Biotechnological improvements of bio-based fuels and lubricants
- characterization of genes for branched chain fatty acids
and mono-acyl esters

By

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Chapter 1. General Introduction

Significant and Rationale

Vegetable oils are extracted from seeds of plants. Biochemically they are triacylglycerol (TAG) molecules. The physical properties, like melting point, viscosity, and pourability of these molecules vary with the structures of the acyl moieties. As important biorenewable raw industrial materials, vegetable oils have wide variety of potential applications as lubricants, plasticizers, paints, ink, and diesel fuel. However, in most of these applications, TAG is first converted to mono-acyl esters because this chemical conversion overcomes the high viscosity of the oil. Subsequent modification on the acyl moiety renders mono-acyl esters, which have specific applications, with specific properties such as higher stability.

When the diesel engine was first designed, its fuel was oil extracted from peanut. The problem with peanut oil is that TAG is too viscous. Subsequently, chemists converted TAG to mono-acyl esters by reacting them with simple alcohols. The fuel properties of the mono-acyl esters produced by this conversion are close to those of petroleum diesel oil. However, this conversion increase the cost of producing biodisel, and it is difficult to find applications for the by-product, glycerol. Therefore, if plants could produce mono-acyl esters, the cost of biodisel production could be reduced. One purpose of the research presented in this dessertation is to identify and characterize genes that can be used for producing mono-acyl esters in plant instead of triacylglycerol.

Another purpose of the project is to overcome two limitations of vegetable oil in its application as a biofuel or as a biolubricant, the oxidative instability and high viscosity of the oil. Firstly, mono-acyl esters generated from vegetable oil that contains unsaturated acyl moieties, are chemically unstable at elevated temperatures. Thus, the quality of the resulting biolubricant is degraded at elevated temperature. To overcome this instability, the vegetable oils are usually partially hydrogenated to reduce the carbon-carbon double bonds and increase the stability of the products at elevated temperatures.

However, saturated fatty acids have higher melting temperature than unsaturated fatty acid due to the different degree of packing of fatty acid molecule. Because the free rotation around carbon-carbon bonds allows greater flexibility of the carbon chain, the carbon chain can be fully extended, and thus saturated fatty acid can pack tightly in nearly crystalline arrays. Unsaturated carbon-carbon bonds, particularly cis-double bonds, cause a kink in the acyl chain, which prevents the tight packing of the molecules. Thus, the melting point of unsaturated fatty acids is lower than that of saturated fatty acids. The reduction of unsaturated carbon- carbon bonds therefore causes another performance degrading characteristic, namely increased viscosity at low temperatures. Hence, to overcome these two limitations of unsaturated fatty acids in vegetable oil, it is necessary to replace the carbon-carbon double bonds of fatty acids with a functional group that is chemically stable to oxidation and maintains liquid state at low temperatures.

These properties are characteristics of branched chain fatty acids (BCFAs) that have no unsaturated carbon-carbon bonds and they display lower melting point than straight-chain

fatty acids. For example, the melting point of normal pentadecanoic acid is 52 °C while that of anteiso pentadecanoic acid is 23 °C [1].

The traditional role of crop plants as a food-source is changing and more crop plant are grown for industrial purposes to provide the specific chemicals, which are needed by industry. Although fatty acids derived from vegetable oils currently have wide applications in industry, their application can still be enhanced if oil-producing crop are genetically engineered to produce structure of value needed by the chemical industry.

Genetic engineering has been successful in generating transgenic plants with desired traits. For example, researchers at DuPont, created transgenic soybean seeds producing oil, that is approximately 80% oleic acid [2]. Long-term goal of the research of my thesis is to identify genes that can be used to generate transgenetic plants to improve the quality of biolubricant and biofuels, and decrease the costs for the production for such applications. This thesis focuses on studying the function of genes, which are capable of generating branched chain fatty acids or mono-acyl esters via biological conversion.

Branched chain fatty acids

Branched chain fatty acids are found in many organisms, such as bacteria, plants, and animals. In bacteria, BCFAs occurs in both gram-positive and gram-negative bacteria. For example, *Staphylococcus* [3], *Streptococcus* [4], *Vibrrioaceae, Micrococcus* [5]*, Bacillus* [5-7]; *Xanthomonas* [8], *Flavobaterium* [9, 10]*,* and *Legionella* [11]. It is thought that a significant role of BCFAs in bacteria is to control the fluidity of their membranes. Small

amount of BCFAs are also found in plants; for example in *Petunia hybrida cv* and tomato [12]. In animals, the biosynthesis of BCFAs is usually a minor process, but there is significant accumulation in the coat wax of sheep [13]. 18-Methyl-eicosanoic acid accounts for 60% of the total fatty acids esterified directly to wool via ester bonds [13]. In all mammalian hairs, 18-methyl-eicosanoic acid comprises 40% or more of the same lipid [13]. However, in other tissues of most mammals, BCFAs are rarely more than 1-2% of the total fatty acids, and they may be generated by the bacteria living in the intestines of animal or are from the consumed food.

Microorganisms live in an environment, which is susceptible to changes of temperature. To adapt to this change, they adjust the acyl components of the membrane lipids. *E. coli* incorporates more unsaturated fatty acids into the membrane lipid at low environmental temperature. When the growth temperature decreases, yeast and fungi also increase polyunsaturated fatty acids such as linoleic and linolenic acids [14-17]. At low temperature *Bacillus* species generate BCFAs to achieve the appropriate fluidity of membrane while they also increase the biosynthesis of unsaturated fatty acids. [18, 19]

The mechanism of low-temperature induction of unsaturated fatty acids is quite clear [20-22]. However, little is known about how bacteria adjust the biosynthesis of BCFAs under the influence of temperature. BCFAs first reported in *Bacillus* in 1960s are mainly anteiso and iso fatty acids [23, 24]. Fifteen-carbon anteiso and iso fatty acids account for more than 50% of total fatty acids in *Bacillus subtilis*. There is experimental evidence that indicate the increase of anteiso fatty acids in *subtilis* is correlated to the drop of growth temperature [25].

Two kinds of fatty acids synthases, type I or type II, are responsible for the biosynthesis of fatty acids. In contrast to the type II fatty acid synthase, which occurs in bacteria and higher plant plastids and is composed of four distinct enzymes [26], a type I fatty acid synthase occurs in eukaryotic organisms and its enzyme components occur as domains on a multifunctional protein(s) [27, 28]. However, in both type I and type II fatty acid synthase systems acyl intermediates of the process are bound to a phosphopantetheine group. The mechanism of the biosynthesis of fatty acid in bacteria has been widely studied in *E. coli* as a model system [29].

This biosynthesis process starts with an acyl-primer, which is elongated, 2-carbons per cycle, using carbon atoms derived from a malonyl moiety. The four sequential reactions that make up this cycle generate 3-ketoacyl-, 3-hydroxyacyl-, and 2-enoyl-thioester derivative intermediates, and finally an acyl-thioester derivative that is 2-carbons longer than the initial acyl primer. In bacteria, typified by the *E. coli* system, these reactions are catalyzed by a dissociable, type II fatty acid synthase that is composed of the enzymes 3-ketoacyl-ACP synthase (KAS), 3-ketoacyl-ACP reductase (encoded by *fabG*), 3-hydroxyacyl-ACP dehydrase (encoded by *fabA*), and enoyl-ACP reductase (encoded by *fabI*). The mechanism of BCFA biosynthesis is similar to this. Except instead of priming with acetyl-CoA, which would generate straight chain fatty acids, iso- or anteiso- bounded acyl-CoA primers are used to generate the respective BCFAs.

There are three types of KAS enzymes, KAS I, KAS II, and KASIII. They differ in their specificities of acyl-thioester substrate, having optimum activities for substrates of different acyl-chain lengths and different thioesters. While KASI and KASII catalyze the condensation between acyl-ACP and malonyl-ACP substrates, KAS III specifically utilizes acetyl-CoA as a substrate for the condensing reaction with malonyl-ACP [30, 31], and thus initiates the biosynthesis of fatty acids. In *Bacillus* both straight-chain fatty acids and BCFAs are produced. In this organism, therefore, both acetyl- and branched-chain acids are used to initiate the fatty acid biosynthesis. The pathway suggested for producing BCFAs in *Bacillus* is illustrated in Figure 1.

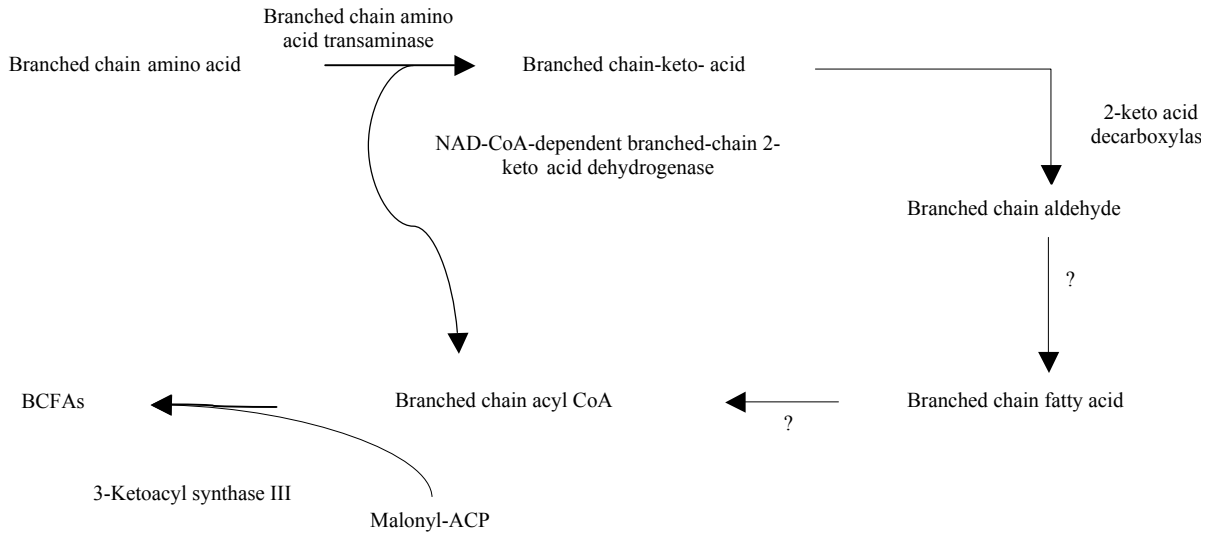

Figure 1. Hypothetical pathway for BCFA biosynthesis in *Bacillus* (Abraham et al., 1993)

The branched chain acyl CoA substrates used in branched chain fatty acids biosynthesis are derived from branched amino acids, leucine, isoleucine, and valine [32]. They respectively

produce 3-methylbutyryl-CoA, 2-methylpropionyl-CoA, and 2-methylbutyryl-CoA by the series of reactions shown in Figure 1. Specifically, each amino acid is deaminated, producing an alpha-keto acid. Following oxidative decarboxylation or dehydrogenation, the resulting short, branched chain acyl CoAs are used as primers to begin the synthesis of BCFAs. Based on the structure of the three amino acids and mechanism of deamination and biosynthetic pathway of BCFAs, iso-odd-fatty acids would be derived from leucine, anteiso-odd-fatty acids would be derived from isoleucine and even-chain iso-fatty acids would be derived from valine [33, 34]. Evidence for this BCFAs biosynthetic pathway is provided by the isolation of a 3-ketoacyl-ACP synthase III enzyme from S. *glaucescens* [35] and *B. subtilis* [36].

In *E. coli* KAS III is encoded by *fabH* gene. The *Bacillus* genome contains two paralogs of *fabH*, called *yhfB* and *yjaX*. The *Bacillus yhfB* is 38 % identical and 48 % similar to *E. coli fabH* gene. The *Bacillus yjaX* is 46 % identical and 54 % similar to *E. coli fabH* gene. The previous researches have shown that KAS III synthase from both S. *glaucescens* and *B. subtilis* are able to use branched chain acyl primers to generate branched chain fatty acids in vitro. But these past studies have made contrary conclusion as to whether KAS III gene is responsible for the BCFA biosynthesis in vivo [37].

In this thesis, the functions of the *Bacillus yjaX* and *yhfB* genes, in branched chain fatty acid biosynthesis were characterized by the combination of "gain of function" and "loss of function" experiments.

Mono-acyl esters synthase

Mono-acyl esters are commercially used in cosmetics, lubricants, polishes, surface coatings, inks, and many other applications [38]. These molecules are mainly obtained from two origins: One is mineral material like brown coat and peat deposits [39], and the other is living organisms. Occurring discreetly in various organisms, mono-acyl esters have a variety of functions in animals [40, 41], plants [42], insects, and bacteria [43, 44]. The desert bush, jojoba, accumulates mono-acyl esters in their seeds. In addition, all plants accumulate mono-acyl esters in their cuticle, which covers the aerial surface of plants to prevent water evaporation [45, 46], and protect plants from diseases [47], and insects [48]. Similar to the function of mono-acyl esters in plants, mono-acyl esters occur in the waxes covering the surface of insects, restricting the water loss across insects' cuticle and preventing desiccation [49]. In most animals sebaceous glands of the skin produce mono-acyl esters for lubricating and protecting the hair and skin, and preventing drying and irritation of membranes [50]. Diverging from the above functions, mono-acyl esters in marine animals are used for buoyancy, insulation, and even echo locating [51]. Even though mono-acyl esters are not common in the prokaryotes, some bacterial species can produce mono-acyl esters. For example *Acinetobacter* sp. generates mono-acyl esters in nitrogen-limited medium [52, 53].

The compositions of mono-acyl esters vary among organisms. In jojoba seeds, those mono-acyl esters are mainly 18:1 (6%), 20:1 (35%), and 22:1 (7%) fatty acids linked to 20:1 (22%), 22:1 (21%), and 24:1 (4%) fatty alcohols [54]. Esters isolated from sheep wool are characterized by *iso*- and *anteiso*-methyl-branched chain fatty acids of mono-acyl ester or sterol. The alcohol moiety of the mono-acyl esters and sterol esters is of 1- and 2-alkanols,

and 1,2-diols sterol, and triterpene alcohols [55] . Honeycomb is built with waxes secreted by bees consisting of 35-80% of mono-acyl esters. Their chain lengths are in the range of 40 to 46 carbons with acyl group based on n16:0 and n18:0 fatty acids, and some of acyl groups are hydroxylated at the ϖ-2 and ϖ-3 positions [56].

$$CH_3\ (CH_2)\ n\ CO\text{-}S\text{-}CoA + HOR \longrightarrow CH_3(CH_2)n\ \ COOR + CoSH$$

Figure 2. The reaction o of mono-acyl esters formation

Those mono-acyl esters are generated by the esterification of an alcohol with fatty acid derived from acyl-CoA (Figure 2). The biosynthesis of alcohol has been studied in the variety of organisms. Two distinct biosynthetic pathways of alcohol have been suggested. One is that alcohol is generated from a four-electron reduction of fatty acyl-coenzyme A (CoA) [40, 41, 43, 57] catalyzed by fatty acyl-coenzyme A reductase (FAR). The other one is that alcohol is produced by the enzymes carrying out two-electron reduction of fatty acyl–CoA. In the former pathway free aldehyde is not detected and the FAR might be an integral membrane protein. In the second pathway free aldehyde is released [58]. In this latter pathway, the enzymes performing the reduction are either soluble or associated peripherally with membranes [59-61].

Fatty acids utilized in the biosynthesis of mono-acyl esters come from either fatty acids generated by *de novo* biosynthesis or elongated fatty acids. Similar to the *de novo* biosynthesis of fatty acids, the elongation of fatty acids is also conducted by four separate enzymes: β-ketoacyl-CoA synthase , β-ketoacyl-CoA reductase, β-ketoacyl-CoA dehydrase,

and β-enoyl-CoA reductase. The repeated iteration of these four-reactions elongates fatty acids by two carbons in each cycle. In contrast to de novo fatty acids biosynthesis, the intermediates of these elongation reactions occur as CoA esters.

