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BIOENERGY/BIOFUELS/BIOCHEMICALS

Physiological characterization of lipid accumulation and in vivo ester formation in *Gordonia* sp. KTR9

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Abstract Previous work has demonstrated the feasibility of in vivo biodiesel synthesis in *Escherichia coli*, however, ethyl ester formation was dependent on an external fatty acid feedstock. In contrast to *E. coli*, actinomycetes may be ideal organisms for direct biodiesel synthesis because of their capacity to synthesize high levels of triacylglcerides (TAGs). In this study, we investigated the physiology and associated TAG accumulation along with the in vivo ability to catalyze ester formation from exogenous short chain alcohol sources in *Gordonia* sp. KTR9, a strain that possesses a large number of genes dedicated to fatty acid and lipid biosynthesis. Total lipid fatty acids content increased by 75 % and TAG content increased by 50 % under nitrogen starvation conditions in strain KTR9. Strain KTR9 tolerated the exogenous addition of up to 4 % methanol, 4 % ethanol and 2 % propanol in the media. Increasing alcohol concentrations resulted in a decrease in the degree of saturation of recovered fatty acid alcohol esters and a slight increase in the fatty acid chain length. A linear dose dependency in fatty alcohol ester synthesis was observed in the presence of 0.5–2 % methanol and ethanol compared to control KTR9 strains grown in the absence of alcohols. An inspection of the KTR9 genome revealed the presence of several putative wax ester synthase/acyl-coenzyme A : diacylglycerol acyltransferase (WS/DGAT) enzymes, encoded by *atf* gene homologs, that may catalyze the in vivo synthesis of fatty acid esters from short chain alcohols. Collectively, these results indicate that *Gordonia* sp. KTR9 may be a suitable actinomycete host strain for in vivo biodiesel synthesis.

Keywords Biodiesel · Triacylglcerides · Fatty acid esters · *Gordonia*

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Introduction

Declining petroleum reserves and the growing demand for energy have led to an increased interest in alternative, renewable fuels. Liquid biofuels, such as ethanol and biodiesel are of the greatest consideration because of their compatibility with the existing transportation fuel infrastructure [3]. However, biodiesel is of particular interest as a renewable fuel because of its high energy content and ability to function as a drop-in replacement for petroleum diesel [10].

According to the U.S. Energy Information Administration, in the United States alone approximately 967 million gallons of biodiesel were produced in 2011 (www.eia.gov). Production largely proceeds via the transesterification of plant derived oils, mainly comprised of triacyglycerides (TAGs), with short-chain alcohols resulting in fatty acid esters (FAE). Alkaline or acidic conditions are used to commercially catalyze these reactions and these processes are energy intensive, often requiring additional cost to recover the desired products. Furthermore, reliance on plant derived oils for biodiesel synthesis has led to concerns about competition with agricultural food production [20]. As a result of some of these limitations, significant research is now focused on utilizing microorganisms as a means of producing renewable biofuels [7, 10, 23].

Towards the goal of producing renewable biofuels in microorganisms, a host of synthetic biology and metabolic engineering approaches are being pursued for de novo biosynthesis of biodiesel in well characterized production hosts, like *Escherichia coli* [6]. As a proof-of-concept, de novo biodiesel synthesis was first reported in a recombinant *E. coli* strain expressing an unspecified wax ester synthase/acyl-coenzyme A : diacylglycerol acyltransferase (WS/DGAT) from *Acinetobacter baylyi* along with genes coding for the synthesis of ethanol [17]. The resulting "microdiesel" comprised up to 26 % of the bacterial dry biomass, however, formation of microdiesel was solely dependent on the addition of an exogenous source of fatty acids. The inability of production strains like *E. coli* to generate and/or utilize de novo fatty acid biosynthesis to provide sufficient quantities of intracellular acyl substrates for biodiesel synthesis has led to a number of efforts aimed at increasing de novo fatty acid synthesis. These strategies include a combination of overexpression and deletion of key genes involved in fatty acid synthesis and degradation, respectively, with the common goal of increasing carbon flux towards free fatty acid synthesis. As a result of some of these optimizations, *E. coli* was able to yield 922 mg/L FAEs without the addition of exogenous fatty acids [11].

