

University of Nevada, Reno

Functional characterization of fatty acyl-CoA reductases from *Drosophila melanogaster*

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science
in Biochemistry

by

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prepared under our supervision by

LEAH P. WICKENBERG

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*Melanogaster***

be accepted in partial fulfillment of the
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MASTER OF SCIENCE

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Abstract

Fossil fuels are a finite resource, making research into alternative energy sources a high priority. Biological hydrocarbon production may contribute to technologies that replace fossil fuels. Hydrocarbon production from fatty acyl-CoA precursors in insects proceeds through a reduction of fatty acyl-CoA to an aldehyde, followed by aldehyde decarbonylation to yield hydrocarbon. This study focused on the reduction mechanism, catalyzed by a fatty acyl-CoA reductase (FAR) through efforts to functionally characterize three FAR enzymes from *Drosophila melanogaster*: CG18031, CG13091 and CG17562. All three FARs were heterologously produced in Sf9 cells using a baculoviral expression system and assayed with a variety of substrates. CG18031 converted 26 acyl-CoA to C26 alcohol and appears to prefer long-chain substrates. No activity was found when CG18031 was assayed with shorter chain (C24 or C24:1) substrates. No activity was observed with any substrate when CG17562 or CG13091 were assayed. These data suggest that insect FARs have narrow substrate ranges, and additional assays must be performed in order to identify an aldehyde producing FAR.

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Introduction

Energy consumption worldwide is increasing every day. Projections according to the World Energy Outlook (WEO) state that the demand for electricity will increase by 70% compared to the current demand, and the current demand for transport fuels will increase by 40% by 2035 (IEA, 2012). This consumption is no longer sustainable by use of fossil fuels alone. In 2007 the United States created a set of renewable fuel standards. These required that 15.2 billion gallons of biofuels be produced by 2012 and 36 billion gallons in 2022. Of the 36 billion gallons, 50% need to be produced via “advanced biofuels” (IEA 2012). Alternative fuel production from biomass is currently the most attractive option to meet increased demand. The compatibility of ethanol with current fuel systems makes bioethanol production from both corn and sugarcane especially attractive (Zhang et al. 2010). Algal based alternatives are also attractive, mainly because of their production quantities. It is estimated that algal based technologies can produce up to 60,000 L of biofuel per hectare per year (Dixon 2012).

As of 2010, most global CO₂ emissions were attributed to heat and electricity production (41%) and the transportation industry (22%) (IEA 2012). In order to efficiently replace fossil fuels, technology needs to supply a comparable fuel source that both retains the chemical properties of current petro-fuels and reduces carbon emissions on a global scale. Natural hydrocarbon biosynthesis seems to be the point at which a focus needs to be made. Natural hydrocarbons are, in some cases, chemically equivalent to modern petroleum products. For example, the cuticular hydrocarbons of ants are mainly composed of methyl branched alkanes ranging from 3-19 carbons (Martin et al.

2009). Standard petroleum diesel is composed of 8 to 20 carbon molecules that are 62-85 vol. % aliphatic hydrocarbons mixed with some aromatic hydrocarbons and olefin molecules (Ott et al. 2008).

There are three known biosynthetic routes converting long chain fatty acid precursors to hydrocarbons distributed among the Cyanobacteria, plants and insects. Although the hydrocarbon biosynthetic pathways are similar, they show important enzymatic differences (Figure 1). Bacteria begin with a fatty acid conjugated to an acyl carrier protein (ACP) which is then converted to a fatty aldehyde and decarboxylated using the *n*-1 model to yield hydrocarbon. The final step is catalyzed by a cytochrome P450 with high sequence and functional similarity to ω -oxidases (Schirmer et al. 2010). Plant hydrocarbon biosynthesis begins with a fatty acyl-CoA that is subsequently

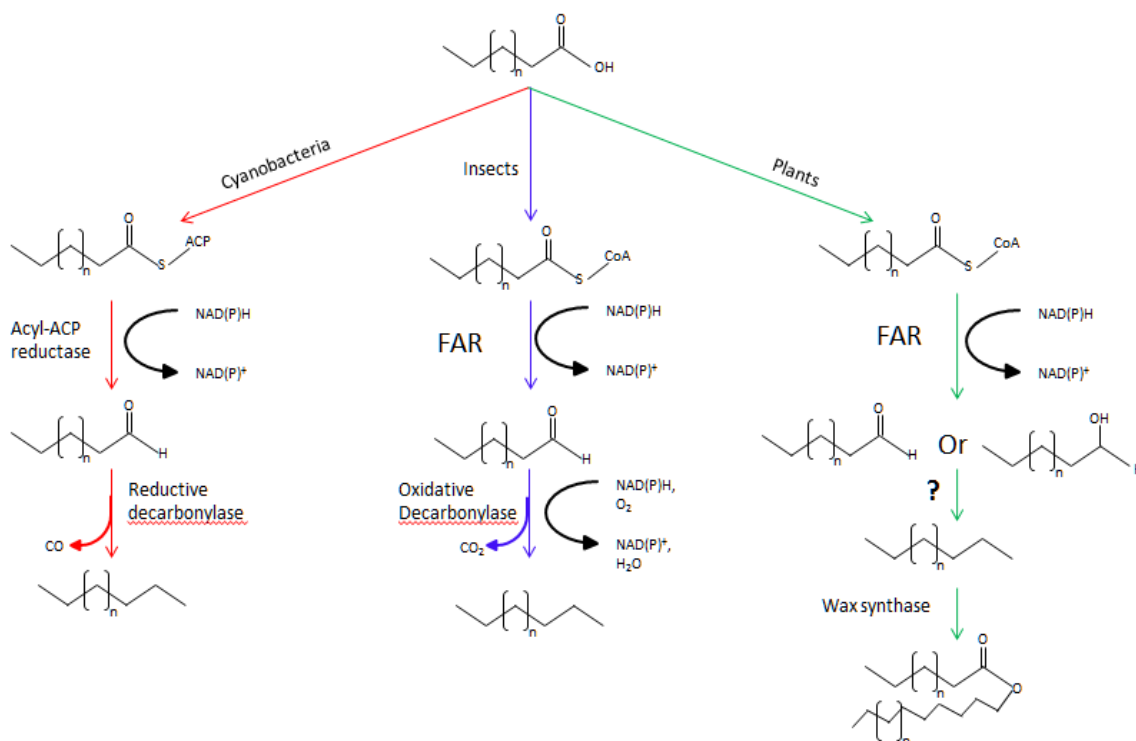


Figure 1. Different mechanisms of hydrocarbon production across different organisms. Decarboxylase activity has not been identified in plants. (ACP- acyl carrier protein)

elongated to the desired chain length and reductively decarbonylated to yield the desired hydrocarbon (Templier et al. 1984). However, a reductive decarbonylase has not been identified, and recent evidence suggests plants may also take advantage of a decarbonylation mechanism similar to the one found within bacteria, but with the use of a fatty acyl-CoA molecule to make the aldehyde or to make a primary alcohol (Metz et al. 2000). Both organisms release CO during the decarbonylation reaction.

Insect hydrocarbon biosynthesis proceeds through a long pathway of desaturases and elongases for fatty acyl-CoA production, before culminating in a two-step reduction and oxidative decarbonylation (Howard et al. 2005). During these final two steps, long chain fatty acyl-CoA molecules are converted to fatty aldehydes by fatty acyl-CoA reductase (FAR). The fatty aldehyde products of FARs are then converted to hydrocarbons by a cytochrome P450 oxidative decarbonylase (Y. Qiu et al. 2012). This final reaction uses NADPH as a cofactor as well as molecular oxygen, and releases CO₂ (Reed et al. 1994). This is in contrast to the proposed pathway in plants that results in the production of CO and does not require molecular oxygen or nucleotide cofactors (Dennis 1992).

Insect hydrocarbon biosynthesis occurs in oenocytes, specialized cells associated with the integument. The oxidative decarbonylase enzyme from the housefly, *Musca domestica*, is highly abundant in these cells. This enzyme has been named Cytochrome P450 4G2 (Cyp4G2; (Y. Qiu et al. 2012)). *Drosophila melanogaster* deficient in the orthologous Cyp4G1 are prone to early death due to desiccation and have reduced cuticular hydrocarbon compared to wild type flies (Y. Qiu et al. 2012). Thus, while

Cyp4G2 clearly catalyzes the final step in hydrocarbon biosynthesis, an aldehyde producing FAR has not yet been characterized.

Fatty acyl-CoA reductases have been functionally defined in many different species. Most characterized FARs are from plants (Rowland et al. 2006; Rowland, 2012; Vioque et al. 1997), though a few have been studied in mammals (Cheng et al. 2004) and birds (Hellenbrand et al. 2011). Plant FARs are utilized to produce the cuticular waxes of plants and mainly yield fatty alcohols from fatty acyl-CoAs. However, there is some evidence that some plant FARs produce an aldehyde intermediate (Rowland et al. 2006). Currently, few FARs from insects have been functionally defined. The honey bee (*Apis mellifera*) utilizes a FAR to produce the alarm pheromone [Z]-11-eicosenol (Teerawanichpan et al. 2010). The silk moth (*Bombyx mori*) utilizes an alcohol producing FAR to produce its pheromone, bombykol ((*E,Z*)-10,12-hexadecadien-1-ol). Bombykol production proceeds through a double desaturation and reduction by the FAR, pgFAR (Moto et al. 2003). There is evidence that the honey bee utilizes the decarbonylase pathway, though it has not been extensively studied, and that the required aldehyde is produced from the alcohol product of AmFAR1 (Teerawanichpan et al. 2010). Thus, aldehyde production may require a FAR to produce an alcohol, and an oxidoreductase to oxidize the alcohol to the aldehyde.

A FAR that directly produces the aldehyde substrate for Cyp4G2 would fulfill the needs of a manufactured hydrocarbon biosynthetic mechanism but has yet to be identified in insects. Preliminary efforts to identify such a FAR were involved comparative sequence analysis, high throughput expression profiling and RNAi experiments. There

are 18 putative FAR gene candidates in *D. melanogaster*, according to the *Drosophila melanogaster* database, Flybase (Flybase.org). Because an aldehyde-producing FAR is necessary to produce the substrate for the oxidative decarboxylase, it is reasonable to assume that aldehyde-producing FAR genes are coordinately regulated with *CYP4G1* in oenocytes.

A comparison of FAR mRNA distribution with that of *CYP4G1* using freely available FlyAtlas (Flyatlas.org) and FlyBase expression data suggested *CG4020*, *CG18031*, *CG17562* and *CG13091* as encoding potential fatty aldehyde producing FARs. All four candidate FARs were highly expressed in the heart, fat body and carcass (Claude Wicker-Thomas, unpublished data), a tissue distribution highly similar to that of *CYP4G1*. Additionally, these FARs are highly expressed in the oenocytes of flies (Joel Levine, personal communication), indicating co-localization with Cyp4G1. These data indicate that these FARs are good candidates to study as partners for Cyp4G1, potentially producing the aldehyde substrate(s) necessary for Cyp4G2 activity.