The first mono-acyl ester synthase was isolated from jojoba embryos. It was expressed in the seed of *Arabidopsis,* which do not normally accumulate mono-acyl esters. These transgenetic seeds were found to be capable of producing large quantities of mono-acyl esters [62]. Using the jojoba wax synthase cDNA sequence as an entry point into sequence data bases, twelve *Arabidopsis* genes were identified that putatively encode mono-acyl ester synthase genes. We hypothesize that each of these genes may encode mono-acyl ester synthase isozymes that are involved in the biosynthesis of different mono-acyl esters from different precursors, (i.e. catalyze the reaction between different fatty acyl-CoAs and different alcohols), or they are differently expressed during plant development.

Organization of dissertation

This dissertation consists of six chapters. Chapter 1 is a literature review, which covers general introduction to the functions and the biosynthesis of BCFAs; and the functions and the biosynthesis of mono-acyl esters.

Chapter 2 is a manuscript in preparation for the submission to the Journal of Bacteriology. The manuscript describes the characterization of *Bacillus yhfB* and *yjaX* knockout mutants. The findings reported in this manuscript include conditional expression of *yhfB* gene, the

thermo regulation of BCFAs biosynthesis, and the effect of the *yhfB* and *yjaX* knockout on the genetic transformation of *Bacillus*. The experiments covered in this chapter were all conducted by Yuqin Jin under the supervision of Dr. Basil Nikolau. The manuscript was also edited by Dr. Basil Nikolau

Chapter 3 is a manuscript, which was submitted to the Proceedings of the National Academy of Sciences U.S.A. The manuscript describes the experiments that explore the interexchangability of different fatty acids in altering membrane fluidity, specifically testing if BCFAs can substitute for unsaturated fatty acids in maintaining membrane fluidity. The experiments indicate that the BCFA generated by recombinant *Bacillus yhfB* gene can substitute for the lack of unsaturated fatty acids in maintaining membrane viability in *E. coli*. The experiments described in this chapter were all conducted by Yuqin Jin under the supervision of Dr. Basil Nikolau. The manuscript was also edited by Dr. Basil Nikolau.

Chapter 4 is a manuscript in preparation for the submission to Journal of Bacteriology. The experiments reported in this chapter show that the effect of deleting and replacing the *fabH* gene in *E. coli* with the *Bacillus yhfB* or *yjaX* genes. The findings of this research show that *E. coli* is still viable in the absence of *fabH* coding KAS III and this result is contrary to previous studies [37]. The experiments covered in this chapter were all conducted by Yuqin Jin under the supervision of Dr. Basil Nikolau. The manuscript was also edited by Dr. Basil Nikolau.

Chapter 5 is a manuscript describing experiments about the mono-acyl ester synthase. Of the 15 mono-acyl ester synthase mutants genotyped in this dissertation, SALK_060303 allele carrying T-DNA insertion in At5g55380 shows growth phenotypes. The gene structure and transcriptions of At5g55380 from SALK_060303 were characterized. The developing and growth morphologic phenotypes were shown in this dissertation. The experiments of paralogous expression were performed by Ling Li, the other experiments covered in this chapter were done by Yuqin Jin under the supervision of Dr. Basil Nikolau. The manuscript was edited by Dr. Basil Nikolau.

Chapter 6 is a general summary and conclusion of the dissertation.

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Chapter 2. Genetic Dissection of the Function of duplicated 3-ketoAcyl-CoA Synthase Genes in Fatty Acid Biosynthesis in *Bacillus*

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Abstract

3-Ketoacyl-ACP synthase III (KASIII) catalyzes the initial condensation reaction of *de novo* fatty acid biosynthesis. Prior *in vitro* experiments have demonstrated that the *Bacillus* genome contains two homologous KASIII genes (*yjaX* and *yhfB*) that catalyze the condensation reaction between a variety of acyl-CoAs and malonyl-ACP. To characterize the specific physiological function of these two paralogs in the synthesis of fatty acids, these genes were individually disrupted and the resulting mutant strains were characterized. While the *yhfB*-knockout strain had no detectable change in growth at either 37 ^{0}C or 16 ^{0}C, relative to the wild type strain, the *yjaX*-knockout strain showed an increased growth rate at the lower temperature. These changes in growth characteristics are coupled with fatty acid compositional changes in the *yjaX*-knockout strain, whereas the fatty acid composition of the *yhfB*-knockout strain is near indistinguishable from the wild type. Studies of the expression of these genes using *lacZ*-gene fusions as reporter of expression, or by western blot analysis of the accumulation of the YjaX and YhfB proteins, indicate that normally only the *yjaX* gene is active, and that the expression of the *yhfB* gene is activated only in the *yjaX*-knockout strain. These observations establish therefore, that the phenotype and chemotype of the *yjaX* mutant strain is due to the replacement of this gene function by the expression of the *yhfB* gene. Because the enhanced growth rate of the *yjaX*-knockout strain at the cooler

temperature is coupled with the increased accumulation of anteiso-branched chain fatty acids, these data indicate that the *yhfB*-coding KASIII has a higher preference for 2-methylbutyryl-CoA as a substrate for initiating fatty acid biosynthesis. Finally, we also determined that the *yjaX* mutant strain looses the natural competence for DNA transformation. This observation indicates a coupling between membrane fatty acid composition and the operation of the Ψ-pilus assembly and/or the ComEC complex, which facilitates the import of the exogenous DNA and the successful expression of natural competence.

Introduction

The biochemical mechanism of fatty acid biosynthesis is universally similar among all organisms. Generally, fatty acids are synthesized by the repeated iteration of four-reactions, which start with an acyl-primer, which is elongated, 2-carbons per cycle, using carbon atoms derived from a malonyl moiety. The four sequential reactions that make up this cycle generate 3-ketoacyl-, 3-hydroxyacyl-, and 2-enoyl-thioester derivative intermediates, and finally an acyl-thioester derivative that is 2-carbons longer than the initial acyl primer. In bacteria, typified by the *E. coli* system, these reactions are catalyzed by a dissociable, type II fatty acid synthase that is composed of the enzymes 3-ketoacyl-ACP synthase (KAS), 3-ketoacyl-ACP reductase (encoded by *fabG*), 3-hydroxyacyl-ACP dehydrase (encoded by *fabA*), and enoyl-ACP reductase (encoded by *fabI*). In contrast to the type II fatty acid synthase, which occurs in bacteria and higher plant plastids and is a dissociable enzyme system composed of four distinct enzymes, [1], a type I fatty acid synthase which occurs in other eukaryotic organisms, these enzyme components occur as domains on a multifunctional

protein(s) [2, 3]. However, in both type I and type II fatty acid synthase systems acyl derivative are bound to phosphopantetheine group.

In the type II fatty acid synthase system, there are three genetically and biochemically distinct KAS isomers, namely KASI (encoded by *fabA*), KASII (encoded by *fab F*), and KASIII (encoded by *fabH*) [1, 4, 5]. Their functions have been studied extensively in *E. coli*. They differ in their specificities of acyl-thioester substrate, having optimum activities for substrates of different acyl-chain lengths and different thioesters. While KASI and KASII catalyze the condensation between acyl-ACP with malonyl-ACP substrates, KAS III specifically utilizes acetyl-CoA as a substrate for the condensing reaction with malonyl-ACP [6, 7], and thus initiates the biosynthesis of fatty acids.

Although the general mechanism of fatty acid biosynthesis in gram positive bacteria, such as *Bacillus subtilis*, is similar to that of *E. coli*, one major difference is the fact that *B. subtilis* produces large quantities of branch chain fatty acids. These fatty acids are branched with methyl groups at the iso- and anteiso positions (i.e., 13-methyltetradecanoic, 12-methyltetradecanoic acid, and 14-methylpentadecanoic acid). These fatty acids are biosynthesized by a type II FAS that has the ability to initiate this process by using branched acyl-CoAs that are derived from the branched chain amino acids, leucine, isoleucine and valine [8]. Thus, the *Bacillus* FAS enzymes must have the capacity to utilize such branched acyl substrates. Genomics-based analysis of the *B. subtilus* genome has lead to the identification KASII [9] and KASIII homologous genes, however, it appears that this bacterium does not contain a sequence-recognizable KASI homolog. KASII is an essential

enzyme in *Bacillus* which is encoded by *yjaY*. Two *Bacillus* KASIII genes, *yhfB* and *yjaX*, have been characterized, and these, unlike the *E. coli* homolog, have the capacity to catalyze the condensation of branched acyl-CoAs with malonyl-ACP [10, 11]. The specific physiological functions of the two KASIII genes are unclear in *Bacillus*. This paper, addresses this question.

Materials and Methods

Bacterial strains and growth conditions. The wild type *B. subtilis* strain 168 used in these studies was obtained from the Bacillus Genetic Stock Center. All *B. subtilis* strains, wild type and mutants, were grown at 37°C or 16°C in Luria Broth (LB) medium or in minimal medium [12], both of which were supplemented with appropriate antibiotics; erythromycin was used at 5μg/ml, and ampicillin at 100 μg/ml. The minimal medium consisted of 1% glucose, 0.2% potassium L-glutamate, 100 mM potassium phosphate buffer (pH=7), 3 mM $MgSO_4$, 3 mM trisodium citrate, 22 mg/L of ferric ammonium citrate, 50 mg/L of L-tryptophan, and 0.1% casein hydrolysate.

Construction of recombinant integration vectors. A fragment of the *yjaX* gene (nucleotide position from 190 to 513) was PCR amplified with the primers, yjaXF and yjaXR (Table1). The yjaXF primer contains a *Hin*dIII restriction site while the yjaXR primer contains a *Bam*HI restriction site. This *yjaX* fragment was cloned into the *HindIII* and *BamHI* restriction sites of pMUTIN4 vector [13]. By the analogous procedure, a *yhfB* fragment (from nucleotide from 415 to 843) was also cloned into the pMUTIN4 vector. The recombinant

pMutin4 plasmids carrying the *yjaX* and *yhfB* gene fragments are named pM4X and pM4B, respectively.

Protein Analysis. To extract proteins, bacterial cells were collected by centrifugation from a 1-ml aliquot of culture, grown overnight. The pellet was then suspended in extraction buffer consisting of 30 mM Tris-HCl, pH 8, 10 mM EDTA and 0.5 mg/ml lysozyme, and incubated at 37 °C for 30 min. Following centrifugation for 1 min at 16,100 g, the supernatant was retained for analysis. Prior to electrophoresis, the extract was adjusted to 10% glycerol, 0.001 % Bromophenol Blue, 1% SDS, 10 mg/ml DTT and boiled for 1 minute. After fractionation by electrophoresis, proteins were either visualized by staining with Coommassie Brilliant Blue, or they were transferred electrophoretically to a nitrocellulose blotting membrane. Immunological detection was achieved by a two-step process, in which the proteins on the membrane were first reacted with a rabbit generated antisera, followed by a goat-anti rabbit IgG conjugated to horseradish peroxidase. The resulting antigen-antibody complexes were visualized with the ECL Western Blotting Detection Reagents and Analysis System (Amersham Bioscience, Piscataway, NJ).

Assays of ß-Galactosidase. β-galactosidase activity was assayed as described [14] using *o*-nitrophenyl-β-D-galactoside as substrate, and the results were presented as Miller units [15].

Fatty acid analysis. Lipids were extracted from bacterial cell pellets using chloroform/methanol [16, 17], and fatty acids were then converted to methyl esters using methanolic-HCl at 80^{o}C for 20 minutes [18-20].The recovered fatty acid methyl esters were

analyzed with GC-MS (6890 series Agilent, Palo Alto, CA), interfaced with a Mass Detector 5973 (Agilent, Palo Alto, CA).

Results

Mutant isolation. Sequence-based comparisons, confirmed by biochemical experimentation have established that the *B. subtilis* genome contains two KASIII paralogs, encoded by the *yhfB* and *yjaX* genes. These two genes code for 325- and 312-residue proteins that share 43% sequence identity (54% sequence similarity). To identify the physiological roles of these two KASIII isozymes individual mutant strains that carried disrupted null alleles of these two genes were constructed. These strains were constructed by inserting subfragments of the *yhfB* and *yjaX* genes into the integration vector, pMUTIN4 [13], to generate plasmids pM4B and pM4X, respectively (Figure 1). These plasmids were transformed into wild type *B. subtilis* strain168 and the desired mutants were selected by virtue of their ability to grow in lethal doses of erythromycin.

To verify that the single cross-over recombination-mediated integration of the vectors occurred as expected, genomic DNA was isolated from erythromycin-resistant colonies. Using this DNA as template, we conducted PCR reactions with primers that would support amplification only from appropriately recombined alleles. Specifically, the disrupted *yhfB* locus would support PCR analysis with the primer pairs BL and P, and not with the primer pairs BL and BR (Fig. 1E). Indeed, this was the case (Fig. 1G), and the nucleotide sequence of this PCR product confirmed that the *yhfB* locus was disrupted by the insertion of the pM4B vector. Similarly for the *yjaX* locus, PCR analysis with primer pairs XL and P

supported the amplification of a DNA fragment whose sequence confirmed the disruption of this gene by the insertion of the pM4X vector (Fig. 1F and H). The resulting recombinant *B. subtilis* strain characterized by the disruption of the *yjaX* gene was named *yjaX-m,* while the strain characterized by the disruption of the *yhfB* gene was named *yhfB-m*.

Growth characteristics of mutant strains. To test if the disruption of the *yhfB* or *yjaX* genes affects cell growth, we cultured the wild-type *B. subtilis* strain 168, and mutant strains, *yjaX-m,* and *yhfB-m* at 37 °C and 16 °C (Fig. 2). As shown in Figure 2, at 37 °C, there is no difference in the growth rate between the wild-type and *yhfB-m* strains, but the *yjaX-m* strain shows a decreased log phase rate of growth (Fig. 2A). However, because this decreased rate of growth can be reversed by the inclusion of IPTG in the growth medium, it is a polar effect of the disruption of this gene. Namely, the insertion of the pM4X sequence in *yjaX*, affects the expression of the down stream essential gene, *fabF* (initially called *yjaY* [9]). This effect is compensated by the addition of IPTG that induces the promoter, P_{spac}, situated at the end of the insertion sequence (Fig. 1F), activating the expression of *fabF* and thereby eliminating the polar effect of the *yjaX-m* mutation. Therefore, all later experiments in this study were conducted by adding IPTG to the media to a final concentration of 1mM. These results indicate that there is no difference in growth rate at 37 °C between the wild-type strain and the strains lacking either of the KASIII paralogs (*yhfB-m* or *yjaX-m* mutant strains).

When these strains are cultured at a lower temperature (16°C, in the presence of IPTG), we observed that the log phase growth rate of *yhfB-m* is indistinguishable from that of the wild type parental strain. However, the log phase growth rate of the *yjaX-m* strain is higher than

that of both the wild type and *yhfB-m* strains (Fig. 2B). Hence, the elimination of the *yhfB*-encoded KASIII appears not to affect the growth of *B. subtilis,* while elimination of the *yjaX*-encoded KASIII appears to promote the growth rate of *B. subtilis* at 16 ºC.

Expression of *yjaX* and *yhfB* genes. To examine if the growth phenotypes observed in the knockout experiments are related to the expression patterns of the *yjaX* or *yhfB* genes, western analyses were conducted using previously generated antisera [21], to ascertain the expression patterns of these two genes. Figure 3A shows that the YjaX protein, but not the YhfB protein, accumulates to detectable levels in wild-type *B. subtilis*. As expected, the accumulation of the YjaX protein is eliminated by the *yjaX* gene-knockout, and is not affected by the *yhfB* gene-knockout. Interestingly, the accumulation of the YhfB protein is induced in the *yhfB-m* strain (Fig. 3B). These data therefore, indicate that whereas the *yjaX* gen expressed in wild type, the *yhfB* gene is normally silent, and its expression is induced in the absence of a functional *yjaX* gene.