As an alternative to using a well characterized bacterial system like *E. coli*, members of the Actinomycetes group of bacteria are known to naturally accumulate TAGs and therefore may be a more suitable bacterial host for de novo biosynthesis of fatty acid esters. Members of the genera *Rhodococcus* and *Gordonia* have been shown to accumulate significant internal reserves of TAGs, as high as 80 % of bacterial dry weight [2, 12, 14]. The metabolic diversity of these organisms [4, 21] makes them especially well suited for biofuels production, in that they may be capable of utilizing alternative feedstocks as a cheap carbon source for TAG production [12]. Collectively, a number of sequenced actinobacteria also encode for a large diversity of putative WS/DGAT enzymes, a key enzyme in the synthesis of fatty acid esters [19]. Furthermore, members of the Actinomycetes have been shown to tolerate a variety of organic solvents including alcohols, which would be desirable for de novo FAE synthesis [8, 9]. Despite these advantages, studies with these organisms have lagged behind model bacterial systems due to limited genetic tools for protein expression and gene deletion analysis. As more of the Actinomycetes are sequenced, insights into their physiology as it relates to biofuels production are emerging. Significant research has focused on understanding the physiological and genetic basis of TAG accumulation in *Rhodococcus* species such as *R. jostii* and *R. opacus* PD630 [2, 15]. In contrast, much less is known about lipid accumulation in *Gordonia*, a genus possessing considerable anabolic capabilities as well as abilities to degrade a wide range of environmental pollutants, xenobiotics and recalcitrant natural polymers [4]. As such, these organisms have a high potential for realizing the "waste to energy" concept. The genome sequence for *Gordonia* sp. KTR9 was recently determined and annotation revealed that these strains possess a large number of genes (9 %) dedicated to fatty acid and lipid biosynthesis [5]. Here we investigate the physiology and associated composition of TAG accumulation along with the ability to catalyze in vivo ester formation from exogenous alcohols in strain *Gordonia* sp. KTR9 [5].

Materials and methods

Bacterial strain and culturing conditions

Gordonia sp. KTR9 was grown on mineral salts medium (MSM) [28] supplemented with 1 g/L NH_4Cl and 20 mM each of fructose, gluconate, and succinate at 30 °C, shaking at 180 rpm. Cultures were also grown on MSM as described above and supplemented with 0.5–8 % methanol, ethanol, propanol, or butanol to determine the ability of the cells to tolerate alcohol and the effect of alcohols on lipid accumulation. All studies were done in 50 ml of MSM media in 250 ml flasks. For nitrogen limited studies, cultures were grown for 3 days then centrifuged at 3220×*g* for 5 min then washed with 5 ml phosphate buffered saline (PBS) and resuspended in fresh, nitrogen free MSM media. Following resuspension, cultures were incubated for 2 days prior to harvesting. Complete depletion of NH_4Cl from the media was verified by analysis with an Aquanal ammonium assay kit (Sigma-Aldrich Corp, St. Louis, MO, USA). Controls were treated the same but resuspended in MSM media with 1 g/L NH_4Cl. All experiments were done in triplicate.

Lipid extraction and analysis

Total lipids were extracted from 40 to 50 mg of dry cells diluted in 200 µl water, 1.5 ml methanol (MeOH) and 5 ml methyl tert-butyl ether (MTBE) following incubation for 1 h at room temperature with constant mixing. The recovered total lipids were then either assayed directly, following methylation, or fractionated on silica gel columns to isolate the neutral or non-polar lipids from the glyco- and polar lipids.

Total lipid analysis—Recovered total lipids were dried under vacuum, then subjected to a strong acid methanolysis using 1 ml of methanol:chloroform:concentrated hydrochloric acid (10:1:1, v:v:v) with heat (80 °C) for 1 h. Fatty acid methyl esters were then extracted in 1 ml of hexane:chloroform (4:1, v:v), dried under vacuum and

re-dissolved in iso-octane containing the internal standard, methyl-nonadecanoate at 50 pmol μl^{-1}. A one microliter sample was injected onto an Agilent DB-5MS capillary column (60 m × 250 μm × 0.25 μm) housed within a Hewlett Packard (HP) 6890 GC coupled to a HP 5973 MS via a HP 7683 autosampler. The injector temperature was maintained at 280 °C, the transfer line at 310 °C, the source at 230 °C and the analyzer at 150 °C. The GC was programmed from an initial temperature of 80 °C (1.75 min hold) to 150 °C at 20 °C/min., then to 325 °C at 4 °C/min. and held there for an additional 1 min. Helium was used as the carrier gas at a total flow of 1.5 ml/min. Sample components were fragmented at 70 eV and areas under the peaks quantified relative to the internal standard. Double bond positions were determined as described in Nichols et al. [22]. Recovered fatty acids are designated as A:BwC, where A is the total number of carbon atoms, B is the number of double bonds, and C is the position of the double bond from the aliphatic (w) end of the molecule. Bond geometry is indicated as either cis 'c' or trans 't'. A prefix of 'n' indicates a normal or un-branched fatty acyl, whereas prefixes of 'i', 'a' and '10me' indicate methyl branching at the iso, anteiso and 10 carbon position. Cyclopropane fatty acids are designated by 'cy'.

Non-polar lipid fraction—Total lipids, extracted from 40 to 50 mg of dried cells as described above, were first dissolved in 200 μl of $CHCl_3$:MeOH (2:1, v:v) and then loaded onto a 0.5 g Waters SPE-silica column (Waters Corporation, Milford, MA, USA) to obtain the non-polar lipid fraction. Non-polar or neutral lipids were then eluted in 4.5 ml of $CHCl_3$ containing 1 % acetic acid to enhance the recovery of free fatty acids. Although not analyzed in this study, glycolipids and polar lipids were subsequently eluted in 7.5 ml of acetone:methanol (9:1, v:v) and 5 ml of methanol, respectively. Recovered non-polar lipids were then dried under vacuum and immediately re-dissolved in iso-octane containing the internal standard. All identified non-polar lipid fatty acid esters were assumed to have formed via de novo synthesis, since this fraction was not subjected to either strong acid or mild alkaline transesterification to produce methyl, ethyl or butyl esters.