Preliminary confirmation that these genes are involved in hydrocarbon production comes from analyses of RNAi-mediated knockdowns of *CG4020*, *CG18031*, *CG17562*, and *CG13091* in *D. melanogaster* oenocytes. RNAi-mediated knockdown of *CG4020* yields an almost 50% decrease in total hydrocarbon production compared to wild type. *CG18031* knockdown flies show approximately 10% reduction in total hydrocarbon, and *CG13091* and *CG17562* show a marginal increase in total hydrocarbon (Claude Wicker-Thomas, unpublished data). These data indicate that *CG4020* is the best candidate FAR for hydrocarbon production and investigation into its activity is underway. However, the

remaining three FARs are also legitimate candidates, particularly if different FARs have different substrate preferences that contribute to variation in adult hydrocarbon profiles. Here, I present data summarizing work to functionally characterize the substrate and product profiles of CG18031, CG13091 and CG17562.

Materials and Methods

Drosophila melanogaster fatty acyl-CoA reductase cDNA clones GH27892 (CG13091), LP02712 (CG18031), RE20520 (CG17562) were purchased from the Drosophila Genomics Center (DGRC) (Bloomington, In.). Long chain fatty acyl-CoA molecules (28:0, 26:0, 24:1 and 24:0) were purchased from Avanti Polar Lipids Inc. (Alabaster, Al.). NADH was purchased from Fisher Scientific. NADPH, Coenzyme A, hexacosanoic acid, octacosanoic acid, hexacosanol, and methanolic HCl were purchased from Sigma Aldrich (St. Louis, Mo.). All oligonucleotide primers were synthesized by Integrated DNA technologies (Coralville, Ia.) or Sigma Aldrich (St. Louis, Mo.)

Transfer to competent cells

Purchased fatty acyl-CoA reductase clones were recovered from Whatman disks according to a modified protocol based on that provided by the DGRC. Fifty μ l of sterile 1X TE was added to the Whatman disk. The disk was rinsed briefly by pipetting up and down twice and TE was removed. The tube was incubated on ice while 50 μ l of Stellar™ Competent Cells (Clonetech, Mountainview, CA) were added and incubated on ice for 30 min. Cells were vortexed briefly at 15 and 30 min as per the suggestion from the DGRC. After 30 min, cells were heat shocked at 37°C for exactly 2 minutes, then placed back on ice for 2 min. The cells were transferred to 400 μ l SOC media instead of the recommended LB, leaving the disk behind. The cell/media mixture was incubated at 37°C for 1 h with shaking and proper aeration. Fifty, 100 and 200 μ l aliquots of the transformation were plated on LB carbancillin (50 μ g/ml) (CG17562) and LB

chloramphenicol (20 µg/ml) (CG18031, CG13091) plates and incubated overnight at 37°C.

Colony Verification

Five colonies were selected from the replica plates to confirm their inserts by standard PCR screening with vector primers, followed by plasmid preparation using Qiaprep Spin Miniprep Kit (Qiagen, Catalog # 27106) and sequencing using the vector and gene specific primers indicated in Table 1 (T7/T3-CG17562, PM001/T7_pOT2-CG13091, CG18031).

Sequencing

All sequencing reactions were carried out by the Nevada Genomics Center (Reno, NV.), using dideoxy sequencing on an ABI 3700 sequencer and analyzed using Vector NTI (Version 9.0.0) software.

Expression Cloning

For all FARs, the selected cDNA was subcloned into the pENTR^{NcoI}- modified GateWay (Invitrogen) plasmid (Sandstrom et al., 2006). The FAR insert was modified for Gibson assembly reaction (Cobb & Zhao 2012) by using specific primers (CG18031 InFusF1 and CG18031 InFusR2; Table 1) designed to add 3' and 5' overhangs on the fragments that permitted cloning into the target vector, pENTR^{NcoI}- (Sandstrom et al. 2006). Purified plasmid DNA was used as the template. One µl template was combined with 5 µl 10X PFU ultra II reaction buffer (Agilent) 2.5 mM MgCl₂, 0.1 mM dNTP, 0.4 pM forward and reverse primers, 0.025 U/ µl PFU Ultra II HS DNA polymerase

(Agilent) and 40.5 μl sterile H_2O for a final reaction volume of 50 μl . Cycling parameters were as follows: 95°C for 1 min, 94°C for 40 s, 56°C for 1 min 30 s, 0.3°/s ramp to 72°C, 72°C for 1 min 30 s. Steps 2-5 were repeated 2 times, then 94°C for 40 s, 67°C for 30 s, 72°C for 1 min 30 s. Steps 7-9 were repeated 34 times. Final annealing time was 72°C for 6 min with a final hold at 4°C until the reaction tubes were removed from the thermocycler. CG17562 and CG13091 inserts were prepared using similar cycling conditions and gene-specific infusion primers (CG13091 inFus F1, CG13091 inFus R1, CG17562 inFus F, CG17562 inFus R; Table 1).

Products from PCR reactions were purified from 1% agarose gels using the Illustra GFX PCR DNA and Gel Band purification kit (GE Healthcare) following the manufacturer's protocol.

Similarly, purified pENTR^{Nco-1} DNA was used as template in 50 μl reactions including: 5 μl 10X PFU ultra II reaction buffer (Agilent), 2.5mM MgCl_2 , 0.1 mM dNTP, 0.4 μM forward and reverse primers (pENTR F4/pENTR R5) 0.0.25 U μl PFU Ultra II HS DNA polymerase (Agilent) and 40.5 μl sterile H_2O . Cycling parameters: 95°C for 1 min, 94°C for 40 s, 67°C for 30 s, 72°C for 45 s. Steps 2-4 were repeated 30 times, then 72°C for 3 min and a final hold at 4°C until the PCR tubes were removed.

For Gibson assembly of insert and plasmid DNA a total of 18.8 μl CG18031 were mixed with one and a half times the amount of linearized plasmid and 10 μl of Buffer 4 (New England Biolabs) and 1 μl of *Dpn1*. The reaction was made up to 100 μl with sterile H_2O and incubated at 37°C for 1 h. Digested products were purified using the Nucleospin gel and PCR Clean-up kit (Machery Nagel) as per the manufacturer's

instructions. Purified insert and vector were combined using the Infusion HD Cloning Kit according to the manufacturer's instructions (Clontech, Mountainview, CA.)

A total of 2.5 μ l of the infusion reaction was used to transform *E. coli* Stellar™ Competent Cells (Clontech, Mountainview, CA) by standard chemical transformation. Transformants were selected following overnight growth on LB-kanamycin (30 μ g/ml) plates at 37 °C. Recombinant colonies were identified using the colony PCR protocol above and the primers pENTR F2 and pENTR R2 as well as gene specific internal primers as appropriate (Table 1). The integrity of the inserts was confirmed by sequencing using pENTRF2/pENTRR2 vector primers and gene specific primers (Table 1).

Transfection of pENTR constructs into Baculovirus

An LR recombination reaction to transfer pENTR insert DNA into Baculo-Direct Baculoviral DNA was carried out according to the manufacturer's protocol (Invitrogen) and confirmed by PCR prior to infection. Infection of Sf9 cells (Gibco) was performed following the manufacturer's instructions with the addition of 100 U/ μ l of penicillin and streptomycin and 0.25 μ g/L of Fungizone. P1 viral stocks were harvested by centrifugation in Beckman GS-6R centrifuge at 3000 RPM (4°C) for 10 min and collecting of the supernatant. The presence of insert DNA was confirmed by PCR using the primers Bac F1 and Bac R2. P2 and P3 viral stocks were produced by successive amplifications according to the manufacturer's instructions, and the integrity of the insert was re-confirmed by sequencing PCR products produced by amplifications of isolated viral DNA using Bac F1 and Bac R2 as well as the appropriate gene specific primers

(Table 1). Baculoviral DNA was isolated using the MasterPure complete DNA and RNA purification kit (Epicenter) according to the manufacturer's instructions. 1:100 dilutions of purified viral DNA were used as template for PCR confirmation of P1, P2 and P3 viral stocks.

Recombinant protein production and harvest

A plaque assay was performed according to the Baculovirus protocol (Invitrogen) to assess the titer of the P3 viral stock. To produce recombinant FARs in Sf9 cells, 50 ml liquid cultures were seeded at a concentration of 0.8×10^6 cells/ml with 10 % fetal bovine serum (FBS) in SF900 unsupplemented media (Life technologies). Cultures were incubated at 27°C with shaking at 1,300 rpm on orbital cell culture shakers for 72 h and then centrifuged at 3000 rpm in the Beckman GS-6R centrifuge for 10 min at 4°C. The supernatant was discarded and the pellet was washed twice by successive resuspension in 5 ml of 100mM Tris-HCl pH 7.0 and centrifugation. The washed pellet was resuspended in cell lysis buffer [(CLB); 10 ml 100mM Tris-HCl pH 7.0 with the addition of 100 µM DTT, 0.5 mM PMSF and 10 µl of protease inhibitor cocktail (Sigma)]. Three ml aliquots of the CLB were used to resuspend the pelleted cells. Suspensions were sonicated using a Branson hand held sonifier (VWR Scientific) with 15 one second bursts repeated 3 times. One ml aliquots were separated and centrifuged at 13,000 rpm in a Biofuge Pico benchtop microcentrifuge (Kendro laboratory products) for 20 min at 4°C. The pellets were discarded and the supernatants were either used directly for functional assays or to purify microsomes.

Recombinant housefly cytochrome P450 reductase (CPR; Wen et al.), CG13091 and CG17562 were prepared similarly and assayed as described below. CPR served as a negative control during functional assays.

For some functional assays and western blots, cell lysate supernatants were centrifuged using a TLA-110 rotor at 53,000 rpm in the Beckman Optima MaxE Ultra centrifuge for 1 h at 4°C. Supernatants were removed, and the microsomal pellets were resuspended in 1 ml CLB. Both supernatants and microsomal fractions were used for functional assays and western blot analysis.

BCA assay

Protein concentrations of cell lysate supernatants in functional assays and microsomal preparations were determined using BCA Protein Assay kit from Thermo Scientific (Product # 23225) following the manufacturer's instructions with Bovine serum albumin as a standard.

Confirmation of protein production

An anti-CG18031 polyclonal antibody was manufactured by Genescript (Piscataway, NJ). Antibody was produced in rabbit as a complete affinity-purified peptide polyclonal antibody with the peptide recognition sequence of CPGVSDMETLSKHGE.