The finding that the expression of the *yhfB* gene is conditional on the absence of the *yjaX*-encoded KASIII was surprising; therefore corroborative evidence for this result was sought using an independent approach. Because the integration event that generated the *yjaX* and *yhfB* knockout alleles fused the *lacZ* gene to each of these respective genes (Fig. 1), it was possible to determine the expression of each gene's promoter by measuring β-galactosidase activities in each mutant strain. As shown in Figure 4, *yjaX* promoter activity is readily detectable throughout the growth curve of the *Bacillus* culture, irrespective of growth temperature or weather it is grown on rich or minimal media. In these experiments, maximal

expression of *yjaX* occurs at early to mid-log phase of growth, coincident with maximal need for membrane lipid deposition. In contrast, *yhfB* promoter activity was near the detection limits of this assay at all experimental points that were taken indicating that the expression of this gene is at least 8-fold lower than the expression of *yjaX*. Hence, the ß-galactosidase reporter assays are consistent with the western blot data and show that *yhfB* gene is minimally expressed, while the *yjaX* gene accounts for the major form of KASIII that is expressed under normal growth conditions of *Bacillus*.

Effect of *yjaX* and *yhfB* gene knockouts on fatty acid composition. Fatty acid compositions of the *yhfB-m* and *yjaX-m* strains were compared to the wild-type progenitor strain. In these experiments, strains were grown in LB medium at either 37 ^{0}C or 16 ^{0}C, and cells were harvested at mid-log phase. These analyses indicate that at either growth temperatures, the fatty acid composition of the *yhfB-m* strain is near identical to that of the wild type (Fig. 5A and 5D). However, there are significant alterations in the fatty acid composition caused by the *yjaX* mutation (Fig. 5A and Fig 5D). These effects are dependent on the temperature at which the *yjaX-m* strain is grown. Specifically, at 37 ^{0}C, the major effect of the *yjaX* mutation is to increase the proportion of n16:0 and anteiso-15:0, at the expense of iso-15:0 and iso-17:0. In addition, there is a modest increase in the ratio of anteiso:iso BCFAs (Fig. 5C). At the lower growth temperature, the effect of the *yjaX* mutation is more dramatic than at 37 ^{0}C. Namely, the proportion of 15-carbon BCFAs increases at the expense of the longer chain lengths (Fig. 5E and 6A), and the ratio of anteiso:iso BCFAs is increased, particularly for the 17-carbon chain length (Fig. 5F and 6B). Finally, we observed that lowering the growth temperature increased the ratio of iso-odd

BCFAs:iso-even BCFAs in both the wild-strain and the *yhfB-m* strain. However, this failed to occur in the *yjaX-m* strain (Fig. 6C).

Effect of *yjaX* and *yhfB* gene knockouts on plasmid transformation. In experiments in which we attempted to create a double mutant strain (i.e., a *yjaX-m, yhfB-m* strain), we noticed that the *yjaX-m* strain was recalcitrant to plasmid transformation. Therefore, we conducted controlled experiments in which we compared the transformation rates of wild-type *B. subtilis* strain 168, with derivative strains, *yjaX-m* and *yhfB-m* using the plasmid PUB110 [22]. In three independent experiments, these strains were transformed with the same amount of this plasmid, and whereas up to 6,000 kanamycin resistant colonies could be recovered with the wild-type strain, the transformation rate with strain *yhfB-m* was reduced to between 1/8 and 1/2, and strain *yjaX-m* was nearly recalcitrant to transformation in these conditions (Table 2).

To examine if this loss in transformation competence is correlated to the change in the composition of fatty acids in these mutants, we compared the of fatty acid compositions of these strains grown in minimum salt medium. As shown in Figure 8, correlated with the loss of transformation competence, we find that the *yjaX-m* strain has a higher proportion of straight-chain fatty acids, than the wild type and *yjaX-m* strains, which are competent for plasmid transformation.

Discussion

The *yjaX* gene is expressed in wild type and *yhfB* gene is expressed in the absence of *yjaX* gene. The occurrence of two KASIII coding genes in the *Bacillus* genome was experimentally demonstrated by gain of function experiments, where the *yhfB* and *yjaX* genes where expressed in *E. coli* and shown to catalyze the condensation of acyl-CoAs with malonyl-ACP [21]. The occurrence of KASIII paralogs in bacterial genomes is an unusual occurrence. Sequence analysis with TBLASTN of the bacterial genome sequences at the NCBI database (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi) indicates that this occurs in this bacterium and a few other bacteria. Although there are a number of potential explanations for this gene redundancy, we considered the possibility that this diversity allows the organism to produce a diversity of fatty acids. Specifically, KASIII catalyzes the initial condensation reaction of fatty acid biosynthesis, thus this enzyme determines the structure of the ω-end of the resulting fatty acid products. Namely, *Bacillus* has the capacity to produce *normal*, iso-, and anteiso-fatty acids, reflecting the priming of fatty acid biosynthesis with acetyl-CoA, isobutyryl-CoA, isovaleryl-CoA, and anteisovaleryl-CoA. Thus, the occurrence of two KASIII paralogs may be a means of regulating this diversity in fatty acid structures.

The experiments presented in this paper disprove this hypothesis, and provides an alternative explanation for the redundancy in genes that code for KASIII in the *Bacillus* genome. Specifically, we demonstrate that while the *yjaX*-encoding KASIII is expressed in the wild type state, the expression of *yhfB*-encoding KASIII was detected only when the *yjaX* gene-function was deleted. The lack of *yhfB* expression in the wild type state also explains the

finding that the growth rate of *yhfB-m* strain (carrying a *yhfB* deletion allele) has identical growth characteristics to that of the wild type strain. In contrast, the difference in log-phase growth rates between *yjaX-m* and the wild-type strain is caused by the conditional expression of *yhfB*-encoding KASIII.

Because both the *yjaX* and *yhfB* genes encode KASIII isozymes, which initiate the biosynthesis of fatty acids, we would anticipate that mutations in each of these genes should alter the acyl composition of the resultant mutant strains. Consequently, such alterations of acyl lipids may affect the growth of these strains. Thus, consistent with the finding that the *yhfB* gene is not expressed in the wild type strain, knocking out this gene does not alter the growth of the mutant strain, and does not alter the fatty acid composition of that strain. In contrast, however, interpretation of the data derived from the *yjaX-m* strain is confounded by the fact that in this mutant the *yjaX*-encoding KASIII is replaced by the expression of the *yhfB*-encoded KASIII. Therefore, the growth phenotype and fatty acid chemotype of the *yjaX-m* strain reflects the functionality *yhfB*-encoded KASIII.

Relationship between KASIII paralogs and thermoregulation of fatty acid composition in *Bacillus*. One of strategies that organisms use to adapt to environmentally low temperature is to alter the acyl-chains of the membrane lipids in order to maintain the appropriate fluidity of membranes. Most organisms achieve this by incorporating into the membrane lipids fatty acids that have lower melting points, such as unsaturated fatty acids [23, 24]. *Bacillus* also uses this mechanism [25-27], but can also control the fluidity of the membrane by modulating the proportion of BCFAs in the membrane lipids [28]. Because

KASIII is the enzyme that would be expected to metabolically determine the nature of the BCFAs that *Bacillus* produces, the mutant strains generated within this study, offered the capacity to investigate the relationship between KASIII paralogs and thermormoregulation of BCFA composition.

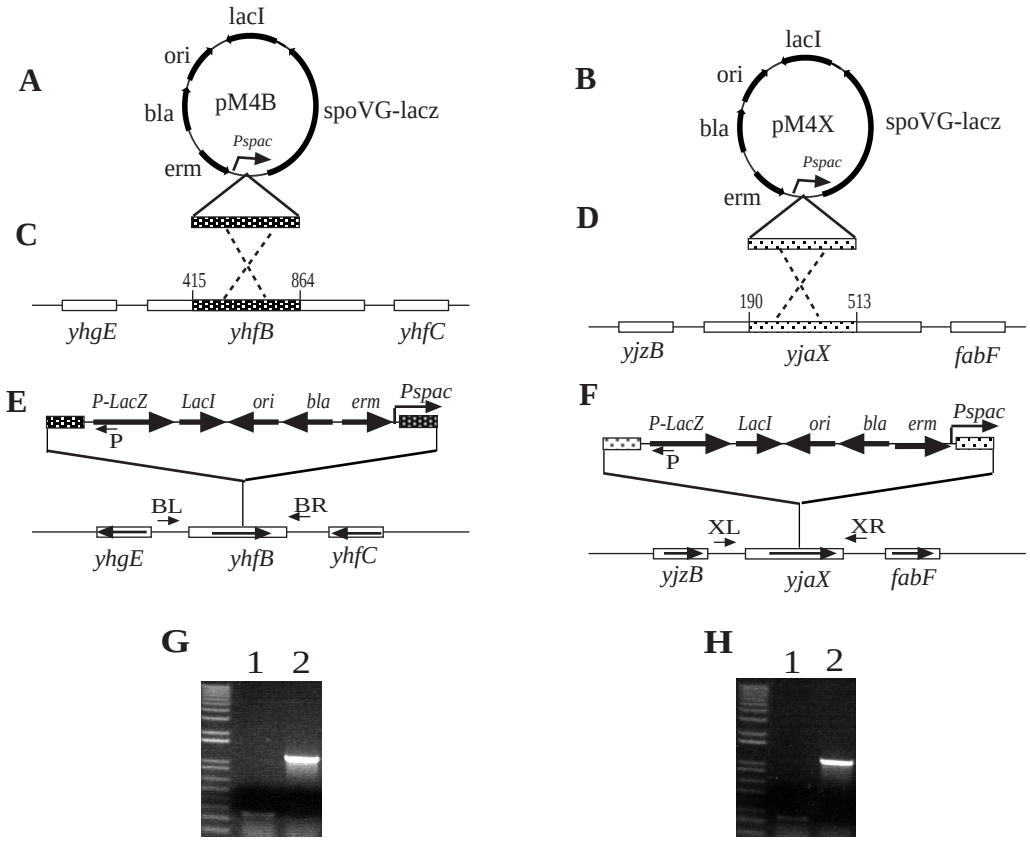
To investigate this relationship we compared the growth phenotype and fatty acid chemotype of each KASIII mutant (strains *yjaX-m* and *yhfB-m*) grown at a normal temperature (37 ^{0}C) and at a lower temperature (16 ^{0}C), and compared these data to the thermoregulation of the expression of each KASIII paralog. These comparisons indicate that at either temperature, the growth phenotype and fatty acid chemotype of the *yhfB-m* strain is indistinguishable from the wild type strain. This is consistent with the fact that the *yhfB*-encoded KASIII expression is not detectable at these two growth temperatures. In contrast, at the lower temperature, the *yhfB-m* strain grows considerably faster than the wild type strain, and this is associated with increased abundance of anteiso-BCFAs and of shorter chain (<15-carbons) fatty acids. Both of these types of fatty acids, have lower melting points [29], and thus these compositional changes would be anticipated to provide fluidity to membrane lipids at lower temperatures. Indeed, earlier studies had indicated that enhanced accumulation of anteiso-BCFAs promotes growth at lower temperatures [28]. Thus, we hypothesize that the enhanced growth rate of this mutant strain at the lower temperature is due to these compositional changes. Furthermore, because in this mutant strain the expression of the *yhfB*-encoded KASIII is induced, we surmise that this KASIII has a higher preference for anteiso-acyl-CoA substrates than the *yjaX*-encoded KASIII, thus generating higher levels of anteiso-BCFAs in the *yjaX-m* mutant strain.

These characterizations have revealed the occurrence of an intriguing mechanism of gene regulation that will need additional experimentation. Specifically, these data indicate that the two KASIII genes in *Bacillus* coordinate their expression in that *the yhfB* gene is inactive, unless the *yjaX* gene function is eliminated. Elucidating the mechanism for this regulation will likely provide insights into the evolutionary advantage of maintaining two functional copies of KASIII in the genome, when one is normally not active.

The knockout of *yjaX* gene affects frequency of transformation. *Bacillus* is a bacterium that shows natural competence for transformation by exogenous DNA without human/artificial manipulation. Natural competence is a physiologically and genetically determined trait, which is very well studied in *Bacillus* [30, 31]. DNA uptake for transformation occurs with the aid of a series of complexes that are composed of over 100 proteins. Central to this process are two integral membrane complexes, the Ψ-pilus assembly and the ComEC membrane channel. The former is thought to bind and pull DNA through the ComEC channel, which is located on the cytoplasmic membrane [30]. Our observations that the *yjaX-m* strain looses natural competence may indicate that membrane lipid composition may be important in this process. Indeed, correlated with this loss of competence, we find that the *yjaX-m* strain shows a fatty acid composition that is enriched in saturated fatty acids. Such a change in membrane lipids would be expected to decrease the fluidity of the membrane, which we hypothesize affects the operation of the Ψ-pilus assembly and/or the ComEC complex, resulting in the loss of competence. Although such a role for the membrane may be obvious from the location of the protein machinery that

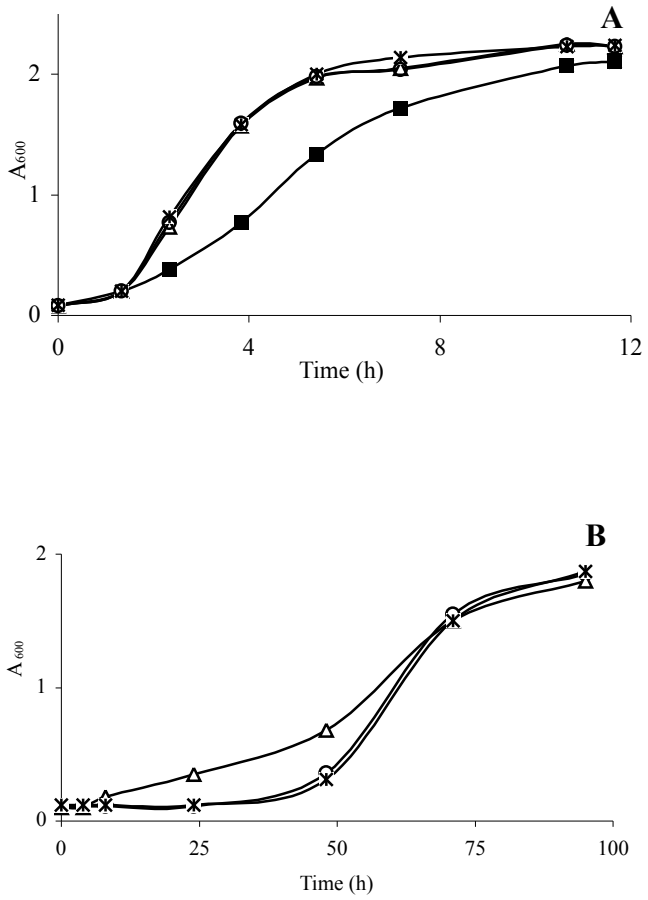
imports the exogenous DNA, it has not been investigated in previous studies of this phenomenon. However, global RNA profiling studies have noted that the expression of the *yjaX* gene is partially dependent on the function of the *comK* gene, which codes for the competence transcription factor that is the master regulator of natural competence [32]. This observation therefore may be in support of our hypothesis that the fatty acid composition of the membrane lipid has a role in the successful expression of natural competence.

Fig.1. Generation of *yhfB* and *yjaX* disruption-alleles. Approximately 350-nucleotide DNA-fragment derived from either the *yhfB* (between nucleotide positions 415 to 843) or *yjaX* (between nucleotide positions 190 to 513) gene was cloned into the vector pMUTIN4 to generate pM4B (A) and pM4X (B), respectively. The position of these fragments that target the recombination event for generating disruption alleles are indicated on the wild-type *yhfB* (C) and *yjaX* (D) locus. A single crossover event, between pM4B and the *yhfB* locus, and between pM4X and the *yjaX* locus would generate disrupted alleles *yhfB-m* (E) and *yjaX-m* (F) in which the pMUTIN4 backbone sequence would be inserted within the respective locus. Arrows labeled, BL, BR, XL, XR and P, indicate the locations and orientations of primers used in diagnostic PCR assays to confirm the disrupted alleles. Diagnostic PCR assays were conducted with DNA template isolated from erythromycin-resistant colonies following transformation with pM4B (G) and pM4X (H). PCR assays with primer combinations that span the entire locus, BL + BR for *yhfB-m* (G, lane 1), and XL + XR for *yjaX-m* (H, lane 1) fail to generate a product due to the insertion of the 8.6-kb pMUTIN4 backbone sequence, but PCR assays with primer combinations BL + P (G, lane 2), and XL + P (H, lane 2) generate products of the appropriate size. The nucleotide sequences of these PCR fragments are consistent with the correct integration of pM4B and pM4X sequences, respectively.