Total TAG content was determined by colorimetric assay using an ABcam Triglyceride Quantification Kit (ABcam, Cambridge, MA, USA).

Results

Growth of *Gordonia* sp. KTR9 on short chain alcohols

Gordonia sp. KTR9 grew in the presence of up to 4 % methanol, ethanol, or propanol. No KTR9 growth was observed on butanol, and 4 % propanol reduced KTR9 growth by 90 % (Fig. 1). Final culture densities with 4 % alcohol concentrations were decreased by 24 and 38 % compared to 1 % alcohol concentrations for methanol and ethanol, respectively. KTR9 was able to grow on ethanol as the sole carbon source but was unable to grow on either methanol or propanol when supplied as the only carbon source (data not shown).

Lipid accumulation and profile analysis

Nitrogen starvation in cultures grown on MSM or 1 % ethanol resulted in a 75 and 70 % increase in total lipid fatty acid (TLFA) content, respectively (Fig. 2). The TAG content in KTR9 increased significantly under nitrogen-starved conditions when grown in MSM, 1 % methanol, and 1 % ethanol, with a nearly twofold increase occurring in cultures grown in the MSM media (Fig. 3). No increase in TAG was observed in cultures grown on 1 % propanol. The highest level of TAG accumulation, 28 nmol/mg cell dry weight, was measured in cultures grown with 1 % ethanol followed by nitrogen starvation.

The KTR9 cultivation under nitrogen limiting conditions and in the presence of alcohols had a significant effect on the resulting TLFA profiles (Fig. 4). A number of fatty acids, n14:0, n15:0, n17:1w8c, n17:0, n18:1w9c, n18:0, and n20:0, increased in relative molar percentage when nitrogen was omitted from the media. Of these fatty acids, n14:0, n15:0, n17:0, and n20:0 showed an additional increase due the presence of 1 % ethanol. In contrast, fatty acids, n14:1w5c, a15:0, i16:0, a17:0, and n10me18:0, decreased in relative molar percentage when the cells were nitrogen starved while n14:1w5c and i16:0 decreased further in the presence of 1 % ethanol. In general, nitrogen starvation induced a shift toward increased monounsaturates and decreased methyl branched saturates with the greatest profile changes occurring within the 18 carbon fatty acid moieties. The presence of 1 % ethanol had no affect on fatty acid production under nitrogen rich growth conditions, but had a significant effect under nitrogen starved conditions with an observed decrease in methyl branched saturates and monounsaturates and an increase in normal saturates (Fig. 7).

In vivo esterification

The in vivo formation of fatty acid esters was observed in KTR9 non-polar lipid fractions when the organism was cultured in the presence of methanol, ethanol, and propanol. Since no derivatization step was undertaken, the occurrence of fatty acid alcohol esters (FAAE) in the recovered non-polar lipid fraction was, presumably, the result of de novo synthesis. Saturated n16:0 and n18:0, monounsaturated n16:1, n17:1 and n18:1, and 10-methyl branched 18:0