SDS-PAGE was performed using Mini Protean TGX Gels, Any KD (Bio-Rad). 1:5 dilutions of microsomal fractions and supernatant fractions of CG18031 and HF-CPR harvested proteins using 1X Lamelli Buffer with β -mercaptoethanol. Following

electrophoresis, proteins were transferred to nitrocellulose overnight using a 15V current at 4°C in transfer buffer (24.8mM Tris base, 193mM Glycine, 20% Methanol).

Membranes were then removed from the transfer apparatus, rinsed with water, and imaged with 1X Ponceau stain. The Ponceau stain was removed by rinsing membrane in TBST-Brij (100 ml 10X TBS, 10 ml 10% Brij, 0.5 ml Tween-20, bring 1L with mpH₂O). Prior to exposure the primary antibody, membrane was blocked with 5% milk in TBST-Brij solution and then washed using the standard method. A 1:2000 dilution of Anti-18031 antibody in 1% milk with TBST-Brij was used as the primary antibody to probe the membrane. Afterwards, membrane was again washed using standard methods. The secondary antibody, goat anti-rabbit (BioRad), was prepared in 1% milk at a 1:10,000 dilution. Standard wash was repeated. The blot was incubated with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Product # 34080), as per the manufacturer's instructions, and imaged with X-ray film (Fuji).

Antibodies were not obtained for CG17562 or CG13091. To infer recombinant protein production, RT-PCR was used to detect recombinant gene transcription. RNA was isolated from infected cells using the RNeasy Plant Mini Kit (Qiagen) following the manufacture's protocol. Prior to kit use, cells were centrifuged at 3000 rpm in the Beckman GS-6R centrifuge for 5 min at 4°C. cDNA was synthesized in a reaction containing 12 µl template RNA, 10 mM random primers (Promega), 0.5 mM dNTPs, 0.005 mM DTT, 4 µl First strand buffer (Invitrogen) and 5 U/µl Superscript III Reverse Transcriptase (Invitrogen), for a final reaction volume of 19.5 µl, with the following cycling conditions; RNA, dNTPs and Random primers were incubated at 65°C for 5 min

then 4°C for 1 min, at this point 1st Strand Buffer, DTT and Superscript II were added. Reaction was incubated at 25°C for 5 min, then 50°C for 1 h, then 70°C for 15 min with a final hold at 4°C until the reaction was removed from the thermocycler. cDNA was then amplified with the appropriate gene specific primers (Table 1). This was performed at a single time point (72 h post-infection) for both genes and was also implemented in a time course fashion for CG13091. For the CG13091 time course, RNA was extracted at 24, 48, 72 and 96 h post-infection and stored at -80°C until all time points were collected. cDNA from all samples was synthesized at the same time and analyzed by PCR as described above.

Functional assays

Harvested protein was used to perform enzymatic functional assays as outlined in Table 2 and summarized below.

For each functional assay involving long chain acyl-CoA molecules, substrate and cell lysate supernatant [suspended in 100mM Tris HCl pH 7.0, 100 µM DTT, 0.5 mM PMSF and 10 µl of protease inhibitor cocktail (Sigma)] were combined to a total reaction volume of 600-1000 µl. The reaction was initiated with the addition of the NAD(P)H cofactor. In the functional assays involving free fatty acids as substrates, cell lysate supernatants were incubated with free fatty acids, MgCl₂, ATP and CoA-SH, for a total reaction volume of 1000 µl, for 30 min. After 30 min, NAD(P)H cofactors were added to initiate FAR activity. After the designated incubation period at 30°C, all assay samples were extracted twice with hexane:ether (50/50, v/v) into glass vials. Samples were dried down to completion under N₂ gas and resuspended in pure hexane for GC analysis.

Fatty acid methyl-ester preparation

Fatty acid methyl esters were prepared in a low-light setting using functional assay samples that had completed the 2 h incubation with substrate and cofactor. Samples were transferred to 10 ml glass tubes with caps. To each was added 500 μ l of methanol followed by 1.5 ml of Methanolic HCl. Samples were flushed with nitrogen gas, capped and incubated at 95°C in a sand bath for 2 h. Samples were cooled to room temperature prior to the addition of 4.0 ml of 0.88% NaCl. The samples were extracted by adding 1.5 ml hexane:ether (50/50, v/v), flushing with nitrogen, capping the tube, vortexing the sample and centrifuging at 1000 x g for 5 min. The organic layer was removed to a separate tube. Extraction was completed twice. Samples were stored overnight at -20°C and dried down completely the next day. Extracts were resuspended in pure hexane for GC analysis.

Gas Chromatography analysis

Extracts from enzyme assays were analyzed using either a DB-5 column (Agilent) or a Shimadzu non-polar polysiloxane column, (catalog number- 220-94536-01, phase-SHR5XLB) (Shimadzu). The conditions for analysis were as follows:

- Assays using 24:0, 24:1 and 26:0-CoA as a substrate- injector 150°C, FID- 300°C. 160°C for 0.2 min, 160°C to 265°C at a rate of 15°C/min, 265°C to 295°C at a rate of 5°C/min and hold for 5 min at 295°C.

- Assays using 26:0 FFA as a substrate- injector 210°C, FID 300°C. 150°C to 275°C at a rate of 10°C/min, 275°C to 295°C at a rate of 3°C/min and a hold at 295°C for 5 min.
- Assays using 26:0 FFA as substrate with FAME preparation- injector 225°C, FID 300°C. 120°C to 265°C at a rate of 15°C/min, 265°C to 295°C at a rate of 2°C/min and a hold at 295°C for 5 min.

Table 1. Oligonucleotide primers. Gene specific primers for *CG18031*, *CG13091* and *CG17562* are listed as well as the vector primers used. Red nucleotides indicate the pENTR portion of Gibson assembly primers.

Primer Name	Sequence
pENTR F4	GGCCGCACTCGAGATATCTA
pENTR R5	CCGGATCCAGTCGACTGAAT
pENTR F2	GCGTTTCTACAAACTCTTCC
pENTR R2	GCAATGTAACATCAGAGATT
CG18031 F1	TGAATCACAATGCACCATC
CG18031 F2	ATCGGGAGTGGTGGGCAAGG
CG18031 F3	TGTCGGAGGCACCTTGGAG
CG18031 F4	GGACATCACGCCGCAGGACA
CG18031 R2	AGAACCCTCGCCGTGCTTGG
CG18031 R3	TGTAGTCTAGAAGAGATGCG
CG18031 inFus F	GTCGACTGGATCCGGGGCT TGAATCACAATGCACCATC
CG18031 inFus R	ATCTCGAGTGC GGCCCCGG TGTAGTCTAGAAGAGATGCG
CG17562 F1	GCCAATTCAAGCGTAAACC
CG17562 F2	GGGAATCAGCGAGAATGATC
CG17562 F3	GCACTGGCGGAGGATGTGAT
CG17562 F4	GCTTTCTTCTATCACACCCTG
CG17562 R1	ACTGCTGAATCAGACTAGG
CG17562 inFus F	GTCGACTGGATCCGGGGC GCCAATTCAAGCGTAAACC
CG17562 inFus R	ATCTCGAGTGC GGCCCCG ACTGCTGAATCAGACTAGG
CG13091 F1	AATGCAGACAGACATATTGC
CG13091 F2	GCCAAGGAGATGAAAGGCT
CG13091 F3	GCCTCTTGTATGTCAATCCT
CG13091 F4	GCTTCTGTTTCCCTTCAATG
CG13091 R1	GCACATTGGATTAATACTTGG
CG13091 inFus F	GTCGACTGGATCCGGGGC AATGCAGACAGACATATTGC
CG13091 inFus R	ATCTCGAGTGC GGCCCCG GCACATTGGATTAATACTTGG
BacF1	AAATGATAACCATCTCGC
BacR2	GTTAGGGATAGGCTTCCC
PM001	CGTTAGAACGCGGCTACAAT
T7_pOT2	AATACGACTCACTATAGG
T7	GCTCTAATACGACTCACTATAGGGCG
T3	GAGCTCAATTAACCCTCACTAAAGGG

tgaatcacaATGCACCATCCACTATCCGGGGAAATGGAGGAATTCTTCGAGGATAGCGAAATATT
 CGTGACGGGCGGATCGGGAGTGGTGGGCAAGGCGCTGGTCGAGAAGCTCCTGCGCTCCTG
 CAACGTTGCGCGCATCTACGTCCTGCTGCGTCCCTGCAAACAGCTAACAGCGGAGCAGAGAC
 TCGTCCGACTGCGGCAGGCCACTGTCTTCCACGTAAGTGGCCGTACAAAAACCCGAAGAGCTG
 GACAAGATCGTGGCGGTCCCGGGCGACGTCTCTTTGCCGGGACTGGGCATCGATCCCTCGAT
 GATGCAGCGTATGAAAGGTGTTTCCCTGGTCTACCACTGTGCCGCCACGGTGGCATTTCGATGA
 GCCGCTTCGCGAGGCAGTACGTCTGAATGTCGGAGGCACCTTGGAGGCGCTTAAGTTCGCC
 GAGACTCTGCCCCAGCTGAGGGCCTTCATCCACGTTTCCACATTCTACAGTAATCCCTATTTGA
 CACGTGTGGAGCCCAAGTACTACTCCTCGCCGATGGACTGGCGGTTGTGCCTCCGAATGATCG
 ACGATGTCGCAGACGACGGGATGCTCAACGCGTTAACAAGAAAGTTGATCATGGGCTTTCCC
 AACACGTATACGTTTACCAAAAACCTGGCCGAATCCCTGGTGAATGACTACCGCCATCGGCTG
 CCAGTAATAGTGTATCGTCTTCCATAGTTCTCTTTGCCGTGGAGGACCCATCGCCGGATTCT
 CGCCATCCTTGATGGGCGCCATGGGACTGTTTCGCGCTGGTCGGCGCTGGAATCCTCAAGACT
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 TTCTCGACTTGCTTCTAAGGATTTTCGGTCAGAAGCCCGTGTGATGAGTGCAGTTCGCAAG
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 TTCGGACATGGAGACGCTGTCCAAGCACGGCGAGGGTTCTGCATTCAACTTCGATGCCTTCA
 ACCATCCGGACATCCATGGCCTGGTGTCCAATCCTGCAGCAACATGCTCCACAGTATCCGAAC
 GCACTTGCTTCGAGAAGATCCGAAAACCTGGAGCGTTCCAAGAGAATCTTAAGGATCAAGG
 TGTTCTCTACCGGCTGCTTCGCCTCTTTTGGCTTTACAAGCTGATCCTCTGGCTTTGGAGAAG
 CTATCTGGCGCATCTCTTCTAActaca

Figure 2a. Full length nucleotide sequence of CG18031. The open reading frame is indicated by uppercase letters and the silent mutation in the stop codon is shown in the red box.