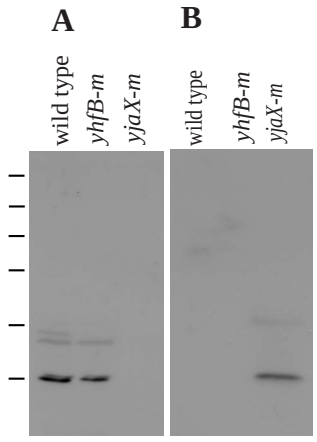
Jin and Nikolau, Figure 1

Fig.2. Effect of disrupting the *yjaX* and *yhfB* genes on cell growth. Cultures were inoculated with 1:100 dilution of an overnight culture, and growth was monitored at A_{600}. Cultures were grown in 50-ml volume of LB medium in a 250-ml Ehlemeyer flask, aerated by shaking at 300 rpm. The wild type strain 168 (○), and the mutant strains *yjaX-m* (■), and *yhfB-m* (∗) were cultured either at 37 ^{0}C (A) or 16 ^{0}C (B) in LB medium. All three strains were also grown in LB media containing IPTG as an inducer of the P_{spac} promoter. Whereas growth rates were unaffected by the inclusion of IPTG for the wild type and yhfB-m strains (data not shown) the growth of the *yjaX-m* strain was enhanced by the inclusion of this inducer in the growth medium (Δ).



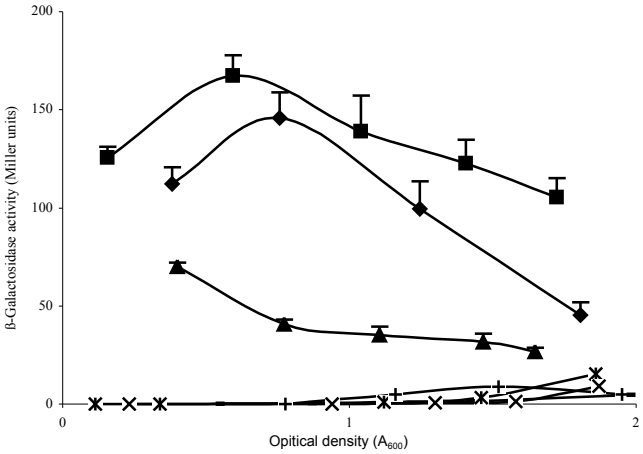
Jin and Nikolau, Figure2

Fig. 3. Effect of disrupting the *yjaX* or *yhfB* genes on the accumulation of the YjaX and YhfB proteins. Aliquots of protein extracts (about 50 μg per lane) from wild type strain 168, and mutant strains *yhfB-m,* and *yjaX-m* were subjected to SDS-PAGE and western blot analysis, using either YjaX (A) or YhfB (B) antisera.



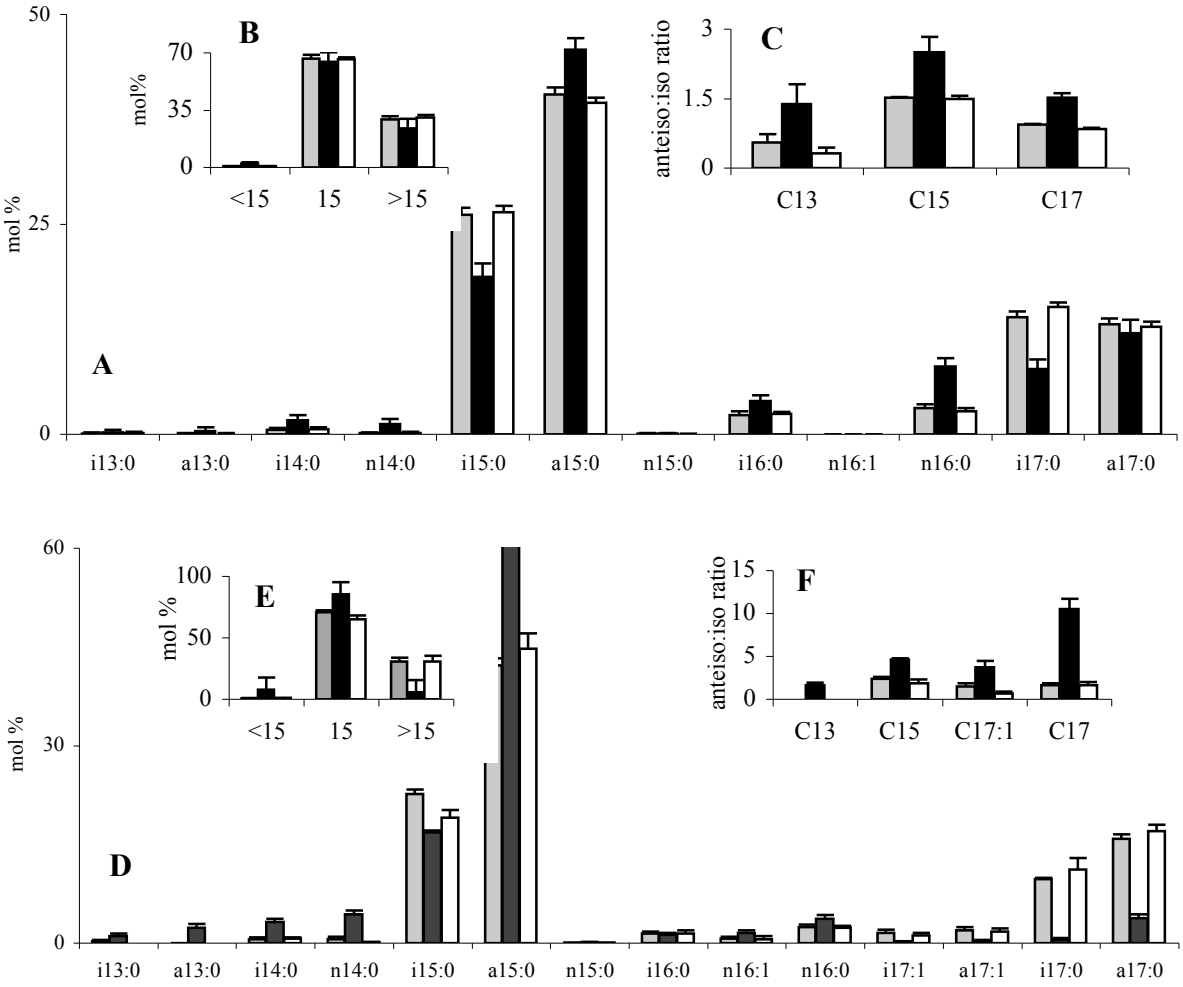
Jin and Nikolau, Figure 3

Fig. 4. Expression of *yhfB* and *yjaX* genes determined by ß-galactosidase activity. In the disrupted alleles *yjaX-m* and *yhfB-m*, the *lacZ* gene is fused with the *yjaX* and *yhfB* genes, respectively (Fig. 1E and F). Therefore, ß-galactosidase activity was used to assay the expression of these two genes. The wild-type strain168 (×), and mutant strains *yjaX-m* (◆, ■), and *yhfB-m* (+, ✻) were grown in LB medium at either 37 ^{0}C (×, ◆, +) or 16 ^{0}C (■, ✻), and the y*jaX-m* strain was grown in minimum salt medium at 37 ^{0}C (▲). At the indicated optical density, aliquots of the cultures were removed and β-galactosidase activity determined.



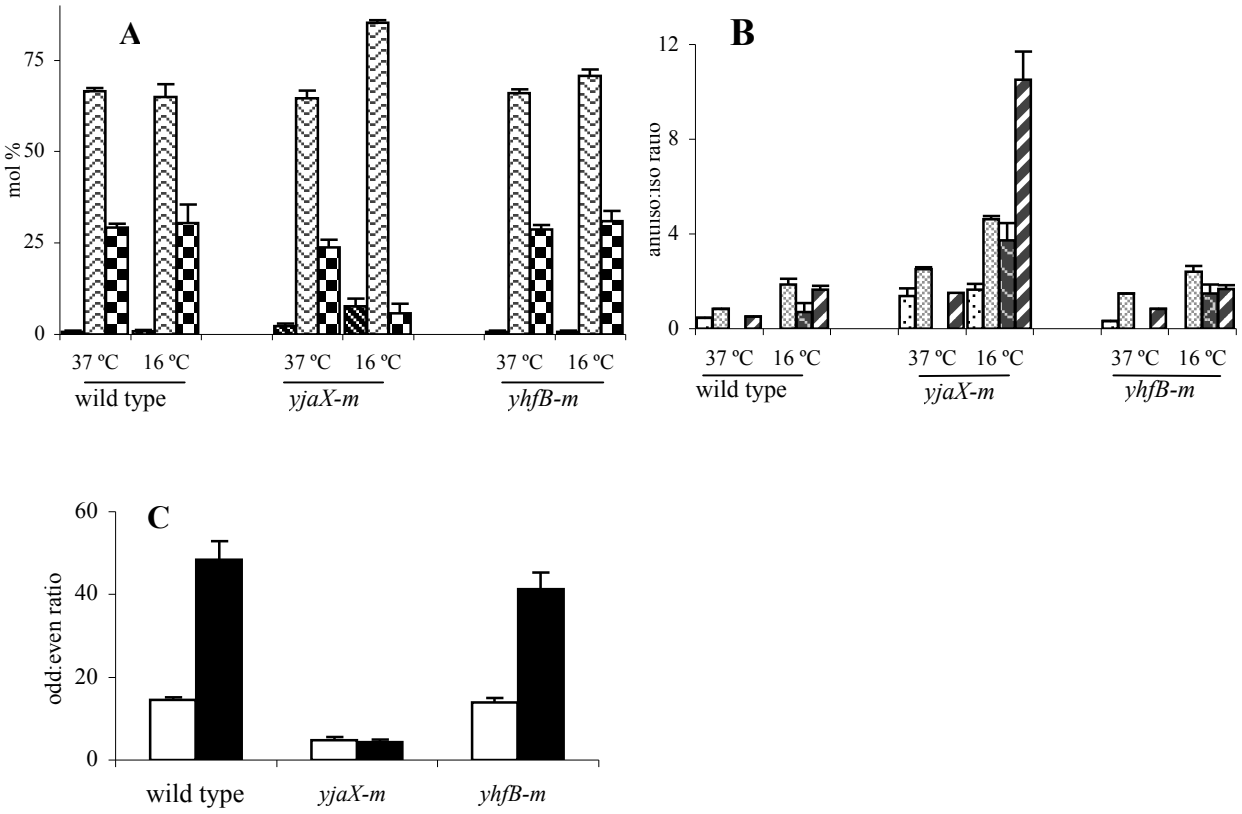
Jin and Nikolau, Figure4

Fig. 5. Effect of disrupting the *yjaX* or *yhfB* genes on fatty acid composition of *Bacillus* cultures grown in rich media. Cultures of wild-type strain 168 (□), and mutant strains *yhfB-m* (■) and *yjaX-m* (□) were grown in LB medium at either 37 ^{0}C (A) or 16 ^{0}C (D), and cells were collected 4 hours (37^{0}C) or 30 hours (16^{0}C) after growth, when A_{600} of the two cultures was about 0.3-0.6. Lipids were extracted from the collected cells, and following transmethylation fatty acid methyl esters were analyzed by GC-MS. From these data we calculated the proportion of fatty acids with chain lengths of <15, 15 or >15 carbon atoms (B, E), and the ratio of anteiso:iso fatty acids (C, F).



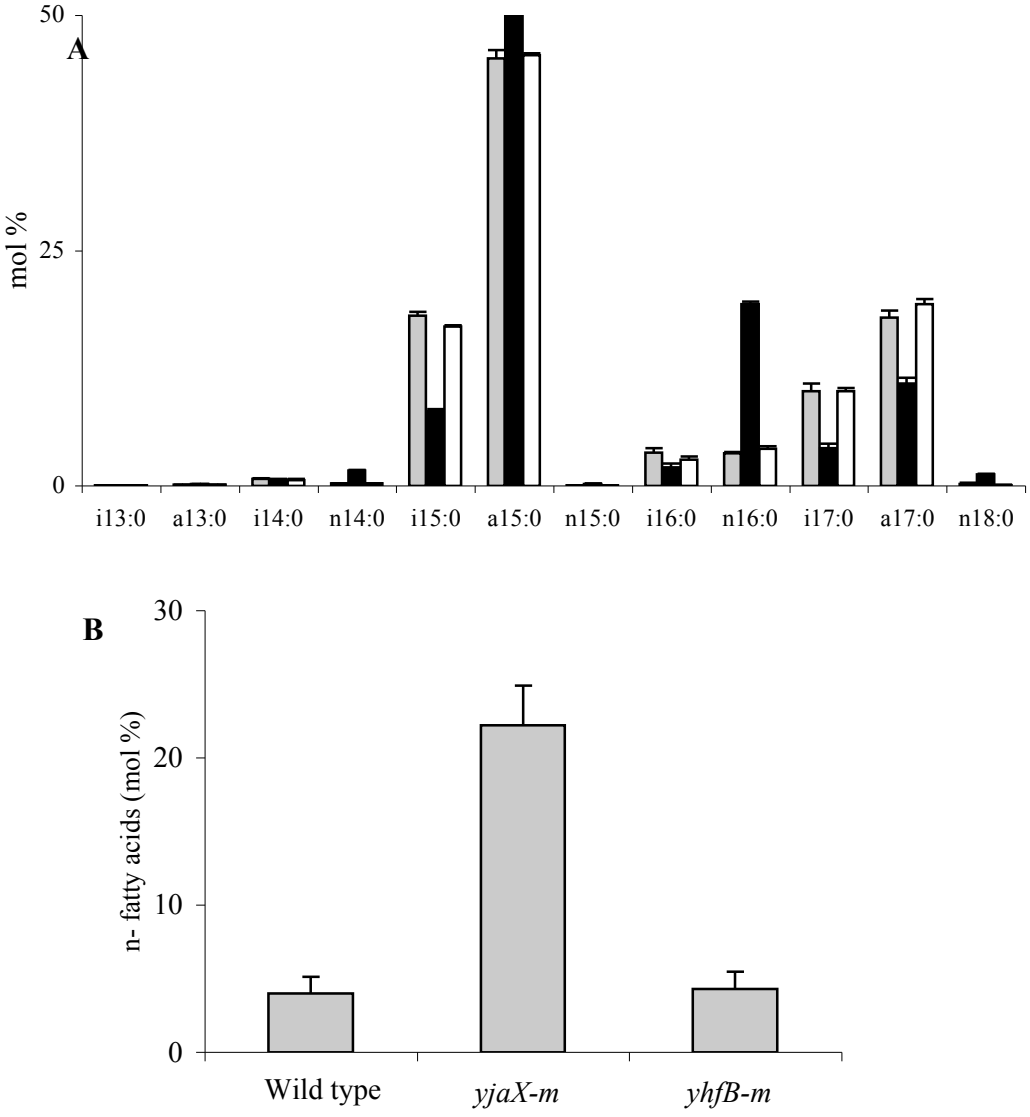
Jin and Nikolau, Figure 5

Fig. 6. Effect of disrupting the *yjaX* or *yhfB* genes on the temperature response in fatty acid composition. From the data presented in Figure 5, we compared the proportion of fatty acids with chain lengths of <15, 15 or >15 carbon atoms (A), the ratio of anteiso:iso branched fatty acids of 13:0, 15:0, 17:0 and 17:1 (B), and the ratio of odd:even number of carbon atoms (C).



Jin and Nikolau, Figure 6

Fig. 7. Effect of disrupting the *yjaX* or *yhfB* genes on fatty acid composition of *Bacillus* cultures grown in minimal salt medium. Cultures of wild-type strain 168 (□), and mutant strains *yhfB-m* (■), and *yjaX-m* (□) were grown in minimal salt medium at 37 ^{0}C, and cells were collected when A_{600} of the cultures was 1.0. Lipids were extracted from the collected cells, and following transmethylation, fatty acid methyl esters were analyzed by GC-MS (A). From these data we calculated the proportion of the sum of the straight chain fatty acids of 14-, 16- and 18-carbon chain lengths (B)



Jin and Nikolau, Figure 7

Table1. Primers used for knockout and checking knockout.