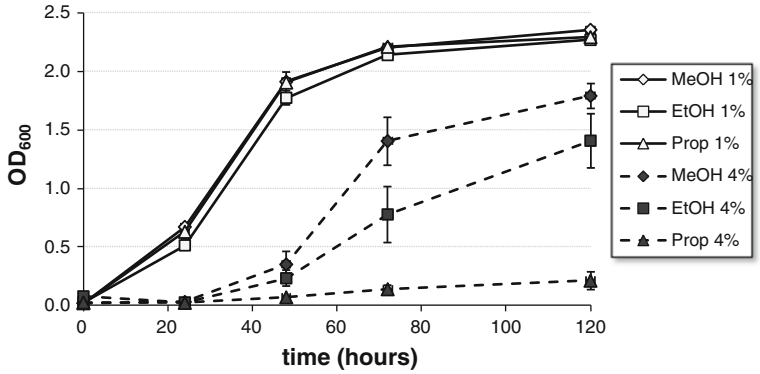

Fig. 1 Growth of KTR9 on MSM supplemented with methanol, ethanol, or propanol

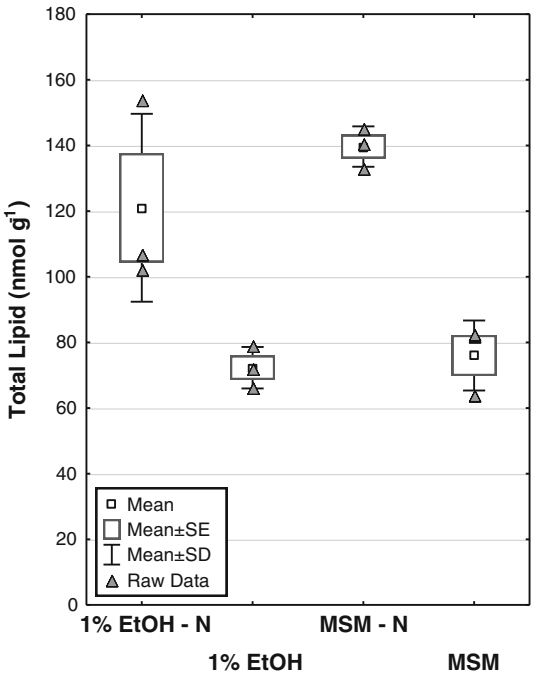

Fig. 2 Total lipid content in nitrogen starved KTR9 cultures and cultures supplemented with 1 % ethanol. Left to right: media supplemented with 1 % EtOH followed by nitrogen starvation for 48 h, media supplemented with 1 % EtOH, no nitrogen starvation, standard MSM media with nitrogen starvation for 48 h, MSM media without nitrogen starvation

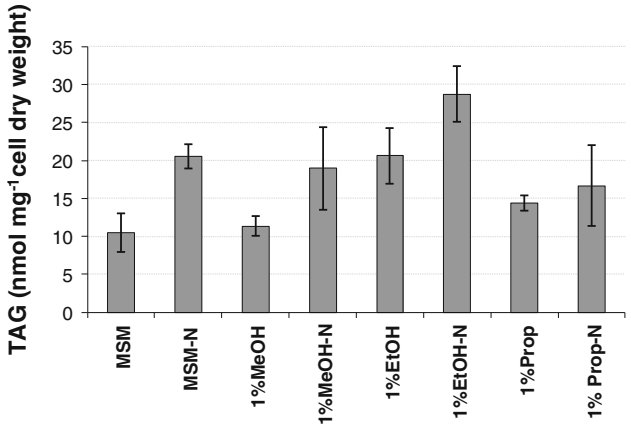

Fig. 3 TAG content in KTR9 cultures grown on 1 % alcohol and 1 % alcohol followed by nitrogen starvation

(tuberculostearic acid) were the predominant FAAE detected (Fig. 5). Relative to growth in the absence of alcohol (0.05 ± 0.01 nmol mg^{-1} cells), the greatest concentrations of methyl esters recovered occurred in cultures grown in 2 % methanol (6.7 ± 1.8 nmol mg^{-1} cells), ethyl esters in cultures grown in 4 % ethanol (5.9 ± 0.8 nmol mg^{-1} cells), and cultures grown in propyl esters in 1 % propanol (2.3 ± 0.2 nmol mg^{-1} cells) (Fig. 6).

Significant changes in FAAE profiles were also observed with increasing alcohol content (Fig. 7). With increasing alcohol concentration, methanol, ethanol, or propanol, the degree of saturation (i.e., monounsaturated to normal saturated ratio) decreased, FAAE chain length increased (i.e., n18:0 to n16:0 ratio) and the ratio of Δ-9 to Δ-10 monounstaurated NLFA decreased. The exception being the Δ9/Δ10 observed at 4 % propanol.

Discussion

Previous work has demonstrated the feasibility of in vivo biodiesel synthesis [17] in *E. coli*, with yields as high as 26 % cell dry weight, however, ethyl ester formation was dependent on an external fatty acid feedstock. In contrast to *E. coli*, Actinomycetes may be ideal organisms for direct biodiesel synthesis because of their capacity to generate a high TAG content, the high frequency of acyltransferase WS/DGAT homologs present in their genomes, and their ability to utilize/tolerate a variety of feed stocks [14, 15, 18]. As a prerequisite to attaining this goal, a better understanding of the physiology of TAG accumulation, TAG composition, tolerance to organic solvents like alcohols, and the capacity for in vivo synthesis of fatty acid esters is warranted. In this study, we investigated the physiology and associated composition of TAG accumulation along with the in vivo ability to catalyze ester synthesis from exogenous alcohol sources in a recently