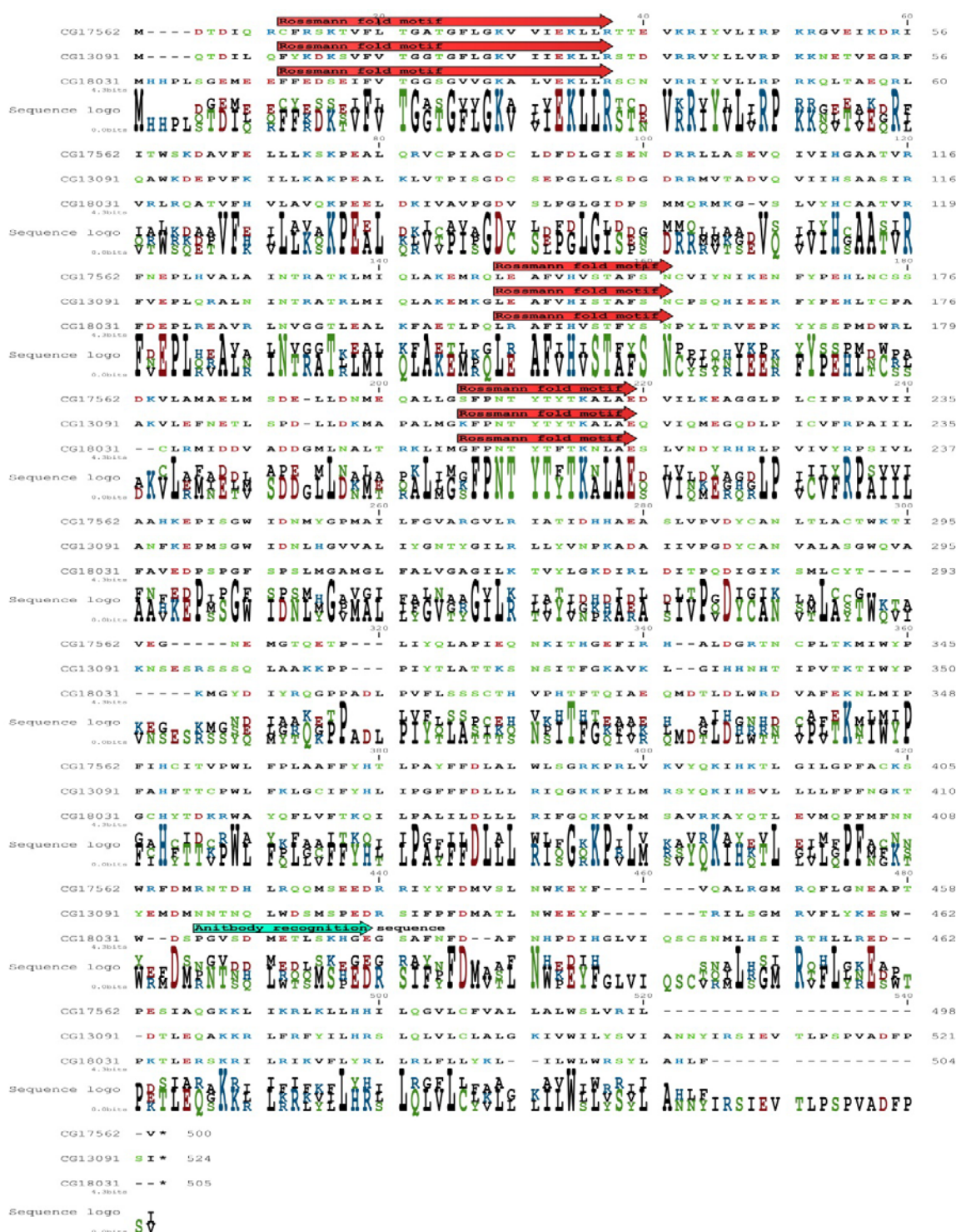


Figure 2b. Alignment of CG17562, CG13091 and CG18031 full length amino acid sequences. Predicted Rossmann fold motifs are indicated with red arrows. The antibody recognition sequence for anti-CG18031 is indicated by the blue arrow. Consensus is shown based on the bit size of the letters underneath the sequence

Table 2. CG18031 functional assays completed using fatty acyl-CoA substrates

Assay	Reagent	Volume added (μ l)	Final Concentration	Incubation at 30°C	Date	Notes
Replicate 1: 26:0-CoA Assay	2 mM 26:0-CoA	6 μ l	19.8 μ M	2 h	5/25/2012	
	7 mM NAD(P)H	200 μ l	2.3 mM			
	Cell lysate supernatant	398 μ l	/			
Replicate 2: 26:0-CoA Assay	2 mM 26:0-CoA	6 μ l	19.8 μ M	2.25 h	6/11/2012	
	7 mM NAD(P)H	200 μ l	2.3 mM			
	Cell lysate supernatant	398 μ l	/			
Replicates 3,4,5: 26:0- CoA Assay	2 mM 26:0-CoA	6 μ l (5.5 μ l)	19.8 μ M	2 h	6/18/2012	Parentheses indicate altered volumes for the ZnDH and CPR negative controls.
	7 mM NAD(P)H	200 μ l (180 μ l)	2.3 mM			
	Cell lysate supernatant	398 μ l (358 μ l)	0.109 μ g/ μ l (.12 μ g/ μ l)			
26:0-CoA	1.5 mM 26:0-CoA	60	150 μ M	2 h	3/18/2013	2 replicates were completed side by side with and without protease inhibitors.
	7 mM NAD(P)H	197	2.3 mM			
	Cell lysate supernatant	343	/			
24:0-CoA	1.5 mM 26:0-CoA	60	150 μ M	2.25 h	4/5/2013	
	7 mM NAD(P)H	197	2.3 mM			
	Cell lysate supernatant	343	/			
24:1-CoA	2.0 mM 26:0-CoA	6	20 μ M	2.25 h	4/5/2013	
	7 mM NAD(P)H	197	2.3 mM			
	Cell lysate supernatant	343	/			

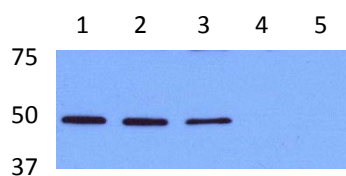


Figure 3. Western blot for MOI optimization.

Anti-CG18031 was used to optimize the MOI of Baculovirus infection. All lanes contain 6 μ g of either the microsomal fraction or the supernatant as determined by BCA assay. Lane 1: CG18031 microsomes, MOI 0.08, Lane 2: CG18031 microsomes MOI 0.16, Lane 3: CG18031 microsomes MOI 0.32, Lane 4: CPR microsomes (negative control), Lane 5: CG18031 supernatant (MOI 0.32).

Table 3. CG18031 functional assays completed with free fatty acid substrates

Assay	Reagent	Volume added (μ l)	Final Concentration	Incubation at 30°C	Date	Notes
26:0 Free Fatty Acid Assay	1 μ M ATP	5.6	5.6nM	2 h (Reactions were incubated with only ATP, CoASH, FFA for 30 minutes. Initiated FAR reaction with addition of NAD(P)H)	7/5/2012	Parentheses indicate concentration of cell lysate supernatant for CPR negative control. Free fatty acid was solubilized in 2mM Triton 100X before addition to the reaction
	25mM CoASH	10	250 μ M			
	25mM MgCl ₂	200	5mM			
	14.4mM hexacosanoic acid	100	1.44mM			
	10mM NAD(P)H	230	2.3mM			
	Cell lysate supernatant	454.4	0.923 μ g/ μ l (1.6 μ g/ μ l)			
26:0 Free Fatty Acid Assay	1 μ M ATP	5.6	5.6nM	2 h (Reactions were incubated with only ATP, CoASH, FFA for 30 minutes. Initiated FAR reaction with addition of NAD(P)H)	7/16/2012	Parentheses indicate concentration of cell lysate supernatant for CPR negative control. Free fatty acid was solubilized in 2mM Triton 100X before addition to the reaction
	25mM CoASH	10	250 μ M			
	25mM MgCl ₂	200	5mM			
	1mM hexacosanoic acid	100	100 μ M			
	10mM NAD(P)H	230	2.3mM			
	Cell lysate supernatant	454	8.8 μ g/ μ l (12.1 μ g/ μ l)			
26:0 Free Fatty Acid Assay with FAME prep	1 μ M ATP	5.6	5.6nM	2 h (Reactions were incubated with only ATP, CoASH, FFA for 30 minutes. Initiated FAR reaction with addition of NAD(P)H)	8/13/2012	After 2 hour incubation samples were prepared to manufacture Fatty Acid Methyl Esters in order to separate the unused free fatty acid substrate and the fatty alcohol product.
	25mM CoASH	10	250 μ M			
	25mM MgCl ₂	200	5mM			
	10mM hexacosanoic acid	100	1mM			
	10mM NAD(P)H	230	2.3mM			
	Cell lysate supernatant	454	/			
28:0 Free Fatty Acid Assay with FAME prep	1 μ M ATP	5.6	5.6nM	2 h (Reactions were incubated with only ATP, CoASH, FFA for 30 minutes. Initiated FAR reaction with addition of NAD(P)H)	8/13/2012	After 2 hour incubation samples were prepared to manufacture Fatty Acid Methyl Esters in order to separate the unused free fatty acid substrate and the fatty alcohol product.
	25mM CoASH	10	250 μ M			
	25mM MgCl ₂	200	5mM			
	10mM hexacosanoic acid	100	1mM			
	10mM NAD(P)H	230	2.3mM			
	Cell lysate supernatant	454	/			

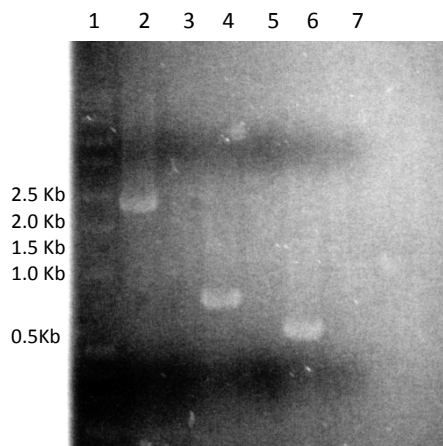


Figure 4a. cDNA synthesis of CG13091 from RNA isolated from infected Sf9 cells. RNA was isolated 72h post infection. cDNA was synthesized using the gene specific primers Bac F1/ Bac R2 (Lane 2, expected band size 1700), Bac F1/13091 R2 (Lane 4, expected band size 720) and 13091 F1/13091 R2 (Lane 6, expected band size 538). No template controls are present in lanes 3, 5 and 7. The molecular weight standards are present in lane 1.