Gene	Name	Position	Primer (5'-3')
Primer used for knockout			
yjaX gene	yjaXF	191-230	AAGCTTAACAAGCTGAAGTGGCTGCT
	yjaXR	513-532	GGATCCATCACTGACTGGCCCGACTA
yhfB gene	yhfBF	415-439	AAGCTTGCCGGAGAGACGTTATCAAA
	yhfBR	824-843	GGATCCCGTGTTTCCGTAGTGCTCAA
Primer for checking knokout			
	P	Pmutin4 vector	GACAGTATCGGCCTCAGGAA
	XL		TGCTGTTCCTCCTCCTTCTC
	XR		TTTGCCGGATATTCTTCAGC
	BL		GGAGTGATTCATATGTCAAAAGCA
	BR		CCCTCGTCATGATTGCACTA

Table 2. Effect of the gene disruption on the rate of cell transformation.

Experiment	Wild type	*yhfB-m*	*yjaX-m*
		Number of colonies /μg DNA	
1	6160	1740	0
2	4200	600	0
3	2560	1320	46

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Chapter 3. Branched-Chain Fatty Acid Biosynthesis Can Genetically Complement Unsaturated Fatty Acid Autotrophy for Maintaining Membrane Viability

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Abstract

It is generally believed that in response to changes in growth temperature, poikilothermic organisms maintain membrane viability by altering the fatty acid composition of the membrane lipids. These alterations in membrane lipid metabolism can manifest increased levels of unsaturated fatty acids when organisms are grown at lower temperatures. However, some microbes, for example *Bacillus subtilis*, may also maintain membrane viability by the use of branched chain fatty acids. This study tested the hypothesis that branched chain fatty acids can substitute for unsaturated fatty acids in maintaining membrane viability at lower growth temperatures. Specifically, the expression of a 3-ketoacyl-ACP synthase III gene of *B. subtilis* that conferred branched chain fatty acid biosynthetic capacity on an *Escherichia coli fabA* mutant, complemented the *fabA* associated unsaturated fatty acid autotrophy. The complemented *fabA* mutant strain accumulated membrane phospholipids that contained branched chain fatty acids. These results indicate that the *E. coli* metabolic enzymes have the capacity to generate and incorporate branched chain fatty acids into lipid membranes, and that these fatty acids can complement the role of unsaturated fatty acids in maintaining membrane viability, presumably by maintaining membrane fluidity.

Introduction

Cellular membranes have critical dynamic functions in maintaining biological integrity [1]. These functions include their role as selective physical barriers between the extracellular environment and the cellular milieu, and between intracellular compartments. In addition, cellular membranes act as the physical matrix upon which complex biochemical processes are organized (e.g., photosynthesis [2]). These membrane-associated functions have to be maintained in response to a wide variety of physical environmental stimuli. The integrity of the membrane and its ability to support complex biochemical processes is to some extent dependent upon the maintenance of membrane fluidity in the face of changing environmental conditions, especially in response to changing temperature [3, 4]. To a first approximation, organisms can maintain membrane fluidity at different temperatures by altering the fatty acid composition of the membrane lipids [5, 6]. Thus, the increased abundance of fatty acids that have lower melting points, translates to maintenance of membrane fluidity at lower temperatures. An assortment of different structures on the alkyl chain of fatty acids can affect the melting characteristics of these molecules. Alkyl-chain modifications that decrease the ability of these molecules to closely pack in the solid-state decreases their melting points. Specifically, the lengths of the alkyl chain, the occurrence of carbon-carbon double bonds that are in the *cis*-configuration or bulky substituents on the alkyl chain, can affect the melting characteristic of these molecules. Presumably, homoiothermic organisms that can control their body temperature, have to a large extent overcome the necessity for maintaining membrane fluidity in response to environmental temperature. However, in poikilothermic organisms, such as plants, microbes and cold-blooded animals, which cannot control body

temperature, mechanisms exist for maintaining membrane fluidity in the face of changing environmental temperatures [6, 7].

Indeed, cold adaptation experiments have revealed that different poikilothermic organisms take advantage of one or more mechanisms for modifying the alkyl-chain of membrane fatty acids to alter membrane fluidity and adapt to lower temperatures [7-9]. For example, yeast, fungi and many gram-negative bacteria respond to alterations in growth temperature by changing abundance of unsaturated fatty acids [8, 9]. Thus, increased abundance of polyunsaturated linoleic and linolenic acids, or of monounsaturated palmitoleic and oleic acids, have been observed in yeasts [10] and fungi [11, 12] grown at lower temperature. Many gram-positive bacteria adapt to temperature fluctuations by altering the abundance of branched chain fatty acids (either iso- or anteiso-methyl branching) and by altering the degree of fatty acid unsaturation [9].

In this study, we investigated whether these different mechanisms for altering membrane fluidity are interchangeable. Specifically, we sought to test the hypothesis that branched chain fatty acids (BCFAs) can substitute for unsaturated fatty acids in maintaining membrane fluidity. These characterizations indicate that expression of BCFA biosynthetic capability can substitute for the lack of unsaturated fatty acids in maintaining membrane viability.

Results and discussion

Complementation of unsaturated fatty acid autotrophy with exogenous branched chain fatty acids. Strains of *E. coli* that carry mutations at the *fabA* locus cannot produce

unsaturated fatty acids and thus cannot grow [13, 14]. The *fabA* gene codes for a bifunctional protein that catalyzes key reactions in the biosynthesis of unsaturated fatty acids [15], the dehydration of (*R*)-3-hydroxydecanoyl-ACP to (*E*)-2-decenoyl-ACP, and the interconversion of (*E*)-2-decenoyl-ACP and (*Z*)-3-decenoyl-ACP. The subsequent elongation of (*Z*)-3-decenoyl-ACP by further rounds of fatty acid biosynthesis generates the unsaturated fatty acids required by *E. coli* for the maintenance of membrane fluidity [16]. A number of *fabA* mutant alleles have been identified [13, 17-19], and some of these are temperature sensitive [13, 17, 18], as is the allele carried by the MH13 strain that we used in our studies [19].

Mutant *fabA* strains can be rescued by the inclusion of unsaturated fatty acids in the growth media [13, 14]. Indeed, MH13 grows at the non-permissive temperature (39 ^{0}C) only in the presence of an unsaturated fatty acid, such as oleic acid (Fig. 1A-C). As with an earlier study [20], we were also able to recover growth of MH13 at the non-permissive temperature, when BCFAs were included in the media, suggesting that BCFAs can replace the essential nature of unsaturated fatty acids in maintaining membrane fluidity. However, we noted that in these complementation experiments, the bacterial growth that was obtained occurred as isolated colonies (Fig. 1 D and E). Moreover, progeny from these recovered colonies could grow at the non-permissive temperature independent of any fatty acid complementation in the growth medium (Fig. 1 F and G). Fatty acid analysis of six such MH13-derived progeny strains, which can grow at the non-permissive temperature in the absence of exogenous unsaturated fatty acids, established that they are able to accumulate unsaturated fatty acids when grown at the non-permissive temperature. As examples, Table 1 shows the fatty acid composition of

two such MH13-derived strains, 12-1 and 13-1, grown at the permissive and non-permissive temperature. These data indicate that the MH13-derived strains that were recovered after growth at the non-permissive temperature may be carrying either wild-type *fabA* revertant or suppressor alleles.

To distinguish between these two possibilities, the *fabA* gene was PCR amplified and sequenced from *E. coli* strain MH13 (which carries the *fabA(ts)* allele), strain M8 (which carries another distinct allele, the *fabA18(ts*) [17, 21] and the two of the MH13-derived, suppressor strains 12-1 and 13-1. These analyses reveal that the *fabA* gene sequence in strains MH13 and MH13-derivatives, 12-1 and 13-1 are identical to each other, but different from that in strain M8, and all of these sequences are different from the wild-type *fabA* sequence of strain K12 [22]. Specifically, in strain MH13 and in the MH13-derived suppressor strains, a C to G mutation at position 214 of the gene sequence results in a G101D change relative to the wild-type sequence, and in strain M8 a C to G mutation at position 214 results in a G95D change (Fig. 2A). Both of these mutations map to the surface of the FabA dimmer protein (Fig. 2B), occurring either near the end of the central helix or in the loop that connects this central helix to the subsequent anti-parallel β-sheet [23]. These results indicate that sequence alterations near this position in the structure of the FabA protein make this enzyme more susceptible to thermal inactivation. In addition, these results establish that strains, 12-1 and 13-1, which are derived from strain MH13, are carrying suppressor alleles that overcome the thermal instability of the FabA protein. Therefore, these MH13-derived *fabA* suppressor strains are probably similar to the class of fabA “revertants” that were

previously characterized as expressing thermally unstable 3-hydroxydecanoyl-ACP dehydrase activity [24].

These results therefore, indicate that our experimental approach of complementing the *fabA*-associated unsaturated fatty acid autotrophy with exogenous BCFAs cannot address the hypothesis that BCFAs can substitute for the essential nature of unsaturated fatty acids in maintaining membrane fluidity. We therefore conducted experiments to genetically test this hypothesis.

Expression of the *B. subtilis yhfB* and *yjaX* genes in *fabA* strain of *E. coli*. In contrast to *E. coli*, which appears to regulate membrane fluidity by manipulating the proportion of unsaturated fatty acids in membrane lipids, many gram positive bacteria, such as *B. subtilis,* appears to accomplish the same task by manipulating the proportion of BCFAs in membrane lipids [25-27]. Hence, we genetically manipulated *E. coli* with *Bacillus* genes to instill the ability to produce BCFAs, a trait that does not naturally occur in the former bacterium.

The BCFA biosynthetic trait appears to be associated with the enzyme 3-ketoacyl-ACP synthase III (KASIII) [16]. KASIII is one of the three KAS isozymes that are required for *de novo* fatty acid biosynthesis, the other two being KASI and KASII [28-30]. These KAS isozymes catalyze the condensation of an acyl-thioester substrate with malonyl-ACP, forming a 3-ketoacyl-ACP product that is 2-carbons longer than initial acyl-substrate. These KAS isozymes differ in their specificity of the acyl-thioester substrate, having optimum activities for substrates of different acyl-chain lengths and different thioesters. Thus, KASI

and KASII both utilize an acyl-ACP substrate, but they differ in the preferred carbon chain length of that acyl-substrate [16]. In contrast, KASIII is specific for a short-chain acyl-CoA substrate [30].

In *normal* (straight) chain fatty acid biosynthesis, KASIII initiates this process, using acetyl-CoA as the acyl substrate [25]. In *E. coli*, KAS III is encoded by the *fabH* gene, but two homologous genes, *yhfB* and *yjaX* encode this enzyme in *B. subtilis* [16]. In contrast to the *fabH*-encoded KASIII, which uses only acetyl-CoA as its acyl substrate, the *yhfB*- and *yjaX*-encoded KASIII enzymes appear to also use short-chain branched acyl-CoA substrates resulting in the biosynthesis of iso- and anteiso-BCFAs [31]. Therefore, to instill BCFA biosynthesis in *E. coli* we expressed these two *B. subtilis* KASIII-coding genes in *E. coli*.

To readily detect the expression of the *yhfB* and *yjaX* genes in *E. coli*, we first produced antibodies directed against each gene product. The *yhfB* and *yjaX* genes were PCR-amplified and cloned into the pET30a expression vector. As shown in Figure 3A, the recombinant, His- and S-tagged, YhfB and YjaX proteins were expressed to relatively high levels as novel 36-kDa and 41-kDa polypeptides. These tagged version of YhfB and YjaX proteins were purified by affinity chromatography and used to generate antibodies in rabbits. Fig. 3B and 3C show the reactions of the resulting antibodies against the recombinant YhfB and YjaX proteins. Antibodies against the YjaX protein mono-specifically react with the 41-kDa recombinant YjaX protein, which is the predicted molecular weight of this protein. However, the YhfB antibodies react with both the YhfB and YjaX proteins, indicating that these antibodies react against common epitopes that are shared between the two proteins

(YhfB and YjaX share 46% sequence identity) [31]. Moreover, whereas the YhfB protein is predicted to be about 41-kDa, the actual recombinant protein produced in this system is smaller than expected, at about 36-kDa; this is either because the recombinant protein is proteolytically unstable in *E. coli*, or its due to an unexpected premature termination in transcription or translation of the recombinant gene.

The expression of *yhfB* or *yjaX* in the *E. coli* strain BL21 (DE3) failed to induce the accumulation of BCFAs to detectable quantities (data was not shown). In the case of the *yhfB* gene, this is probably due to the inability to express the full-length YhfB protein (Fig. 3C). However, the failure to accumulate BCFAs in the *yjaX*-expressing BL21 (DE3) strain is more difficult to explain, but this result is consistent with a previous report that no detectable levels of BCFA were produced in *E. coli* strain BL21 expressing the *S. glaucescens* KASIII homologous [32], and may be due to the unavailability of the appropriate short branched chain acyl-CoA substrates. Another potential explanation may be the fact that the *E. coli* fatty acid and lipid biosynthetic machinery may not be able to facilitate the stable accumulation of BCFA-containing lipids. But this contrasts with a previous study that successfully achieved BCFA accumulation in this *E. coli* strain [31].

Can BCFAs substitute for unsaturated fatty acids? To test whether BCFAs can substitute for unsaturated fatty acids, the *yhfB* or *yjaX* genes were expressed in an *E. coli* strain carrying a temperature-sensitive *fabA*-allele (lysogenized strain MH13 (DE3); see Materials and Methods). In these experiments, heterologous expression was achieved using the pET11d and pET17b expression vectors, which were designed to express the full-length mature YhfB

or YjaX proteins without any additional tags. Figure 4 shows Western blot analyses with either YhfB or YjaX antibodies of *E. coli* strain MH13 (DE3) transformed with non-recombinant, control pET17b vector, recombinant pET17b vector expressing the *yjaX* gene, or recombinant pET11d vector expressing the *yhfB* gene. Consistent with the expected molecular weights of the YhfB (35.9-kDa) and YjaX (34.4-kDa) proteins, these blots reveal the occurrence of the appropriately sized immunologically-reactive proteins in all the strains carrying the recombinant vectors (Fig. 4A and 4B). However, a weakly reactive protein is also detected in the control *E. coli* strain MH13 (DE3), which has a molecular weight indistinguishable from that of the YhfB and YjaX proteins. Based on the sequence similarity between the *E. coli* KAS III protein, and YjaX (45% identical) and YhfB (38% identical) proteins, we believe this immunologically identified protein is the *E. coli* KAS III. Because in each expression experiment, the intensity of the immunologically-detected band was considerably greater in the recombinant *E. coli* strains than in the control strain, we conclude that *yhfB* (Fig. 4A) and *yjaX* (Fig. 4B) gene were successfully expressed in strain MH13 (DE3).

To ascertain whether these genetic manipulations resulted in the accumulation of BCFAs, lipids were extracted from the control and recombinant *E. coli* MH13 (DE3)-derived strains that are expressing the *yhfB* or *yjaX* genes, which were grown in LB medium at the permissive temperature (30ºC). GC-MS analysis of fatty acid methyl esters prepared from these lipid extracts showed that the *E. coli* strain expressing the *yhfB* gene produced *iso*-16:0 and *iso*-14:0, while the control strain did not accumulate detectable levels of any BCFAs (Table 2). The only BCFA that accumulated in the *yjaX*-expressing strain was *iso*-16:0. The

identity of *iso*-16:0 and *iso*-14:0 was authenticated by co-elution with commercially available standards and by their mass-spectra.