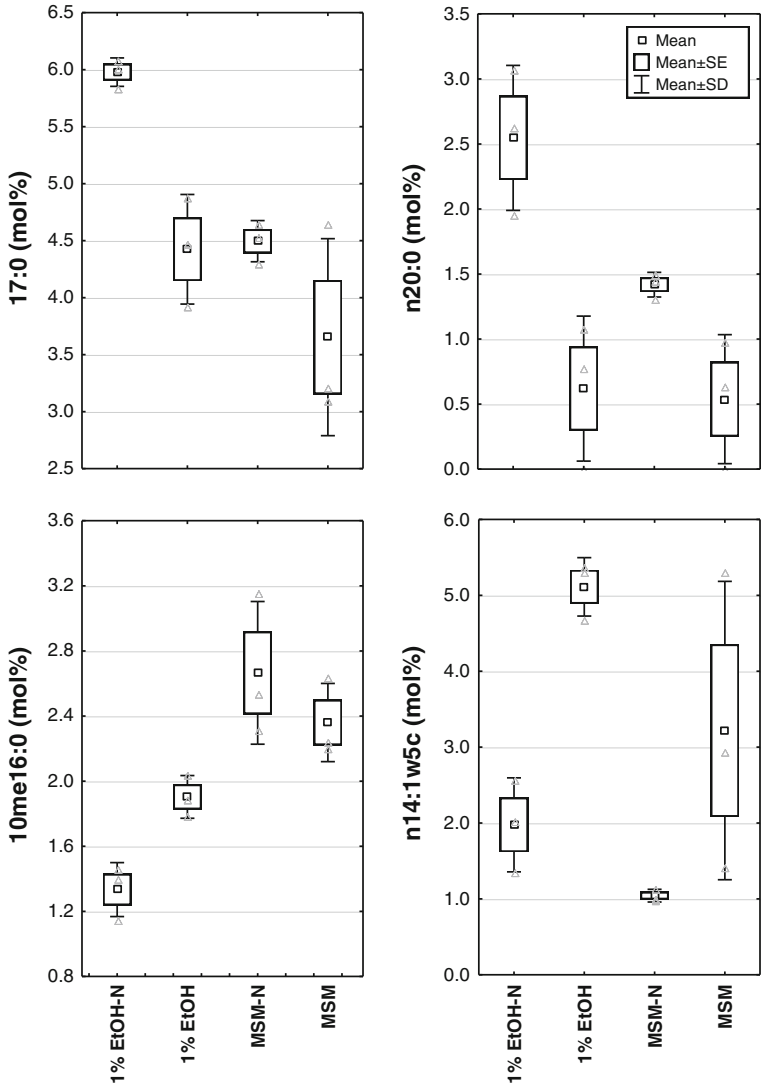

Fig. 4 Effect of 1 % ethanol and nitrogen limitation on total lipid fatty acid profiles. Only those fatty acids significantly affected by the presence of 1 % ethanol, independent of nitrogen starvation, are illustrated

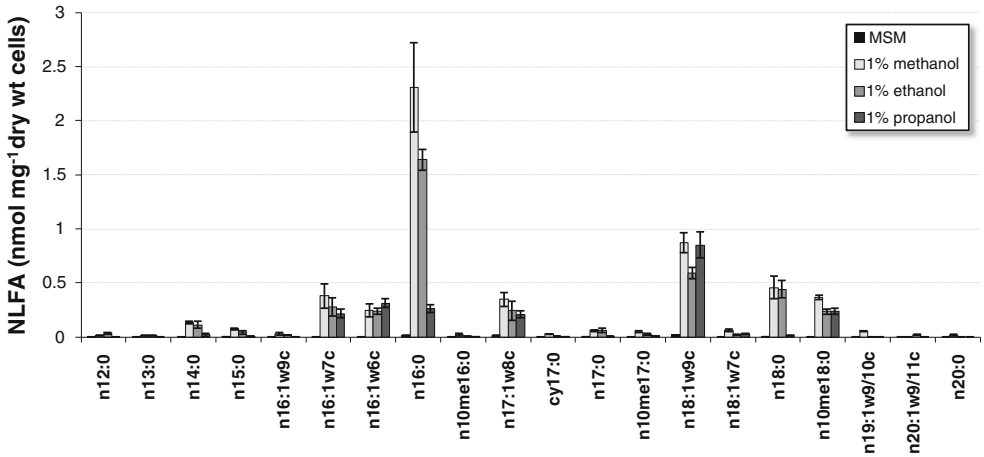

Fig. 5 Comparison of non-polar fatty acid profiles for KTR9 cultures grown in MSM supplemented with 1 % methanol, ethanol, or propanol

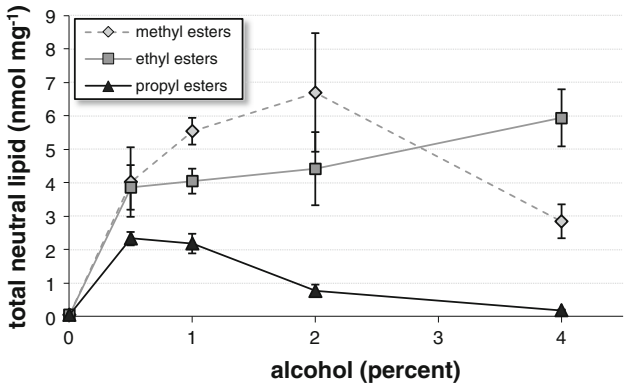

Fig. 6 Total fatty acid ester content at increasing alcohol concentrations showing in vivo ester synthesis in strain KTR9