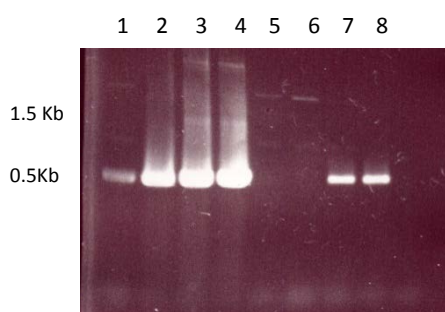


Figure 4b. cDNA synthesis from CG13091 RNA isolated during a 96 h time course. RNA was isolated from CG13091 infected Sf9 cells every 24 hours. CG13091; time 1 (24h) (Lane 1), time 2 (48h) (Lane 2), time 3 (72h) (Lane 3), time 4 (96h) (Lane 4). CPR negative control; time 1 (Lane 5), time 2 (Lane 6), time 3 (Lane 7), time 4 (Lane 8). Expected band size for CG13091 isolation is 538 bp, all lanes were amplified with 13091F1/CG13091R2.

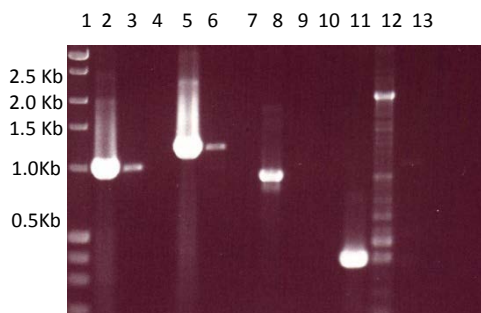


Figure 4c. cDNA synthesis from CG17562 isolated RNA. RNA was isolated from CG17562 infected cells 72h post infection and cDNA was synthesized using the gene specific primers CG17562 Real F1/CG17562 R2 (Lane 2, expected band size 900 bp), CG17562F1/CG17562 R2 (Lane 5, expected band size 1200 bp), CG17562 F2/CG17562 R2 (Lane 8, expected band size 1000 bp), CG17562 F1/CG17562 Real R1 (Lane 11, expected band size 350 bp). CPR negative controls are present in lanes 3, 6, 8 and 12. No template controls are present in lanes 4, 7, 10 and 13.

Table 4. CG17562 completed functional assays

Assay	Reagent	Volume added (μ l)	Final Concentration	Incubation at 30°C	Date	Notes
26:0-CoA Assay	2 mM 26:0-CoA	6 μ l	19.8 μ M	3 h	5/21/2012	
	7 mM NAD(P)H	200 μ l	2.3 mM			
	Cell lysate supernatant	398 μ l	/			
24:0, 26:0 & 28:0 Free Fatty Acid Assay	1 μ M ATP	5.6	5.6 mM	2 h (Reactions were incubated with only ATP, CoASH, FFA for 30 minutes. Initiated FAR reaction with addition of NAD(P)H)	7/23/2012	Free fatty acid was solubilized in 2 mM Triton 100X before addition to the reaction
	25 mM CoASH	10	250 μ M			
	25 mM MgCl ₂	200	5 mM			
	1 mM hexacosanoic acid	100	100 μ M			
	10 mM NAD(P)H	230	2.3 mM			
Cell lysate supernatant	454	/				

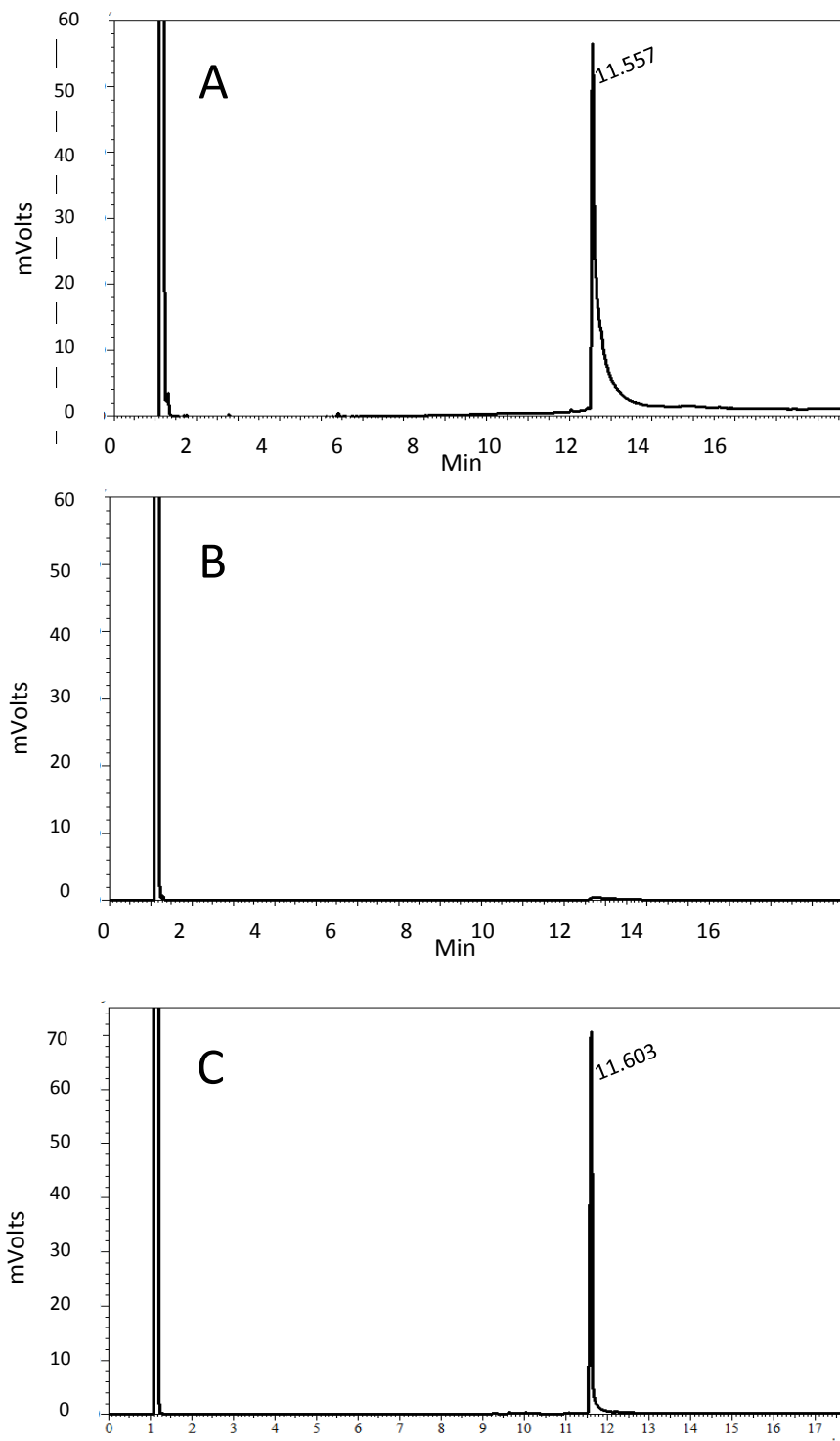
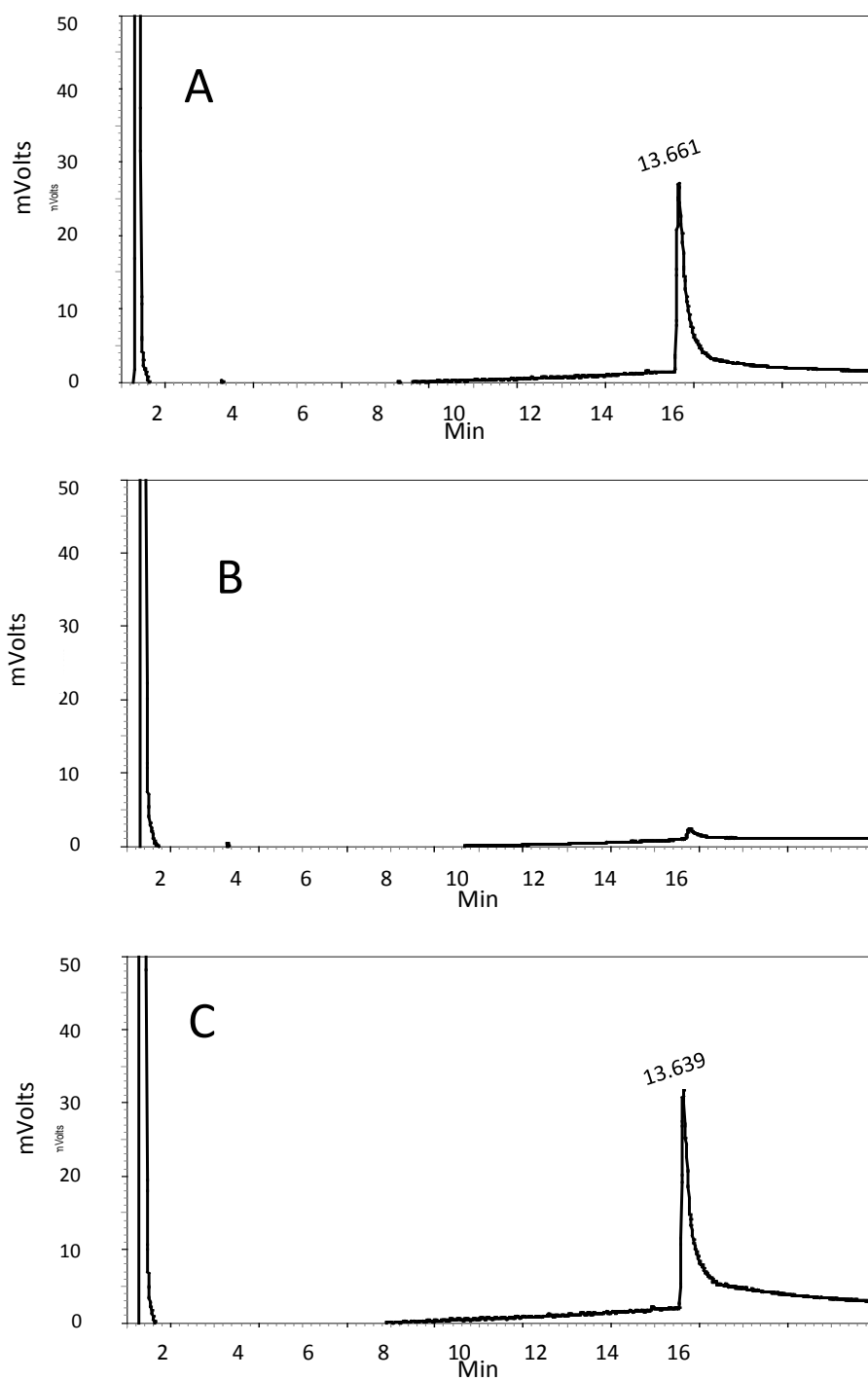


Figure 5. Gas chromatography of CG18031, NADH and 26:0-CoA. Reactions contained; 150 μ M substrate, 2.3mM NADH and cell lysate supernatant. CG18031 experimental (A), CPR negative control (B) and hexacosanol standard (C).