To optimize accumulation of BCFAs, we examined the accumulation pattern of these molecules during the growth of the recombinant culture. These experiments revealed that in both the *yjaX*- (data not shown) and *yhfB*-expressing strains, maximum BCFA accumulation occurred 24-hours after induction of the recombinant genes, and that maximum level of accumulation occurred with the *yhfB*-expressing strain, at 2.5% of the total fatty acids (Table 3). To ascertain whether the BCFAs were successfully incorporated into membrane phospholipids, lipid extracts were fractionated by TLC, and the fatty acids associated with the purified major phospholipids, phosphatidylglycerol and phosphatidylethanolamine, were analyzed. These analyses confirmed that at least in the *yhfB*-expressing strain, BCFAs were incorporated into these phospholipids (Table 4). Moreover, because in these experiments enriched lipid fractions were analyzed, we were able to detect BCFAs that were not detectable when fatty acids were analyzed from a total lipid extract (Table 4). Thus, these analyses demonstrated that in addition to the accumulation of iso-16:0 and iso-14:0, the *yhfB*-expressing strain also accumulates anteiso-16:0 (Table 4). The finding of anteiso-16:0 was somewhat surprising, because these fatty acids are thought to be generated by the priming of *de novo* fatty acid biosynthesis with anteiso-acyl-CoA derived from isoleucine metabolism. However, the expected product from such a process is an anteiso-fatty acid of odd-numbered carbon atoms. Thus, the finding of an even-numbered anteiso-BCFA may suggest alternative metabolic processes, such as α-oxidation [33, 34] (which would remove a single carbon atom from the acyl chain and thus generate an even numbered anteiso-BCFA) or the involvement

of an α-ketoacid elongation pathway [35] (which elongates fatty acids by a single carbon atom).

Having successfully expressed the *Bacillus yhfB* and *yjaX* genes in an *E. coli* strain that cannot synthesize unsaturated fatty acids, and demonstrated that these transgenic manipulations confer BCFA biosynthesis in a host that normally does not synthesize these molecules, we were able to directly test the hypothesis of whether BCFAs can substitute for unsaturated fatty acids. Specifically, the MH13 (DE3) control strain and derivative strains that are expressing the *yhfB* or *yjaX* genes were grown on solid LB medium without any fatty acid supplements at either the permissive (30 ºC) or non-permissive (39 ºC) temperatures (Fig. 5). As expected, all strains grew at the permissive temperature (data not shown), however, at the non-permissive temperature, only the *yhfB*-expressing strains showed any growth (Fig. 5B), whereas because of the temperature sensitive nature of the *fabA* mutant allele, the control strain did not grow in the absence of unsaturated fatty acids (Fig. 5A).

Similar growth characteristics were observed when these strains were grown in liquid LB media without any fatty acid supplementation. Namely, at the non-permissive temperature, the recipient strain, MH13 (DE3), did not show any signs of growth, unless it was transformed with the *yhfB*-expressing plasmid, in contrast, the *yjaX*-expressing strain did not grow (Fig. 5D). Interestingly, although the initial growth rate of the *yhfB*-harboring MH13 (DE3) strain was similar to that the MH13 (DE3) strain grown in the presence of unsaturated fatty acids, the former culture reached stationary phase at lower saturation that the latter.

This is probably a consequence of the fact that exogenous unsaturated fatty acids can more completely supplemented the *fabA*-deficiency than the endogenously produced BCFAs.

These results establish that the BCFAs produced by the expression of the *yhfB* gene can complement the lack of unsaturated fatty acids. The inability to complement this unsaturated fatty acid deficiency with the BCFAs produced by the *yjaX* gene maybe either because the composition of the BCFAs produced by the *yjaX*-coding KASIII does not meet the unsaturated fatty acid deficiency, or because the *yjaX* transgene does not produce sufficient quantities of BCFAs to complement the unsaturated fatty acid autotrophy. Because fatty acid analysis of these transgenic strains, grown at 30 ^{0}C, demonstrate that the *yhfB*-expressing strain produces more than twice the amount of BCFAs as does the *yjaX*-expressing strain, with little difference in BCFA composition, the latter explanation is most likely correct.

One of the surprise findings of this study was the fact that a relatively small amount of BCFA was sufficient to complement the deficiency in unsaturated fatty acids; as little as 2.6 mol % of BCFAs could support the growth of strain MH13 (DE3) (the amount detected in the *yhfB* expressing strain), but 0.8 mol % was not sufficient to support growth (the amount detected in the *yjaX* expressing strain). These findings contrast with earlier studies, which established that the minimum amount of unsaturated fatty acids that is required for viability of *E. coli* membrane fluidity is approximately 15% [24]. The difference between the minimal amounts of unsaturated fatty acids and BCFAs that are required for viability may be associated with different physical properties of these fatty acids, in comparison to straight chain fatty acids. One comparison that may have relevance to this discussion is the melting point of the

different fatty acids. Although there are limited data on the melting points of BCFAs, it appears that branching at the ω-3 position, as occurs in anteiso-BCFAs, is more effective in lowering the melting point of a fatty acid than branching at the ω-2 position (for example, the melting point of *n*-15:0, iso-15:0 and anteiso-15:0 is 52 ^{0}C, 51.7 ^{0}C, and 23 ^{0}C, respectively) [36]. Yet, branching of the alkyl chain is not as effective in lowering the melting point of the fatty acid, as is the insertion of a double bond (e.g., insertion of a *cis*-double bond in a fatty acid of 16- and 18- carbons results in the lowering of the melting point by about 60 ^{0}C). These comparisons may be suggestive of a specific structural role for BCFA in maintaining membrane viability in the absence of unsaturated fatty acids that is beyond the melting point characteristics of these acids.

Materials and Methods

Reagents. All fatty acids, methanol, chloroform, and other chemical reagents were purchased from Sigma Chemical Company (St. Louis. MO). Isopropyl β–D-thiogalactoside (IPTG) was purchased from USB Corporation (Cleveland, Ohio).

Bacterial strains. *E. coli* strain MH13 (a gift from Dr. John Shanklin, Brookhaven National Labs, Upjohn, NY) is a derivative of strain DC308 [19]. The full description of the strain is *fabA(ts) pyrD zcb::TN10 fadR::Tn5*. The wild-type *B. subtilis* strain 168 was used in these studies (Bacillus Genetic Stock Center, Columbus, OH).

DNA amplification. Genes were PCR amplified from *B. subtilis* or *E. coli* genomic DNA isolated with Purelink Genomic DNA Purification Kit (Invitrogen, Carlsbad, CA). The

sequence of the primers used for amplification of the *yhfB* gene are 5'-CGCGGATCCATTCATATGTCAAAAGC-3' and 5'-AGGGAAGCTTCAGAAGAACAGCCGG-3'. The sequence of the primers used to amplify the *yjaX* gene are 5'-CATGCCATGGTAATGAAAGCTGGAATAC-3' and 5'-GCGGGATCCGGAGATAATGCTCCAAG-3'. The primers used for amplification of the *E. coli fabA* gene are 5'-CGCTGAATCAAAGGTCACAA-3' and 5'-AACCATCCAGGAACGTATCG-3'. The authenticity of all PCR products was confirmed by sequencing them directly. Products were subsequently cloned into expression vectors and the resulting plasmids were also confirmed by sequencing.

Expression of recombinant proteins and acquisition of antibodies. In order to generate antigen for antibody production, *E. coli* strain BL21 (DE3) carrying T7 RNA polymerase-based pET expression plasmids (Novagen, San Diego, CA) for the *yhfB* or *yjaX* genes was grown in Luria Broth (LB) medium containing the appropriate antibiotic. When the culture reached an OD600 of 0.9, it was adjusted to 1 mM IPTG to induce expression. Cells were harvested and the proteins were purified by affinity chromatography on a Ni-NTA agarose column (Qiagen, Valencia, CA). Antibodies directed against the yhfB and yjaX proteins were generated by injecting each recombinant protein into a rabbit using Freund's Complete and Incomplete Adjuvants (Pierce, Rockford, IL).

In order to test the biochemical function of the *yhfB* and *yjaX* genes, they were expressed in the *E. coli* strain MH13, using pET11d and pET17b expression vectors, respectively. To achieve this, the MH13 strain was first lysogenized with the bacteriophage λ (DE3) that

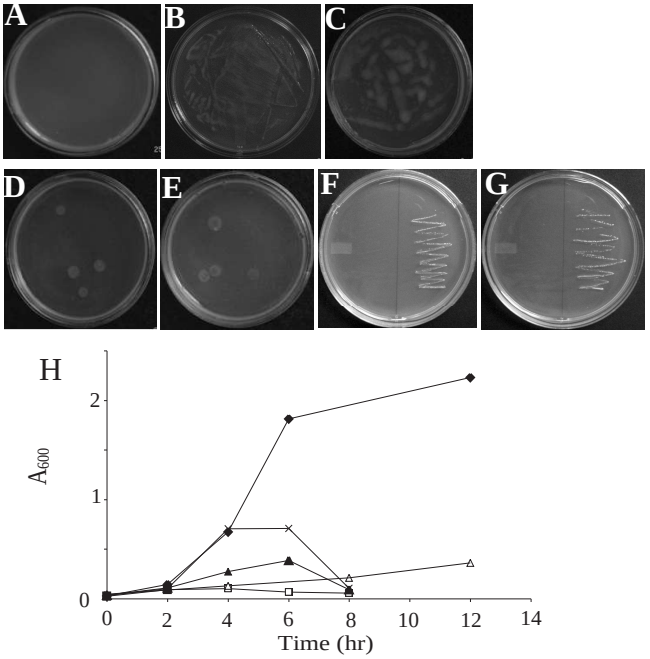
expresses the T7 RNA polymerase gene (Novagen, San Diego, CA). Thus, strain MH13 (DE3) carrying either the pET11d (*yhfB*) or pET17b (*yjaX*) plasmid was grown at either 30°C or 39°C in LB medium containing the appropriate antibiotic. The expression of the recombinant genes was induced by adding IPTG to the growth medium to final concentration of 1.7 mM.

Analysis of proteins. Expressed proteins were analyzed by SDS-PAGE and western blot analyses. Bacterial cell pellets were suspended in extraction buffer consisting of 2% (w/v) sodium dodecyl sulfate, 20 mM Tris-HCl (pH 7.0), 50 μg/ml phenyl methyl sulfonyl fluoride, and the suspension was boiled for 10 minutes. Following centrifugations for 1 min at 16,100 g, the supernatant was retained for analysis. Prior to electrophoresis, the extract was adjusted to 10% glycerol and 0.001 % Bromophenol Blue and fractionated by electrophoresis. After electrophoresis, proteins were either visualized by staining with Coommassie Brilliant Blue, or they were transferred electrophoretically to nitrocellulose blotting membrane. Immunological detection was achieved by a 2-step process, in which the membrane was first reacted with the rabbit generated antisera, followed by a goat-anti rabbit IgG conjugated to horse radish peroxidase. The resulting antigen-antibody complexes were visualized with ECL Western Blotting Detection Reagents and Analysis System (Amersham Bioscience, Piscataway, NJ).

Analysis of fatty acids. Lipids were extracted using chloroform/methanol [37]. The extracted lipids were fractioned by thin-layer chromatography with the solvent mixture chloroform:methanol:acetic acid (65:25:8). Fatty acid were converted to methyl esters using

methanolic-HCl at 80^{o}C for 20 minutes [38], and the recovered fatty acid methyl esters were analyzed by GC-MS (6890 series Agilent, Palo Alto, CA), interfaced with a Mass Detector 5973 (Agilent, Palo Alto, CA).

Fig. 1. Chemical complementation of the *fabA*-associated unsaturated fatty acid autotrophy. Strains of *E. coli* were grown either on solid (A-G) or liquid (H) LB media to which various fatty acid supplements were added. Without exogenous fatty acids in the media, *E. coli* strain MH13 that carries a temperature-sensitive *fabA* allele cannot grow at the non-permissive temperature (39 ^{0}C) (A), but can grow at the permissive temperature (30 ^{0}C) (B). However, at the non-permissive temperature, growth of this strain is supported by the inclusion in the media of an unsaturated fatty acid, such as oleic acid (C). The inclusion of BCFAs, such as anteiso-16:0 (D), and anteiso-14:0 (E) support the growth of this *E. coli* strain, however, the resultant growth is recovered as isolated colonies. These recovered colonies (e.g., strains 12-1 and 13-1) appear to be suppressors of the *fabA(ts)* allele as they are able to grow at the non-permissive temperature in the absence of any exogenous fatty acids. Strains 12-1 (F) and 13-1 (G) (on right hand side of the plates) and strain MH13 (on left hand side of the plates in panels F and G) where grown on media without any exogenous fatty acids at the non-permissive temperature (39 ^{0}C). Strain MH13 was grown in liquid media (H) without exogenous fatty acids at the permissive (Δ) and non-permissive (□) temperature, or at the non-permissive temperature but with the inclusion of oleic acid (♦), anteiso-16:0 (×), or anteiso-15:0 (▲), and growth was monitored by the optical density of the culture at 600 nm.



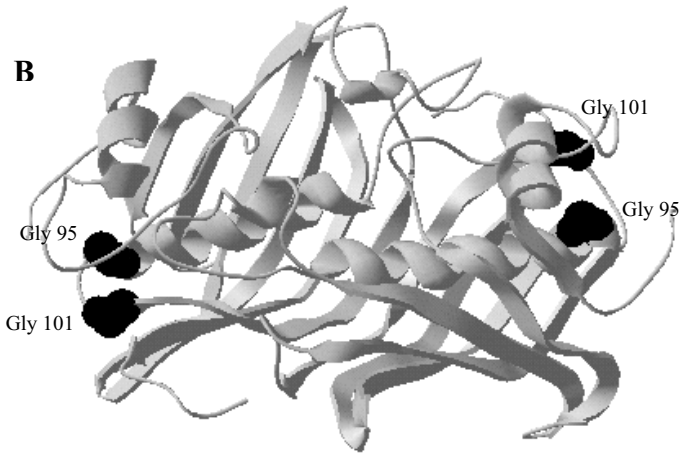
Jin and Nikolau, Figure 1

Fig. 2. Structural characterization of the temperature-sensitive *fabA* alleles. A. Comparison of the amino acid sequence of the wild-type FabA protein from *E. coli* strain K-12 to those deduced from the sequences of the *fabA(ts)* and *fabA18(ts)* alleles PCR amplified from strains MH13 and M8. Mutant amino acid residues at positions 95 and 102 are labeled with an asterisk. B. The mutated residues in the FabA protein encoded by the *fabA(ts)* and *fabA18(ts)* alleles are mapped on to the ribbon-diagram of the crystal structure of the FabA protein (MMDB 4545).

