utilizes eicosane, octadecane, hexadecane, and tetradecane as carbon sources in wax ester biosynthesis (7). The number of carbon atoms in the wax esters produced from these various alkanes (of length *n*) were 2*n*, 2*n*-2, and 2*n*-4. The fatty alcohol portion of the wax ester was always the same length as the alkane used as the carbon source, while the fatty acid segment was *n*, *n*-2, or *n*-4 carbons in length (7). The data presented in Table 2 are consistent with these results in that the Acr1 protein is able to reduce acyl-CoAs containing between 14 and 22 carbons, with a strong preference for C_{14} to C_{18} fatty acyl-CoAs.

Alkane oxidation in *A. calcoaceticus* has been suggested to be catalyzed either by the action of an oxidase or by a cytochrome P-450 oxygenase to yield the corresponding fatty alcohol (4). Assuming this to be the case, the resulting fatty alcohol should serve as a substrate for wax ester biosynthesis by an acyl-CoA (or acyl-ACP) fatty alcohol transferase to give a wax ester product. This suggests that the Wow15 mutant should be complemented by the addition of hexadecane to the growth medium. However, this was not observed to be the case. A total of six class I (Wax^-) mutants were isolated, and these were placed into three groups based on genetic complementation experiments (20). The cosmid 4A-55 was found to complement three of the class I mutants, including Wow15. None of these mutants were phenotypically complemented when grown in the presence of hexadecane. These observations lend support to the suggestion by Finnerty (9) that fatty alcohol represents an end product rather than a metabolic intermediate in alkane oxidation by *Acinetobacter*. He has suggested that the initial step in alkane metabolism is the formation of a peroxy acid which is converted directly to the corresponding aldehyde.

Comparison of the Acr1 amino acid sequence with sequences in the public databases revealed that it had significant homology to a product of unknown function encoded by an open reading frame (ORF2) in *M. tuberculosis* H37Ra (27). Interestingly, an enzymatic activity similar to that described here for the Acr1 protein has been described by Wang et al. for *M. tuberculosis* H37Ra (26). They reported that crude protein extracts from *M. tuberculosis* were able to catalyze the conversion of palmitoyl-CoA directly to fatty alcohol via a fatty alde-

hyde intermediate and ultimately into wax esters. However, based on its location between *cma1*, a gene involved in the cyclopropanation of mycolic acids, and ORF3, which encodes a possible trifunctional hydratase/dehydrogenase/epimerase, it was suggested that ORF2 may have a role in mycolic acid synthesis. A possible reductase function for the ORF2 product is also suggested by sequence similarity (30% identity over 188 amino acids) of the ORF2 product with a known β-ketoacyl reductase from *S. cinnamonensis* which is involved in chain elongation during polyketide biosynthesis (27). Based on the strong sequence similarity of the ORF2 product with Acr1 (44% identity over 264 amino acids), it seems likely that ORF2 encodes some sort of reductase acting on a fatty acyl substrate. Some likely roles include functioning in mycolic acid biosynthesis as a reductase during mycolic acid elongation and functioning in the reduction of mycocerosic acids prior to esterification to phthicerol A. Both of these compounds play important roles as cell wall components of *M. tuberculosis*.

Another class I (Wax^-) mutant, Wow1, has been complemented with the cosmid genomic library. This mutant is known to be the result of a mutation at a locus other than *acr1* because the complementing cosmid does not hybridize to cosmid 4A-55, which carries the *acr1* gene. Wax ester synthesis by Wow1 is restored by exogenous aldehydes and alcohols but not by alkanes or acids. Thus, the mutation in this line appears to be the result of a lesion in an acyl-CoA synthetase or some other enzyme upstream of the wax ester biosynthetic pathway illustrated in Fig. 1. These observations are consistent with preliminary results from the nucleotide sequence analysis of the regions flanking the *acr1* gene, which indicate that it is not contained in an operon of functionally related genes (20).

ACKNOWLEDGMENT

This work was supported in part by grant DE-FG02-94-ER-20133 from the United States Department of Energy.

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