Table 5. Functional assays completed with CG13091

Assay	Reagent	Volume added (μ l)	Final Concentration	Incubation at 30°C	Date	Notes
26:0-CoA	2 mM 26:0-CoA	6 μ l	19.8 μ M	3 h	5/25/2012	
	7 mM NAD(P)H	200 μ l	2.3 mM			
	Cell lysate supernatant	398 μ l	/			
24:0, 24:1 Free Fatty Acid Assay	1 μ M ATP	5.6	5.6 nM	2 h (Reactions were incubated with only ATP, CoASH, FFA for 30 minutes. Initiated FAR reaction with addition of NAD(P)H)	8/24/2012	Free fatty acid was solubelized in 2mM Triton 100X before addition to the reaction
	25mM CoASH	10	250 μ M			
	25mM MgCl ₂	200	5mM			
	1mM hexacosanoic acid	100	100 μ M			
	10mM NAD(P)H	230	2.3mM			
	Cell lysate supernatant	454	/			



n=2

Figure 6. Gas chromatography of CG18031, NADPH and 26:0-CoA. Assay contained $19.8\mu\text{M}$ 26:0-CoA, 2.3 mM NADPH and cell lysate supernatant. CG18031 experimental (A), CPR negative control (B), Hexacosanol standard (C).

Table 6. Summary of results of all completed functional assays.

FAR	Substrate	Co-factor	Products
CG18031	26:0-CoA	NADH	26:0 Alcohol
CG18031	26:0-CoA	NADPH	26:0 Alcohol
CG18031	26:0-FFA	NADH	26:0 Alcohol ?
CG18031	26:0-FFA (FAME prep)	NADPH	28:0 FAME
CG18031	28:0-FFA (FAME prep)	NADH	26:0 FAME
CG18031	24:0-CoA	NAD(P)H	none
CG18031	24:1-CoA	NAD(P)H	none
CG13091	24:0/24:1 FFA	NAD(P)H	none
CG13091	26:0-CoA	NAD(P)H	none
CG17562	24:0/26:0/28:0 FFA	NAD(P)H	none
CG17562	26:0-CoA	NAD(P)H	none

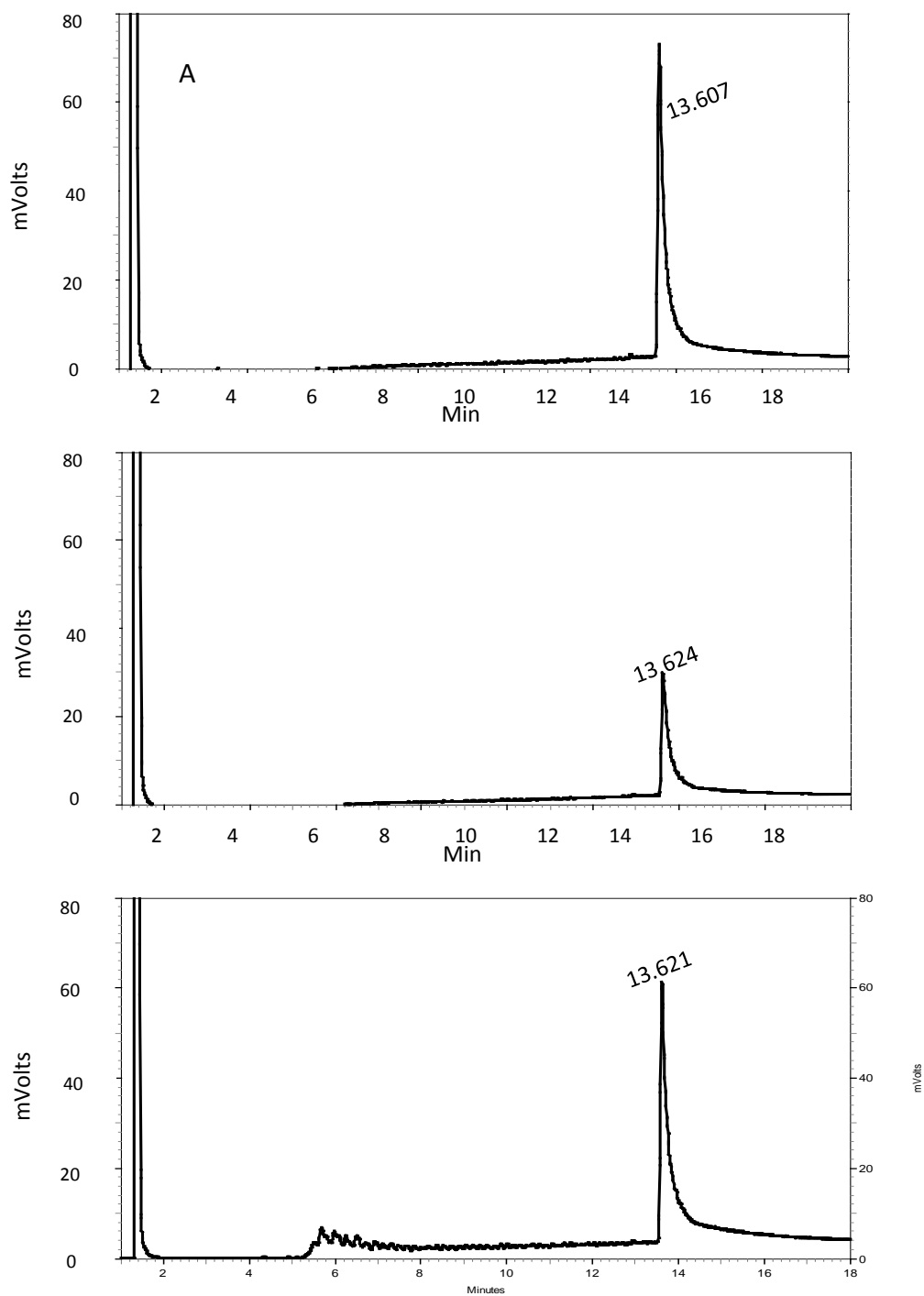


Figure 7. Gas chromatography of CG18031, NADH and 26:0 free fatty acid (FFA). Functional assays were analyzed using gas chromatography. Assays included 1.44mM 26:0 FFA, 2.3mM NADH, 5.6nM ATP, 250 μ M CoASH, 5mM MgCl₂ and cell lysate supernatant. CG18031 experimental (A), CPR negative control (B), 28:0 FAME standard (C).

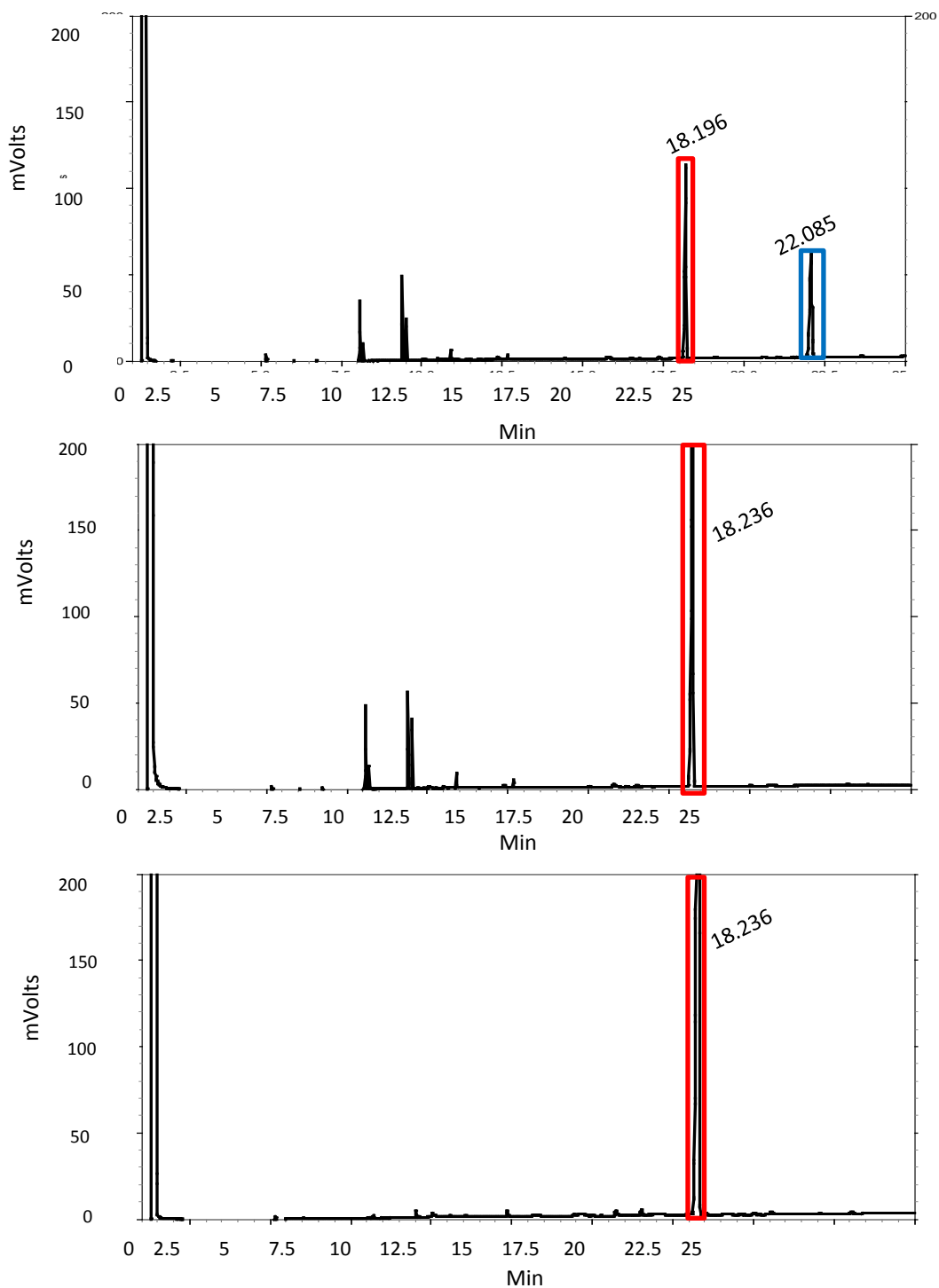


Figure 8. Gas chromatography of CG18031, NADPH and 26:0 free fatty acid (FFA). Data shown contains 1mM 26:0 FFA, 2.3mM NADPH, 5.6nM ATP, 250 μ M CoASH, 5mM MgCl₂ and cell lysate supernatant. After functional incubation the unused substrate was converted to a fatty acid methyl ester (FAME). CG18031 experimental (A), CPR negative control (B), 26:0-FAME standard (C).