A

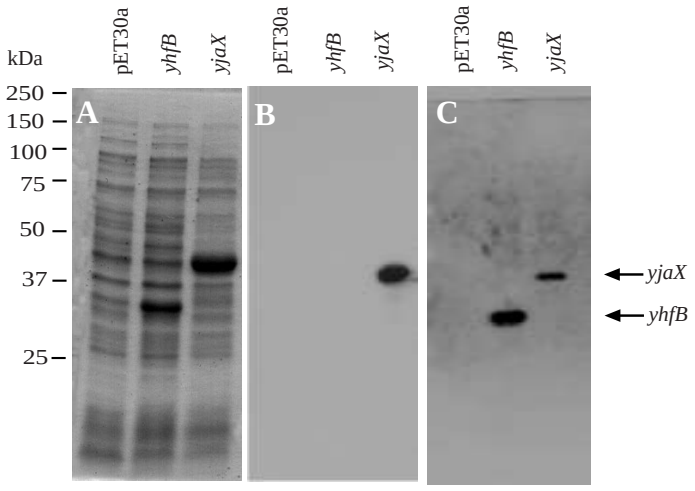
```
      1                                          40
fabA  MVDKRESYTK EDLLASGRGE LFGAKGPQLP APNMLMMDRV
MH13  MVDKRESYTK EDLLASGRGE LFGAKGPQLP APNMLMMDRV
M8    MVDKRESYTK EDLLASGRGE LFGAKGPQLP APNMLMMDRV

      41                                         80
fabA  VKMTETGGNF DKGYVEAELD INPDLWFFGC HFIGDPVMPG
MH13  VKMTETGGNF DKGYVEAELD INPDLWFFGC HFIGDPVMPG
  M8  VKMTETGGNF DKGYVEAELD INPDLWFFGC HFIGDPVMPG

      81              *      *                 120
fabA  CLGLDAMWQL VGFYLGWLGG EGKGRALGVG EVKFTGQVLP
MH13  CLGLDAMWQL VGFYLGWLGG EDKGRALGVG EVKFTGQVLP
M8    CLGLDAMWQL VGFYLDWLGG EGKGRALGVG EVKFTGQVLP

      121                                        160
fabA  TAKKVTYRIH FKRIVNRRLI MGLADGEVLV DGRLIYTASD
MH13  TAKKVTYRIH FKRIVNRRLI MGLADGEVLV DGRLIYTASD
  M8  TAKKVTYRIH FKRIVNRRLI MGLADGEVLV DGRLIYTASD

      161       172
fabA  LKVGLFQDTS AF
MH13  LKVGLFQDTS AF
M8    LKVGLFQDTS AF
```

B



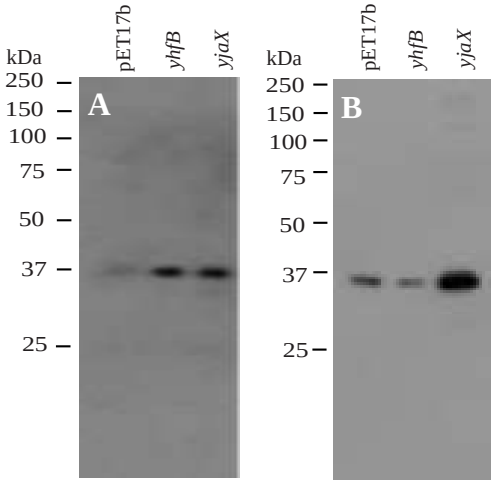
Jin and Nikolau, Figure 2

Fig. 3. Expression of the *B. subtilis yhfB* and *yjaX* genes in *E. coli* and production of antisera. SDS-PAGE (A) and western blot analysis (B and C) of *E. coli* strain BL21(DE3) transformed with non-recombinant pET30a vector, and recombinant pET30a vector harboring the *B. subtilis yhfB* or *yjaX* genes. Western blots were analyzed with antisera directed against the recombinantly produced YhfB (B) and YjaX proteins (C).



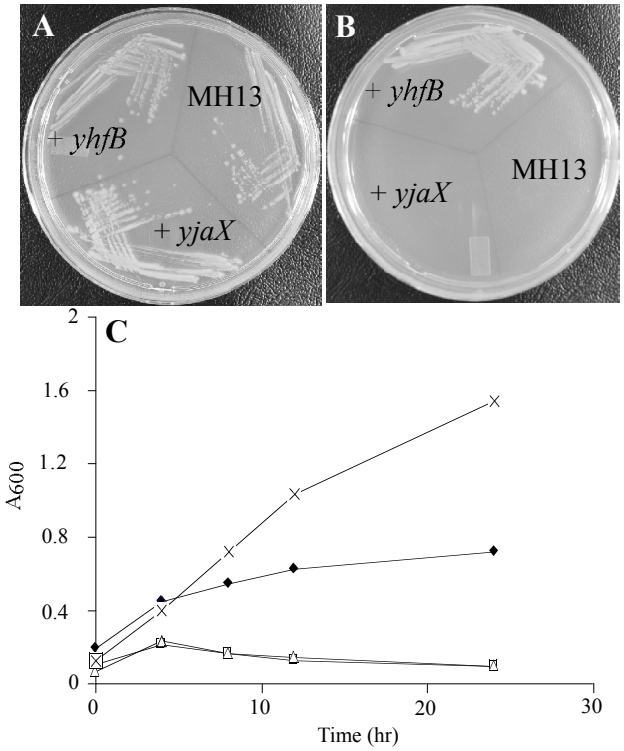
Jin and Nikolau, Figure 3

Fig. 4. Expression of the *B. subtilis yhfB* and *yjaX* genes in *E. coli* strain MH13 (DE3). Western blot analysis of *E. coli* strain MH13 (DE3) transformed with non-recombinant pET17b vector or recombinant pET17b vector harboring the *B. subtilis yhfB* or recombinant pET11d vector harboring the *B. subtilis yjaX* genes. Western blots were analyzed with antisera directed against the recombinant YhfB (A) and YjaX (B) proteins.



Jin and Nikolau, Figure 4

Fig. 5. Expression of the *B. subtilis yhfB*, but not *yjaX*, complements the unsaturated fatty acid autotrophy associated with the *fabA(ts)* allele. Strains were grown either on solid (A, B) or liquid (C) LB media. *E. coli* strain MH13 (DE3) was transformed with either the non-recombinant pET17b, recombinant pET17b vector harboring the *B. subtilis yhfB* gene, or recombinant pET11d vector harboring the *B. subtilis yjaX* gene were cultured at 30 ºC (A) or 39 ºC (B). C. Growth of *E. coli* MH13 (DE3) transformants in liquid LB media. Strain MH13 (DE3) was transformed with the non-recombinant pET17b vector (Δ, ×), the recombinant pET17b vector harboring the *B. subtilis yhfB* genes (◆) or recombinant pET11d vector harboring the *yjaX* gene (□). These strains were cultured at either 39ºC (Δ, ◆, □), or 30 ºC (×). The optical density of the culture at 600 nm was used to monitor growth.



Jin and Nikolau, Figure 5

Table 1. Fatty acid composition of *E. coli* strain MH13 and derivative *fabA* suppressor strains that grow in the absence of exogenous fatty acids.

	30 ºC			39 ºC	
	MH13	12-1[a]	13-1 [a]	12-1	13-1
	mol %			mol %	
n-12:0	4.5 ± 0.4	4.5 ± 0.1	5.7 ± 0.7	6.2 ± 0.2	8.4 ± 2.4
n-14:0	10.6 ± 1.3	7.5 ± 0.2	8.3 ± 1.4	21.8 ± 2.2	26.3 ± 0.8
n-15:0	0.30 ± 0.02	0.23 ± 0.13	n.d. *	1.1 ± 0.3	0.9 ± 0.1
n-16:1	8.1 ± 1.2	18.5 ± 1.4	17.9 ± 2.1	7.9 ± 1.9	5.4 ± 0.8
n-16:0	53.2 ± 3.9	47.8 ± 1.6	49.2 ± 0.4	53.5 ± 0.9	49.5 ± 5.1
cyc-17:0	16.8 ± 1.7	9.2 ± 1.4	9.2 ± 2.4	7.6 ± 0.8	8.1 ± 2.6
n-18:1	3.0 ±1.2	11.6 ± 2.5	9.6 ± 0.4	2.0 ± 0.5	1.40 ± 0.04

[a] Strains 12-1 and 13-1 are derivatives of MH13, which unlike the parental strain can grow at the non-permissive temperature in the absence of exogenous fatty acids. Strains were grown at either 30 ºC or 39 ºC and harvested at late log-phase of growth, A_{600} = 1.3-1.5. Average of 3 determinations ± standard error.

* not detected

Table 2. Fatty acid composition of *E. coli* strain MH13 (DE3) and MH13(DE3) expressing either the *B. subtilus yhfB* or *yjaX* genes

	MH13	*yjaX*	*yhfB*
		mol %	
n-12:0	4.3 ± 0.2*	4.2 ± 0.4*	4.4 ± 1.2*
n-14:0	11.0 ± 1.0	12.3 ± 2.5	14.1± 1.8
iso-14:0	n.d. *	n.d. *	0.11 ± 0.04
n-14:1	0.55 ± 0.11	0.41 ± 0.10	0.40 ± 0.05
n-15:0	0.27 ± 0.04	0.56 ± 0.17	0.81 ± 0.20
n-16:0	57.9 ± 1.2	51.8 ± 2.3	47.0 ± 2.4
iso-16:0	n.d. *	0.80 ± 0.70	2.6 ± 0.6
n-16:1	9.5 ± 0.7	7.8 ± 0.4	1.3 ± 0.4
cyc-17:1	14.4 ± 0.9	17.2 ± 3.5	27.2 ± 1.8
n-18:1	2.1 ± 0.4	4.3 ± 2.6	1.9 ± 0.6

E. coli strain MH13(DE3) transformed with non-recombinant pET17b or recombinant pET17b expressing the *yhfB* gene or recombinant pET11d vector expressing the *yjaX* gene were cultured in LB medium at 30°C. Twenty-four hours after IPTG induction, cells were collected, lipids were extracted, fatty acids were transmethylated and analyzed by GC-MS. Average of 3 determinations ± standard error.

* not detected.

Table 3. Fatty acid composition of *E. coli* strain MH13 (DE3) expressing the *B. subtilus yhfB* gene.

	6 hr	24 hr	51 hr
		mol %	
n-12:0	4.7 ± 0.3	4.5 ± 1.2	3.2 ± 0.7
n-14:0	17.6 ± 1.5	14.1 ± 1.8	14.4 ± 1.0
iso-14:0	n.d. ∗	0.11 ± 0.04	0.12 ± 0.05
n-14:1	0.93 ± 0.13	0.40 ± 0.05	0.52 ± 0.17
n-15:0	1.7 ± 0.2	0.81 ± 0.20	0.66 ± 0.18
n-16:0	42.1 ± 1.9	47.0 ± 2.4	50.0 ± 0.9
iso-16:0	1.6 ± 0.4	2.6 ± 0.6	1.6 ± 0.7
n-16:1	2.0 ± 0.4	1.3 ± 0.4	8.3 ± 4.0
cyc-17:0	25.6 ± 0.2	27.2 ± 1.8	19.4 ± 3.1
n-18:1	2.8 ± 0.1	1.9 ± 0.6	1.8 ± 0.4

E. coli strain MH13 (DE3) expressing the *yhfB* gene was cultured at 30°C and its fatty acid composition was determined at the indicated time after IPTG induction.

∗ not detected

Table 4. Fatty acid composition of isolated phosphatidylethanolamine and phosphatidylglycerol from *yhfB*-expressing *E. coli* strain MH13 (DE3).

	Phosphatidylethanolamine	Phosphatidylglycerol
	mol %	
n-12:0	1.5 ± 0.50	1.6 ± 0.2
n-14:0	22.8 0 ± 0.04	18.8 ± 0.2
n-15:0	1.92 ± 0.38	1.28 ± 0.09
n-16:0	58.6 ± 0.2	60.2 ± 2.3
iso-16:0	0.67 ± 0.14	0.48 ± 0.03
anteiso-16:0	0.17 ± 0.79	0.29 ± 0.11
cyc-17:0	11.6 ± 1.7	16.4 ± 1.3
n-17:0	0.17 ± 0.01	n.d. *
n-18:0	2.5 ± 0.9	0.91 ± 0.10

E. coli strain MH13 (DE3) expressing the *B. subtilis yhfB* gene was cultured at 37 ºC in LB medium, and cells were harvested 24 hours after induction with IPTG. Lipids were extracted, fractioned by TLC, and the fatty acids associated with the isolated phospholipids were recovered as methyl esters and analyzed by GC-MS.

*not detected.

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Chapter 4. Are *Bacillus Subtilis* and *Echerichia coli* 3-Ketoacyl-ACP Synthase III Enzymes Interchangable?

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Abstract

Genetic characterization of the *fabH* locus of *E. coli* indicates that this is not an essential gene for growth. Deletion of this gene, however, affects growth and fatty acid composition of the membrane lipids. The biochemical function of *fabH* is unique in fatty acid biosynthesis, encoding the 3-ketoacyl-ACP synthase III isozyme that catalyzes the condensation reaction between acetyl-CoA and malonyl-ACP, which is the initial condensation reaction of this pathway. Therefore, the viable nature of *fabH* mutant allele indicates that there must be an alternative means of initiating fatty acid biosynthesis. However, there is a growth penalty associated with this alternative mechanism. The growth deficiency of the *fabH* mutant can be partially complemented by the ectopic over-expression of one of the two *Bacillus* KASIII-coding genes (the *yhfB* gene) and complemented by the *E. coli fabH* gene. However, over-expression of the second *Bacillus* KASIII gene (*yjaX)* fails to complement this growth phenotype. Because strains expressing either different KASIII versions, or different doses of these genes show different fatty acid compositions, we surmise that there is a dose-response between fatty acid composition and the amount of KASIII

enzyme activity expressed. Finally, we assess why expression of *Bacillus* KASIII enzymes in *E. coli* fail to induce accumulation of detectable levels of branched chain fatty acids.

Introduction

In bacteria such as *E. coli*, fatty acids are biosynthesized by a dissociable type II fatty acid synthase enzyme system [1]. In this system, there are three genetically and biochemically distinct ketoacyl synthase (KAS) isozymes, namely KASI (encoded by *fabA*), KASII (encoded by *fabF*), and KASIII (encoded by *fabH*) [1-3]. Their functions have been studied extensively in *E. coli*. All theses enzymes catalyze the condensation reaction between an acyl-thioester substrate and malonyl-ACP, resulting in the generation of a 3-ketoacyl-ACP intermediate that is 2-carbon longer than the starting acyl-thioester substrate. But these isozymes differ in their specificities of the acyl-thioester substrate by having optimum activities for substrates of different acyl-chain lengths and different thioesters. While KAS III initiates the biosynthesis of fatty acids mainly by utilizing acetyl-CoA as a substrate for the condensing reaction with malonyl-ACP [4, 5]; KASI and KASII catalyze the condensation between acyl-ACPs having acyl chain longer than two carbons, and the malonyl-ACP substrate.

Although the general mechanism of fatty acid biosynthesis in gram positive bacteria, such as *Bacillus subtilis*, is similar to that of *E. coli*, fatty acids in *Bacillus* are in large quantity branched, with methyl groups occurring at the iso- and anteiso positions (i.e., 13-methyltetradecanoic, 12-methyltetradecanoic acid, and 14-methylpentadecanoic acid). These fatty acids are biosynthesized by distinct type II fatty acid synthases. Namely, it is thought

that theses fatty acids are biosynthesized by a fatty acid synthase system that can use branched chain acyl-CoAs as initiating substrates. These branched chain acyl-CoAs would be derived from the metabolism of the branched chain amino acids, leucine, isoleucine and valine [6]. This suggests that *Bacillus* express KAS III isoenzyme (s) that can use branched chain acyl –CoA as substrates for initiating fatty acids biosynthesis.

Genomics-based analysis of the *B. subtilus* genome has identified two KAS III coding genes, *yjaX* and *yhfB* gene. Both of these enzymes have been shown to be capable of catalyzing the condensation of branched chain acyl-CoAs with malonyl-ACP [7, 8]. One of the purposes of this study is to test if *Bacillus* KAS III isozymes can replace the *E. coli fabH* encoded KAS III, and ascertain whether such strains generate different fatty acid compositions. A second purpose of this research is to test if the *E. coli fabH* gene is essential for growth.

Materials and Methods

Materials. Methanol and chloroform were purchased from Sigma Chemical Company (St. Louis. MO). Isopropyl β–D-thiogalactoside (IPTG) was purchased from USB Corporation (Cleveland, Ohio).

Growth Conditions and Mediums. Bacteria were grown in Luria Broth (LB) medium or M9 minimum medium at 37ºC. M9 medium consists of 12.8 g/l Na_2HPO_4, 3 g/l KH_2PO_4, 0.5 g/l of NaCl, 1g/l NH_4Cl, 2 mM $MgSO_4$, 0.1 mM of $CaCl_2$, and 2 g/l glucose.

Plasmids. *E. coli fabH* gene was PCR amplified from wild type *E. coli* strain MG1655 using the pair primers, forfabh and Revfabh (Table 2). The PCR product was cloned into pENTR/D-TOPO vector using pENTRY Directional TOPO Cloning Kits (Invitrogen, Carlsbad, CA). Then LR recombination reaction was performed between the pENTRY clone containing *fabH* gene and pDEST 17 vector. Resulting plasmid was named pDESTfabH. The authenticity of this plasmid was confirmed by DNA sequencing.