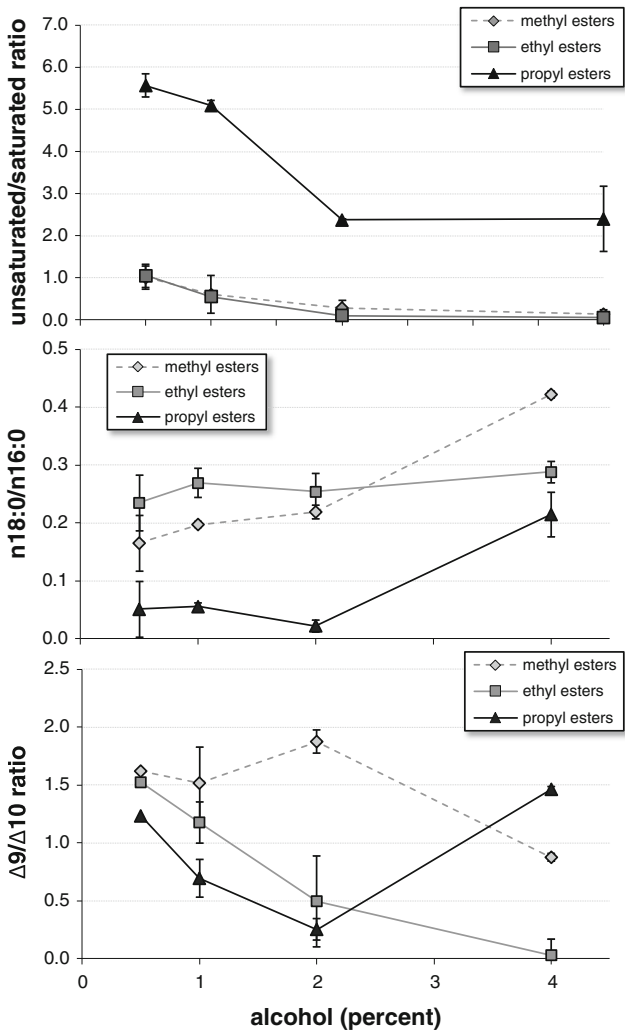

Fig. 7 Unsaturated:saturated fatty acid ratio, C18:0/C16:0 ratio, and Δ9/Δ10 ratio

sequenced environmental strain *Gordonia* sp. KTR9 that possessed a large number of genes dedicated to fatty acid and lipid biosynthesis [5].

Previously, stressors such as nitrogen and oxygen limitation have been shown to increase TAG accumulation in bacteria [1, 2]. In this study, total lipid fatty acids content and TAG content significantly increased by 75 and 50 %, respectively, during nitrogen starvation. Additional increases in TAG content in nitrogen starved cultures supplemented with 1 % ethanol were also observed. Increased TAG production in the presence of ethanol may be a universal stress response adaptation or the result of unbalanced growth caused by additional input of carbon, since it has been also observed in eukaryotic cells [24]. Under the growth conditions described above, strain KTR9 was also found to contain a fairly unique monounsaturated FAME, 16:1ω6c, which has previously been reported in Type X and Type I methanotrophs, *Methylococcus capsulatus* and *Methylomonas methanica*, respectively [13].

Perhaps the most significant finding in this study was the ability of strain KTR9 to synthesize fatty esters in vivo in the presence of exogenous alcohols, thus demonstrating an enzymatic capacity for in vivo production of FAEs. The observed dose-dependent responses, in terms of fatty acid alcohol ester content, differed dependent upon the alcohol added (Fig. 6). Addition of the single carbon methanol and a three carbon propanol appeared to have threshold limits of 2 and 1 %, respectively. Above these concentrations, the organism readjusted membrane fluidity through an increase in chain length (n18:0/n16:0 ratio) and degree of saturation (unsaturated/saturated ratio) (Fig. 7). The two-carbon ethanol addition, however, appeared to induce a linear increase in FAAE content up to a 4 % solution concentration. Similar to the methanol addition, linear increases in chain elongation and degree of saturation were observed as the ethanol concentration in solution increased. This result suggests that KTR9 favors the assimilation of two carbon substrates and is capable of doing so at high solution concentrations. Interestingly, an inspection of the KTR9 genome revealed the presence of multiple putative *atf* homologs encoding for WS/DGAT activity, one of which, KTR9_3844, showed significant homology (20–29 % sequence identity on the amino acid level) to functionally characterized *atf* homologs that have been shown to have some specificity to ethanol as an acyl acceptor [26]. Significantly, these sequences also contain the highly conserved HHxxxDG active site motif, which is believed to be essential for esterification activity between the fatty acid coenzyme A and alcohol substrates (Fig. 8) [11, 24]. The presence of KTR9_3844 coupled with the ability of *Gordonia* KTR9 to synthesize ethyl esters, suggests KTR9 may contain at least one functional WS/DGAT. Furthermore, KTR9 transcriptome studies conducted under nitrogen starvation conditions indicate that this ORF is expressed (Indest, unpublished data).