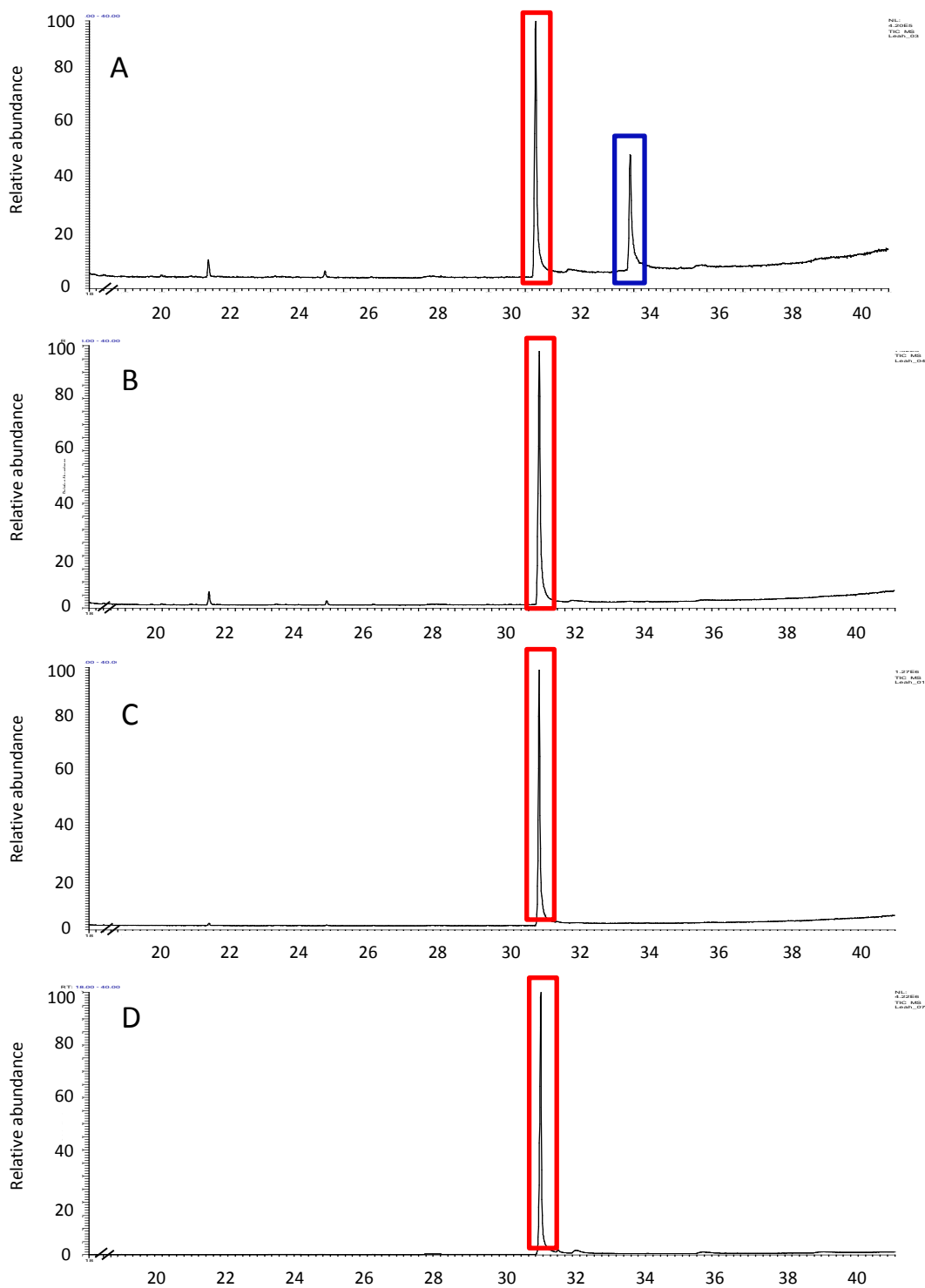


Figure 9. GC/MS analysis of CG18031 functional assay. Analysis of functional assay completed in Figure 8. CG18031 experimental (A), CPR negative control (B), CG18031 with NADH experimental (C), 26:0 FAME standard (D). The 26:0 FAME substrate is boxed in red and the 28:0 FAME product is boxed in blue.

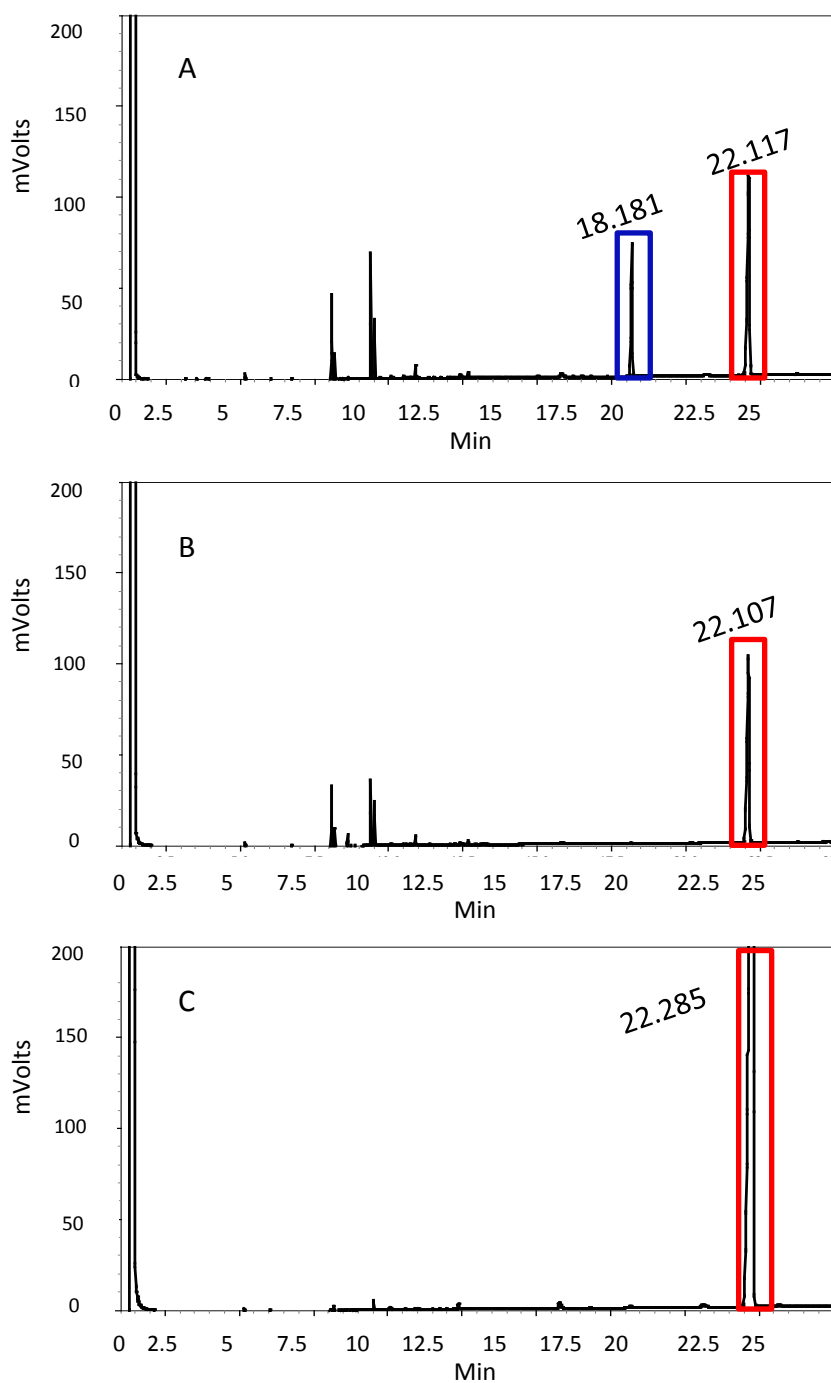


Figure 10. Gas chromatography of CG18031, NADH and 28:0 free fatty acid (FFA). Functional assays were analyzed using gas chromatography. Assays included 28:0 FFA, NADH and cell lysate supernatant. After functional incubation unused substrate was converted to fatty acid methyl ester (FAME). CG18031 experimental (A), CPR negative control (B), 28:0 FAME standard (C). The 28:0 FAME substrate is boxed in red and the 26:0 FAME product is boxed in blue.

Results

Sequence analysis:

The full length nucleotide sequence of *CG18031*:pENTR was identical to that provided by the Drosophila Genomics Resource Center (DGRC), with the exception of a silent mutation in the stop codon (Figure 2a). The full length predicted translation encodes a 504 amino acid (a.a.) protein with Rossmann fold motifs that are common to fatty acyl-CoA reductases (Figure 2b). The pENTR sequences of CG17562 and CG13091 were identical to those provided by the DGRC. CG17562 and CG13091 are predicted to encode for 499 a.a. and 523 a.a. sequences respectively and are shown aligned with the CG18031 a.a. sequence in Figure 2b.

There is a marked difference between the amino acid sequence of the three FARs presented in this study. The highest percent identity is between CG17562 and CG13091 (49%), CG13091 and CG17562 have a 27% and 28% identity with CG18031. The most conserved regions are at predicted Rossmann fold motifs. The polypeptide recognized by the anti-CG18031 antibody is indicated in Figure 2b. This peptide sequence is unique to CG18031 when compared to CG13091 and CG17562.

Recombinant protein production:

High titer P3 viral stocks were generated with all three candidate FARs. CG18031, CG13091, CG17562 had titers of 4×10^6 pfu, 2.5×10^8 pfu and 3×10^7 pfu respectively. The optimal multiplicity of infection (MOI) for CG18031 was between 0.08 and 0.16 (Figure 3).

Subcellular localization of recombinant CG18031 was analyzed using differential centrifugation and western blotting. Recombinant CG18031 localized to the microsomal fraction (Figure 3). Functional expression of recombinant CG13091 and CG17562 was inferred by RT-PCR. RT-PCR with appropriate gene specific primer pairs showed distinct single bands at the appropriate molecular weight for both cDNAs (Figure 4a0, c). A time course of recombinant CG13091 showed an increase in CG13091 mRNA over time, suggesting increased protein production (Figure 4b).

Functional assays:

Functional assays utilizing 26:0-CoA as a substrate and NADH as a cofactor showed strong activity. GC analysis of hexane:ether extracts yielded a peak at 11.557 min, consistent with that of an authentic hexacosan-1-ol standard (Figure 5). This peak was not present in control reactions (Figure 5B). This product was also seen when NADPH was used as a cofactor (Figure 6).

Two functional assays were successfully completed using 26:0 free fatty acid (FFA) as a substrate. In the presence of NADH, CG18031 yielded a peak at 13.621 min which is comparable to the peak of hexacosan-1-ol (Figure 7). In order to confirm an alcohol product and avoid the possibility that the substrate and product had the same GC elution time, a second assay separated unused substrate (26:0 FFA) and product (26:0-OH) by converting the unused substrate to a fatty acid methyl-ester (FAME). Gas chromatography revealed two distinct peaks at 18.193 min and 22.085 min in the sample where NADPH was used as a cofactor (Figure 8). A single functional assay with 28:0 FFA as substrate yielded two peaks (18.181 min and 22.117 min) in reactions where

NADPH was used as a cofactor (Figure 10). Both the 26:0 FFA assay and 28:0 FFA assay with FAME preparation showed similar peaks (18 and 22 min in Figures 8 and 10) in the experimental samples. The negative controls indicate that these reactions were prepared with different substrates.

The identity of the peaks in the reaction using 26:0 FFA (Figure 8) was confirmed by GC/MS analysis at the Nevada proteomics center (Reno, NV.) Results indicated that the second peak was consistent with an octacosanoic methyl ester (28:0 FAME) (Xcalibur GCMS analysis software) (Figure 9a-b)

Recombinant CG18031 was also separately assayed using 24:0-CoA and 24:1-CoA as substrates. No activity was seen with 24:0-CoA (n = 2) or 24:1-CoA (n=2) when analyzed with gas chromatography (data not shown). Recombinant CG13091 was separately assayed with 26:0-CoA, 24:0 FFA and 24:1 FFA as substrates under identical conditions to those used with CG18031. No activity was seen with any of these substrates when samples were analyzed with gas chromatography (data not shown). Recombinant CG17562 was separately assayed with 26:0-CoA, 28:0 FFA, 26:0 FFA and 24:0 FFA as substrates under identical conditions to those used with CG18031 and no activity was seen when analyzed with gas chromatography (data not shown).

Discussion

When comparing the amino acid sequences of the three FARs it is not surprising that they are markedly different. Most characterized FARs have less than a 60% sequence identity with one another. For example, all FARs identified in *Arabidopsis thaliana* shared a 28-54% identity with the well-studied FAR from jojoba (Rowland et al. 2006). The differences in the sequences of these FARs could possibly suggest different three dimensional folding properties. This could have an effect on the substrate range and specificity of each FAR, lending credit to the idea that CG17562 and CG13091 have different substrates than CG18031.

Functional assays show CG18031 was able to utilize both NADH and NADPH as cofactors (Figure 5 & 6). There seems to be a preference for NADH as there were fewer examples of a successful NADPH reaction; more than five replicates have shown activity with NADH whereas only 2 replicates of this assay show activity with NADPH. This is different from the few FARs that have been characterized in insects, which typically show activity with NADPH, but not NADH (Teerawanichpan et al. 2010; Moto et al. 2003). However, FARs from other organisms do show activity with both cofactors. For example, both a fatty aldehyde producing FAR and a fatty alcohol producing FAR have been discovered in *Pisum sativum* (Pea leaves). Both enzymes will utilize both cofactors but, there seems to be a cofactor preference based on product synthesis or possibly a product preference based on the cofactor that is used (Vioque et al. 1997). Alcohol producing FARs seemed to prefer NADPH, while aldehyde producing FARs seem to prefer NADH in this system. Thus, CG18031's apparent ability to use both NADH and

NADPH is reasonable, especially given that relatively few insect FARs have been functionally characterized; indeed, there is not yet enough data to determine whether use of either cofactor is a common characteristic for insect FARs or not. Further assays and kinetic analysis are required to confirm the apparent preference for NADH by CG18031.

Apis mellifera uses AmFAR1 to produce a mixture of long chain alcohols. This FAR requires NADPH as a cofactor and there is no evidence that this FAR activity proceeded through an aldehyde intermediate (Teerawanichpan et al. 2010). AmFAR1 also has a broad substrate range, with octadecanoyl-CoA being its preferred substrate (Teerawanichpan et al. 2010) though it readily accepts 16:0, 20:0, 22:0 and 24:0 saturated fatty acid substrates as well as 16:1 and 18:1 unsaturated fatty acids. CG18031 appears to have a minimum substrate length requirement of 26 carbons because activity was observed for C26 or longer substrates, but not for 24:0-CoA or 24:1-CoA. CG18031 likely has a narrower substrate range than AmFAR1. In summary, the multiple biological and technical replicates presented here indicate that CG18031 accepts a C26 fatty acyl-CoA as a substrate and produces a C26 alcohol.

Long chain acyl-CoA molecules are preferred as substrates for *in vitro* FAR enzyme assays. However, these molecules are expensive, making the development of an assay that utilizes the endogenous enzymes of the Sf9 cells for substrate production a more attractive proposition. The Sf9 cells used for recombinant protein expression are clonal isolates of *Spodoptera frugiperda* which have fatty acyl-CoA synthase activity (Bernal et al. 2010). The metabolic pathways of Sf9 cell have been scrutinized and most of the common pathways such as glycolysis and the TCA cycle have been well

characterized. Though the fatty acyl-CoA biosynthetic pathway has not been explicitly studied, it is almost certainly active within Sf9 cells (Bernal et al. 2010). Therefore, we reasoned that cells supplied with CoA-SH, ATP, heavy metal cations and the appropriate length free fatty acid, should synthesize long chain acyl-CoA molecules.

Assays with free fatty acid substrates yielded mixed results (Figures 7-10). Figure 7 shows the first assay with 26:0 FFA. This was completed without any derivitization of the products or unused substrate. With further scrutiny, there was a concern that the unused fatty acid substrate and the fatty alcohol product might have identical GC retention times. Indeed, subsequent control analyses using the 26:0 FFA and 26:0 alcohol standards confirmed our concerns. In order to distinguish products and substrates, we chose to derivitize the unused substrate in the reaction, making fatty acid methyl esters out of the unused FFA. Figure 8 shows the apparent success of the derivitization in regards to separating the substrate and the product.

CG18031 also produced an alcohol when given 28:0 FFA as a substrate, suggesting it can also accept molecules larger than 26 carbons as a substrate. Interestingly, the retention times of both the product and the substrate correspond to 28:0 and 26:0 fatty acid methyl ester (FAME) standards in both 26:0 FFA assay and the 28:0 FFA assay, respectively. This is also confirmed in Figures 9a and 9b, showing the GCMS results of the 26:0 FFA assay. GCMS data calls for the second peak at 22 min to be a 28 carbon FAME, despite the assay having been performed with 26:0 FFA as a substrate.

The presence of products with apparently different chain lengths compared to their corresponding substrates was unexpected and is difficult to explain. There are at

least two possibilities. First, the FAME protocol may have added two methyl groups to the alcohol instead of the usual one that is added to FFA, producing fatty acid ethyl esters. Finally, there may have been contamination between substrates, as the two assays were completed at the same time. However, this last scenario is highly unlikely as negative controls for both assays show the predicted GC peak for the designated (“correct”) substrates with no sign of a second (contaminating) peak. Therefore, the first option is the most viable alternative. A fatty alcohol would be expected to react differently when subjected to the FAME protocol in comparison to free fatty acids. These data are open to interpretation and need to be replicated in order to solidify the answer.

CG13091 was assayed under the same conditions as CG18031. These FARs are from the same organism and show similar tissue expression patterns according to FlyAtlas (C. Tittiger, personal communication), which suggests that they should have similar if not identical functional environments. The fact that there was no activity seen with 26:0-CoA, 24:1 or 24:0 FFA implies that CG13091 requires a longer chain length as a substrate. CG13091 also has a marked sex bias being expressed 17 fold higher in males than in females (Joel Levine, personal communication). In general the hydrocarbon profile of *D. melanogaster* ranges from C20 to C40. There are differences between subspecies, with some having broader hydrocarbon ranges and some having more concise hydrocarbon profiles (Ferveur 2005). The fact that C20 is the minimum hydrocarbon chain length found in *D. melanogaster*, paired with the included data showing no activity with chain lengths of 24:0, 24:1 or 26:0, indicate that CG17562 and CG13091 probably require longer chain length substrate molecules than CG18031.

CG17562 was also assayed for activity with 26:0-CoA and 24:0, 26:0 and 28:0 FFA under identical conditions as CG18031. No activity was seen for any of these assays, suggesting that the appropriate substrate was not used or that CG17562 requires a different functional environment. There are some instances where FARs from the same organism require different functional environments. For example, the FARs isolated from *Pisum sativum* have different functional pH ranges. The alcohol producing FAR requires a slightly acidic pH and the aldehyde producing FAR requires a slightly basic pH (Vioque et al. 1997). In any case, more work needs to be done to characterize CG17562 and CG13091.

There are precedents that show that different FARs within the same organism specifically require different substrate chain lengths. This is evident in FARs in birds. There is a distinct substrate specificity for each FAR studied in birds by Hellenbrand et al. in 2011. They show that multiple FARs require different substrates in order to produce different chain length alcohol products (Hellenbrand et al. 2011). They also show that there are preferences based on the degree of methyl branching within the substrates between the studied FARs. Therefore, we reason that CG18031, CG17562 and CG13091 will all require different chain length substrates. It would also be interesting to investigate the effects of methyl branching and desaturation with respect to insect FAR activity.

Future Directions

Substrates need to be identified for CG17562 and CG13091. However, this can only be accomplished if the free fatty acid assay can be optimized for insect FARs due to the fact that substrates longer than C26 acyl-CoA cannot be purchased commercially. The other alternative is to attempt to synthesize acyl-CoA molecules longer than 26 carbons. Also, an assay supplying CG18031 with the putative C26 alcohol product and NAD(P)⁺ needs to be completed. This will assist in confirmation of the C26 alcohol product, if successful. The pH optimum needs to be investigated for all three of these FARs. There are some cases in which aldehyde and alcohol production is influenced by different pHs (Vioque et al. 1997). There could be a pH where product production is influenced in CG18031, CG17562 or CG13091. Additionally, assays should be completed assessing the complementarity of any of the candidate FARs with CYP4G2 in the interests of completing a heterologous hydrocarbon production system. This could be completed with co-expression studies of both proteins.

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