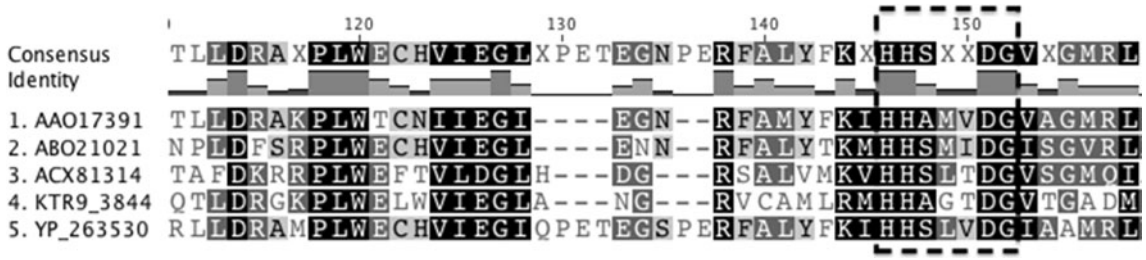

Fig. 8 Alignment of the putative amino acid sequence of the *atf* gene KTR9_3844 from *Gordonia* KTR9 with other characterized *atf* genes with known activity with ethanol. Dashed box shows the HHxxxDG active site motif

There are limited studies regarding growth on and/or tolerance to short chain alcohols in Actinomycetes with most studies focused on the *Rhodococcus* [8, 9]. *Gordonia* sp. KTR9 proved to be tolerant of various short chain alcohols showing growth in up to 4 % methanol and ethanol and 2 % propanol. Alcohols are known to affect membrane integrity by increasing fluidity resulting in uncontrolled solute transport [16]. As a result, many microorganisms counteract alcohol toxicity by increasing the degree of lipid saturation or by incorporating more complex lipids [16]. A similar response was observed in KTR9, which responded to increasing alcohol concentrations by reducing membrane fluidity as evidenced by the increase in fatty acid saturation and chain length. The effect of alcohol on fatty acid dehydrase activity was also indicated. Assuming KTR9 utilizes anaerobic desaturation for fatty acid synthesis, dehydration of ß-hydroxydecanoyl-ACP by different FabA homologs appears likely due to the presence of Δ-9 (16:1ω7c) and Δ-10 (16:1ω6c) monounsaturated fatty acids. The ratio of these two FAAE declined significantly with increasing ethanol media concentrations, suggesting a shift towards the FabA homolog producing the Δ-10 monounsaturate (Fig. 7).

Direct synthesis of biodiesel from microorganisms would be advantageous in that it eliminates the TAG purification step as well as the need for a separate chemical transesterification step. Collectively, these advantages raise the possibility of a more cost effective, cleaner system of biodiesel synthesis. However, before these advantages can be realized, a number of strategies need to be considered to improve fatty acid ester production in the Actinomycetes. For example, acyltransferase WS/DGAT exhibits both WS and DGAT activity [27] and screening/engineering enzymes with predominately WS activity and a high specificity for ethanol could be an approach to improving FAE yield. A variety of gene deletion and over expression strategies could be incorporated to shift metabolism of the cells toward fatty acid ester production. For example, improving the rates of malonyl-CoA production, which is the first key step in fatty acid biosynthesis [26], could lead to an increase in fatty acid ester production. In addition, deleting the *poxB* gene which encodes for a pyruvate dehydrogenase is known to decrease acetate production in *E. coli* and shift the acetyl-CoA flux toward lipid synthesis [25]. These strategies combined with better tools for the genetic manipulation will be a viable means of increasing fatty acid ester synthesis in *Gordonia* sp. KTR9 for biofuels production.

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References

1. Alvarez HM, Steinbuchel A (2002) Triacylglycerols in prokaryotic microorganisms. Appl Microbiol Biotechnol 60(4):367–376
2. Alvarez H, Steinbuchel A (2010) Physiology, Biochemistry, and Molecular Biology of Triacylglycerol Accumulation by *Rhodococcus*. In: Alvarez HM (ed) Biology of *Rhodococcus*, Microbiology Monographs. Springer-Verlag, pp 263–290
3. Antoni D, Zverlov V, Schwarz W (2007) Biofuels from microbes. Appl Microbiol Biotechnol 77(1):23–35
4. Arenskötter M, Baumeister D, Kalscheuer R, Steinbüchel A (2003) Identification and application of plasmids suitable for transfer of foreign DNA to members of the genus *Gordonia*. Appl Environ Microbiol 69(8):4971–4974
5. Chen H-P, Zhu SH, Casabon I, Hallam SJ, Crocker FH, Mohn WW, Indest KJ, Eltis LD (2012) Genomic and transcriptomic studies of an RDX (Hexahydro-1,3,5-Trinitro-1,3,5-Triazine)-degrading actinobacterium. Appl Environ Microbiol 78:7798–7800
6. Clomburg JM, Gonzalez R (2010) Biofuel production in *Escherichia coli*: the role of metabolic engineering and synthetic biology. Appl Microbiol Biotechnol 86(2):419–434
7. Connor M, Atsumi S (2010) Synthetic Biology Guides Biofuel Production. J Biomed Biotechnol 2010:1–9
8. de Carvalho C, Parreño-Marchante B, Neumann G, Fonseca MMR, Heipieper HJ (2005) Adaptation of *Rhodococcus erythropolis* DCL14 to growth on n-alkanes, alcohols and terpenes. Appl Microbiol Biotechnol 67(3):383–388
9. de Carvalho C, da Cruz AA, Pons M-N, Pinheiro HM, Cabral JM, da Fonseca MM, Ferreira BS, Fernandes P (2004) Mycobacterium sp., *Rhodococcus erythropolis*, and *Pseudomonas putida* behavior in the presence of organic solvents. Microsc Res Tech 64(3):215–222

10. Demirbas A (2009) Progress and recent trends in biodiesel fuels. Energy Convers Manage 50(1):14–34
11. Duan Y, Zhu Z, Cai K, Tan X, Lu X (2011) De novo biosynthesis of biodiesel by *Escherichia coli* in optimized fed-batch cultivation. PLoS ONE 6(5):e20265
12. Gouda MK, Omar SH, Aouad LM (2008) Single cell oil production by *Gordonia* sp. DG using agro-industrial wastes. World J Microbiol Biotechnol 24(9):1703–1711
13. Guckert J, Ringelberg D, White D, Hanson R, Bratina B (1991) Membrane fatty acids as phenotypic markers in the polyphasic taxonomy of methylotrophs within the Proteobacteria. Microbiology 137(11):2631–2641
14. Hernández MA, Mohn WW, Martínez E, Rost E, Alvarez AF, Alvarez HM (2008) Biosynthesis of storage compounds by *Rhodococcus jostii* RHA1 and global identification of genes involved in their metabolism. BMC Genomics 9(1):600
15. Holder JW, Ulrich JC, DeBono AC et al (2011) Comparative and functional genomics of *Rhodococcus opacus* PD630 for biofuels development. PLoS Genet 7(9):e1002219
16. Huffer S, Clark ME, Ning JC, Blanch HW, Clark DS (2011) Role of alcohols in growth, lipid composition, and membrane fluidity of yeasts, bacteria, and archaea. Appl Environ Microbiol 77(18): 6400–6408
17. Kalscheuer R (2006) Microdiesel: *Escherichia coli* engineered for fuel production. Microbiology 152(9):2529–2536
18. Kalscheuer R, Stoveken T, Malkus U, Reichelt R, Golyshin PN, Sabirova JS, Ferrer M, Timmis KN, Steinbuchel A (2007) Analysis of storage lipid accumulation in *Alcanivorax borkumensis*: evidence for alternative triacylglycerol biosynthesis routes in bacteria. J Bacteriol 189(3):918–928
19. Kalscheuer R (2010) Hydrocarbons, oils and lipids: diversity, properties and formation. In: Handbook of hydrocarbon and lipid microbiology, vol 1. Springer-Verlag, Heidelberg, pp 527–535
20. Li Y, Horsman M, Wu N, Lan CQ, Dubois-Calero N (2008) Biofuels from microalgae. Biotechnol Prog 24(4):815–820
21. McLeod MP, Warren RL, Hsiao WW, Araki N, Myhre M, Fernandes C, Miyazawa D, Wong W, Lillquist AL, Wang D, Dosanjh M, Hara H, Petrescu A, Morin RD, Yang G, Stott JM, Schein JE, Shin H, Smailus D, Siddiqui AS, Marra MA, Jones SJ, Holt R, Brinkman FS, Miyauchi K, Fukuda M, Davies JE, Mohn WW, Eltis LD (2006) The complete genome of *Rhodococcus* sp. RHA1 provides insights into a catabolic powerhouse. Proc Natl Acad Sci USA 103(42):15582–15587
22. Nichols P, Guckert J, White D (1986) Determination of monosaturated fatty acid double-bond position and geometry for microbial monocultures and complex consortia by capillary GC-MS of their dimethyl disulphide adducts. J Microbiol Methods 5(1):49–55
23. Peralta-Yahya P, Keasling J (2010) Advanced biofuel production in microbes. Biotechnol J 5:147–162
24. Polokoff MA, Iwahashi M, Simon FR (1983) Ethanol treatment increases triacylglycerol and cholesteryl ester content of cultured hepatoma cells. J Lipid Res 24(8):1030–1038
25. Santala S, Efimova E, Kivinen V, Larjo A, Aho T, Karp M, Santala V (2011) Improved triacylglycerol production in *Acinetobacter baylyi* ADP1 by metabolic engineering. Microb Cell Fact 10:36–46
26. Shi S, Valle-Rodríguez JO, Khoomrung S, Siewers V, Nielsen J (2012) Functional expression and characterization of five wax ester synthases in *Saccharomyces cerevisiae* and their utility for biodiesel production. Biotechnol Biofuels 5(1):7–17
27. Stoveken T, Kalscheuer R, Malkus U, Reichelt R, Steinbuchel A (2005) The Wax Ester Synthase/Acyl Coenzyme A:diacylglycerol Acyltransferase from *Acinetobacter* sp. Strain ADP1: Characterization of a Novel Type of Acyltransferase. J Bacteriol 187(4):1369–1376
28. Thompson K, Crocker F, Fredrickson H (2005) Mineralization of the Cyclic Nitramine Explosive Hexahydro-1,3,5-Trinitro-1,3,5-Triazine by Gordonia and Williamsia spp. Appl Environ Microbiol 71(12):8265–8272