Genetic Manipulations. In order to express genes of interest with pET vectors (Novagen, San Diego, CA), and pDEST17 vector (Invitrogen, Carlsbad, CA), which carries T7 promoter, *E coli* strain BW25113, JW1077, MG1655, and kfabh were lysogenized with the bacteriophage (DE3), which contains the T7 RNA polymerase gene (Novagen, San Diego, CA). PI transduction was conducted as previously described [9].

Diagnostic PCR assays. Diagnosis PCR assays were conducted to show that mutants have correct structure. Using genomic DNA, extracted from the cells of interest as template, PCR products were generated with these locus-specific pair primers.

Fatty acid analysis. Lipids were extracted from bacterial cell pellets using chloroform/methanol [10, 11], and fatty acids were then converted to methyl esters using methanolic-HCl at 80^{o}C for 20 minutes [12-14]. The recovered fatty acid methyl esters were analyzed with a GC (6890 series Agilent, Palo Alto, CA), interfaced with a Mass Detector 5973 (Agilent, Palo Alto, CA).

Results

Verification of *fabH* deletion. An *E. coli* strain, JW1077, carrying a Kan^R gene fabH-delation allele was obtained from Keio Collection of gene knockout mutant strains (http://ecoli.aist-nara.ac.jp/gb5/Resources/archive/archive.html) [15]. The mechanism for deleting the *fabH* gene to create *E. coli* strain JW1077 is schematically described in Fig. 1A [16]. Diagnostic PCR assays were conducted to verify the deletion of the *fabH* gene. The sequence of the PCR product with pair primers fabHch5 and fabHch3 generated using genomic DNA from JW1077 strain as template was identical to the Kan^R sequence as expected. In contrast, the PCR product generated by the identical reaction buffer using the parental strain BW25113 genomic DNA as template confirmed the *fabH* gene's sequence (Fig. 1B). Thus, this experiment indicated that *fabH* gene was replaced by the Kan^R gene. Furthermore, because strain JW1077 is viable without a functional *fabH* gene, this gene appears not to be essential.

However, it was previously reported that the *fabH* gene is essential for viability [17]. Therefore, a question arises if there is another copy of the *fabH* gene in the genome of stain JW1077, which complements the deleted *fabH* gene in the normal location of chromosome. For example, it has been reported that in the Wanner paper [16], which was used to generate the systematic deletion strains of *E. coli*, such as strain JW1077 [15], transient local genome duplications can occur that complement phenotypes of knockout alleles. The PCR analyses shown in Figure 1B would suggest that this is not the case. Additional genetic evidence was obtained by evaluating the stability of the Kan-resistant trait when strain JW1077 was propagated without selection. If a duplicated region of the chromosome carried a wild-type

fabH allele, we would expect to recover, at rates of >1:500, strains that have lost the Kan-resistant strain via recombination. After 20 generations of growth in a medium without kanamycin, individual colonies were rescued on a medium without selection and their kanamycin-resistance trait was ascertained by replica plating on to a kanamycin-containing media. Of one thousand colonies that were so assayed, all 1000 could grow in the absence of or presence of kanamycin. Thus, these data indicated that the kanamycin resistance marker did not segregate; and therefore it is unlikely that the strain JW1077 carries duplicated wild type *fabH* allele.

Another potential explanation for the viable nature of the *fabH* knockout allele in strain JW1077, is that this strain may carry a suppressor mutation that complements the deficiency in *fabH*-function. To show the strain JW1077 was free of such suppressor mutation, PI transduction was performed to move the fabH-knockout allele from strain JW1077 to the wild type strain MG1655. Following PI transduction, *E. coli* strain kfabh was selected via kanamycin resistance, indicating that the kanamycin marker was moved from JW1077 to the wild type *E. coli* MG1655 background. Sequencing of the PCR amplified DNA fragment from strain kfabh and MG1655 confirmed that the former strain carried the kanamycin resistance sequence, whereas the latter carried the wild type *fabH* sequence. Thus, the fact that strain kfabh is viable in the absence of a functional *fabH* gene indicates that this gene is not essential for growth.

Characterization of the *fabH* mutants. To examine if the disruption of the *fabH* gene affects the growth of *E. coli*, isogenic strains that carried either the wild type *fabH* allele

(strain MG1655) or the *fabH(Kan^R)* disrupted allele (strain kfabh) were compared. On solid LB media, there is a clear difference in growth rates between these two strains, as evidenced by the smaller colony morphology of the kfabh strain (Fig. 2A). That this growth reduction is due to the disruption of the *fabH* gene is evidenced by the fact that the growth difference between these two strains is eliminated when the wild type *fabH* gene is expressed ectopically from a pET-based vector (Fig. 2B). Thus, although the KASIII-lacking strain (strain kfabh) is viable, there is a growth penalty associated with the lack of this gene function. Because KASIII is thought to catalyze the initial condensation reaction of fatty acid biosynthesis, we ascertained the effect of KASIII deficiency on fatty acid composition. In these experiments the isogenic strains of *E. coli* in the K12 background shown in Figure 2A and B were cultured at 37 ^{0}C in liquid LB media, and cell aliquots were removed in triplicate for fatty acid analysis. Lipids were extracted from the isolated cell pellets, and fatty acid composition was determined by GC-MS analysis following transmethylation of the isolated lipids. The results of these fatty acid analyses are shown in Figure 2C. These results indicate that there is a clear difference in fatty acid composition between the wild type state and the strain that is lacking the KASIII function (strain kfabh). In addition, when the *fabH* gene is ectopically over-expressed, in either of the *fabH* mutant background (strain kfabhH) or in the wild-type background (strain MGfabH), fatty acid compositions were also altered. Therefore, there is no equivalence in fatty acid compositions between any of the strains that we evaluated, and there is no *fabH*-genotype equivalence among these four strains, we conclude that there is a dose-response between fatty acid composition and the amount of KASIII that is expressed in these strains.

Can *Bacillus* KASIII-genes replace the *E. coli* KASIII functions? Having shown that there is a growth deficiency associated with the lack of KASIII function in *E. coli*, we tested whether the *Bacillus* KASIII genes could rescue this growth phenotype. Inherent in the rationale for this experiment, is the test to ascertain whether the *Bacillus* KASIII genes can instill branch chain fatty acid biosynthesis on *E. coli*, a trait that has been associated with these two *Bacillus* genes [7, 18]. To conduct this experiment, isogenic strains were transformed with recombinant pET-derived vectors that were expressing the *Bacillus yhfB* or *yjaX* genes in the BW25113 background. These isogenic strains were carrying either the wild type *fabH* allele (i.e., derivatives of strain BW25113) or the *fabH(Kan^R)* mutant allele (i.e., derivatives of strain JW1077).

As illustrated previously in the MG1655 background, in both solid (Fig. 3A) and liquid (Fig. 3B) LB media, there is a clear growth differential between a strain that is not expressing KASIII and one that is expressing this enzyme. Thus, on LB plates, the mutant strain JW1077 shows smaller colony morphology than the parental wild type strain (BW25113). This growth differential could be partially overcome by the ectopic expression of the *Bacillus yhfB* gene, but not by the *yjaX* gene (Fig. 3A and B). These growth phenotypes appeared to be conditional in that they were different when these strains were grown on minimal salt media using glucose as the carbon source (Fig. 3C). Specifically, the *fabH(Kan^R)* mutant strain grew more efficiently than the wild type progenitor strain. As with the results obtained in LB media, ectopic expression of the *Bacillus yjaX* gene in the mutant strain did not affect the growth rate of this strain, expression of a functional *yhfB* KASIII, reduced the rate of

growth of this strain, but not to the level seen with the strain expressing the wild type *fabH* allele (i.e., strain BW25113).

In parallel experiments we ascertained the effect of these KASIII genetic manipulations on fatty acid composition. The isogenic strains that were analyzed were genetically identical with the exception of the alleles carried at the *fabH* locus and the ectopic alleles that were expressed from pET-based vectors. Specifically, these strains carried either a wild type *fabH* allele (strain BW25113) or the Kan-resistant, *fabH::Kan*^{R} mutant allele (strain JW1077), and each of these strains was transformed with pET-based vectors that ectopically expressed one of the *Bacillus* KASIII genes: 1) the *YhfB*-encoding KASIII (strain JWyhfB); and 2) the *YjaX*-encoding KASIII (strain JWyjaX). Control strains for these expression experiments were transformed with non-recombinant pET17b vectors (strain JWpET17b and BWpET17b). Finally, to ensure that the ectopic KASIII genes could be expressed from the T7 RNA polymerase promoter carried by the pET-vectors, all strains were lysogenized with bacteriophage λDE3, which carries the T7 RNA polymerase gene, under the control of an IPTG-inducible promoter.

All strains were grown in parallel in liquid LB media at 37 ^{0}C, and aliquots were removed at 6, 24 and 48 hours after inoculation when the culture was at log phase, stationary phase, and late stationary phase. Lipids from the resulting samples were extracted, and fatty acids analyzed by GC-MS following conversion to methyl esters. The resulting data are presented in Figure 4. These analyses identified 21 different fatty acids that ranged between 12- and 18-carbon chain lengths, with different degrees of unsaturation.

To assess the role of the *fabH*-encoded KASIII in determining fatty acid composition, we calculated the fatty acid compositional differences associated with the strains in which the sole genetic difference is the presence or absence of a functional KASIII protein, expressed from the chromosomal *fabH* locus. That is, we compared the data from strains BW25113 and JW1077, BWyhfB and JWyhfB, and BWyjaX and JWyjaX (Fig. 5). These difference plots reveal a consistent difference in fatty acid composition associated with the loss of a functional *fabH* gene. Namely, an increased abundance of 16:0, cyclic-17:1, one of the three isomers of 18:1, and a decreased abundance of another 18:1 isomer, and cyclic-19:1.

Similar analyses were conducted to assess the difference associated with the expression of the *Bacillus* KASIII genes (Fig. 6). In these plots were compared the differences in fatty acid composition between strains JW1077 and JWyhfB, and JW1077 and JWyjaX; the former comparison determines the effect of expressing the *YhfB*-encoded KASIII, whereas the latter determines the effect of expressing the *YjaX*-encoded KASIII, in a KASIII null background. These plots reveals that the expression of the *yjaX*-encoded KASIII is less effective in altering fatty acid composition than the expression of the *yhfB*-encoded KASIII; this is particularly evident at the latter phases in the growth of the cultures. Thus, at the log-phase of growth, replacement of the *fabH* gene with either of the *Bacillus* KASIII enzymes affects the abundance of both 16:1 and cyclic-17:1, but the addition of the *yhfB*-encoded KASIII primarily affects the abundance of 18:1. As the culture progresses into the later stage of growth, the addition of the *yhfB* gene, but not of the *yjaX* gene affects the abundance of additional fatty acids, and these alterations are of higher magnitude than those observed with

the *yjaX* gene (Fig. 6 B and C). One of the surprising findings herein is that the expression of either of the two *Bacillus* KASIII genes did not induce the accumulation of branched chain fatty acids.

An alternative strategy that was used to interpret these data is that of Principal Component Analysis (PCA) (Fig. 7). Such statistical analysis of the entire multidimensional dataset, identifies two principle components that can distinguish all genotypes that carried the wild type *FabH* allele, from those that carry the *fadH::Kan*^{R} mutant allele, irrespective of the stage of culture, or the ectopic expression of either the *Bacillus yhfB* or *yjaX* genes (Fig. 7A). Of these two principle components, principle component 1, which is composed of the fatty acids n12:0, n13:0, n14:0, n15:1, n16:0, cyclic-17:1, n17:0, 18:1, n18:0 and cyclic-19:1, is most significant in distinguishing these two genotypes. As would be expected, these are the fatty acids whose abundance is most dramatically affected by the *fabH* mutation (Fig. 5).

Similar PCA analyses were conducted to compare the strains in which the ectopic expression of the *Bacillus yhfB* and *yjaX* genes was undertaken in the KASIII-deficient background (i.e., strain carrying the *fadH::Kan*^{R} mutant allele) (Fig. 7B), or in the strain carrying the wild type *fabH* allele (Fig. 7C). Consistent with the fact that expression of the *yhfB*-encoded KASIII complements the growth phenotype associated with the KASIII-deficiency (Fig. 3), the PCA analysis indicates that the fatty acid composition associated with the *yhfB*-expressing strain is distinguishable from the strain lacking KASIII (Fig. 7B). In contrast, and consistent with the fact that expression of the *yjaX*-encoded KASIII does not complement the growth phenotype associated with the KASIII-deficiency, PCA analysis cannot distinguish any differences in

the fatty acid composition between these two strains (Fig. 7B). Similarly, PCA analysis cannot distinguish differences in fatty acid composition between these strains expressing the *Bacillus* KASIII isozymes, when the endogenous *fabH*-encoded KASIII is in the wild type state (Fig. 7C).

Discussion

***fabH* is not an essential gene.** The experiments conducted in this study have shown that in *E. coli* the *fabH* gene is not essential for growth. This is in contrast to a previous report [17], but consistent with the findings from a systematic genome–wide deletion mutant strains [15] developed by a homologous recombination method [16]. One of the concerns associated with the results of the latter study, is the possibility that the apparent viability of *fabH* mutant strain may be due to the existence of a duplicated and viable copy of this gene elsewhere in the genome, that can complements the deletion allele [19, 20]. Another potential mechanism for recovering a viable *fabH* mutant strain is that of a suppressor mutation at an independent gene that complements the deficiency of a functional *fabH* gene. In our research, three lines of evidence argue against these potential mechanisms for *E. coli* strain JW1077 being viable despite the *fabH* mutation. First, PCR analysis using genomic DNA isolated from strain JW1077 with *fabH*-specific primers, failed to support amplification, indicating that this genome does not carry *fabH*-related sequence. Second, following propagation of the strain in non-selective media, the antibiotic resistance marker which marks the mutant *fabH* allele was not lost due to recombination, an event that would be expected to occur if the genome carried a duplicated wild type *fabH* locus that could complement the deleted allele. Finally, to eliminate the possibility that strain JW1077 carries a suppressor mutation that overcame the

potential lethality associated with the mutant *fabH* allele, we used P1 transduction to move the allele to an independent strain, which would not be expected to carry such a suppressor. Because the MG1655-derived tranduced strain (kfabh) is as viable as the original mutant strain (JW1077), it is unlikely that such suppressor mutations can explain the viability of the *fabH* mutation. Therefore, we conclude that *E. coli fabH* gene is not essential for growth.

The finding that the KASIII enzyme is not essential for growth suggests some intriguing metabolic consequence. The biochemical function of this enzyme is to catalyze the initial condensation reaction of fatty acid biosynthesis, the reaction between acetyl-CoA and malonyl-ACP, to form acetoacetyl-ACP [5]. This intermediate of the process is reduced to butyryl-ACP by the subsequent three reactions of fatty acid synthesis, which is then ready for the next condensation reaction. The subsequent reiterations of the condensation reactions of fatty acid biosynthesis involve malonyl-ACP reacting with acyl-ACPs, and KASI, and KASII isozymes are thought to catalyze these reactions. Therefore, the unique capability of KASIII to catalyze the reaction between acetyl-CoA and malonyl-ACP, and begin fatty acid biosynthesis process was thought to be consistent with the earlier findings of the essential nature of the *fabH* gene [17]. Therefore, our findings that *fabH* is not essential indicates that there must be another biochemical mechanism for overcoming the metabolic block associated with the absence of the KASIII function. Potential mechanisms include the possibility that KASII and/or KASI have the capacity to catalyze this initial condensation reaction. This reaction need not be between acetyl-CoA and malonyl-ACP, but may be between acetyl-ACP and malonyl-ACP, which would necessitate invoking the involvement of an acetyl-CoA:ACP transacetylase. Indeed, such a reaction was thought to be required for

initiating fatty acid biosynthesis, prior to the discovery of KASIII [3, 21]. Other mechanisms that could be invoked for bypassing the KASIII-block also involve transacylation reactions, in which acyl-ACP intermediates down stream of acetoacetyl-ACP are generated to enable fatty acid biosynthesis to proceed normally, examples include 3-hydroxybutyryl-ACP, butyryl-ACP, hexoyl-ACP etc. These latter mechanisms need not invoke that KASI or KASII utilize an acyl-CoA substrate, a property that is not associated with these enzymes.

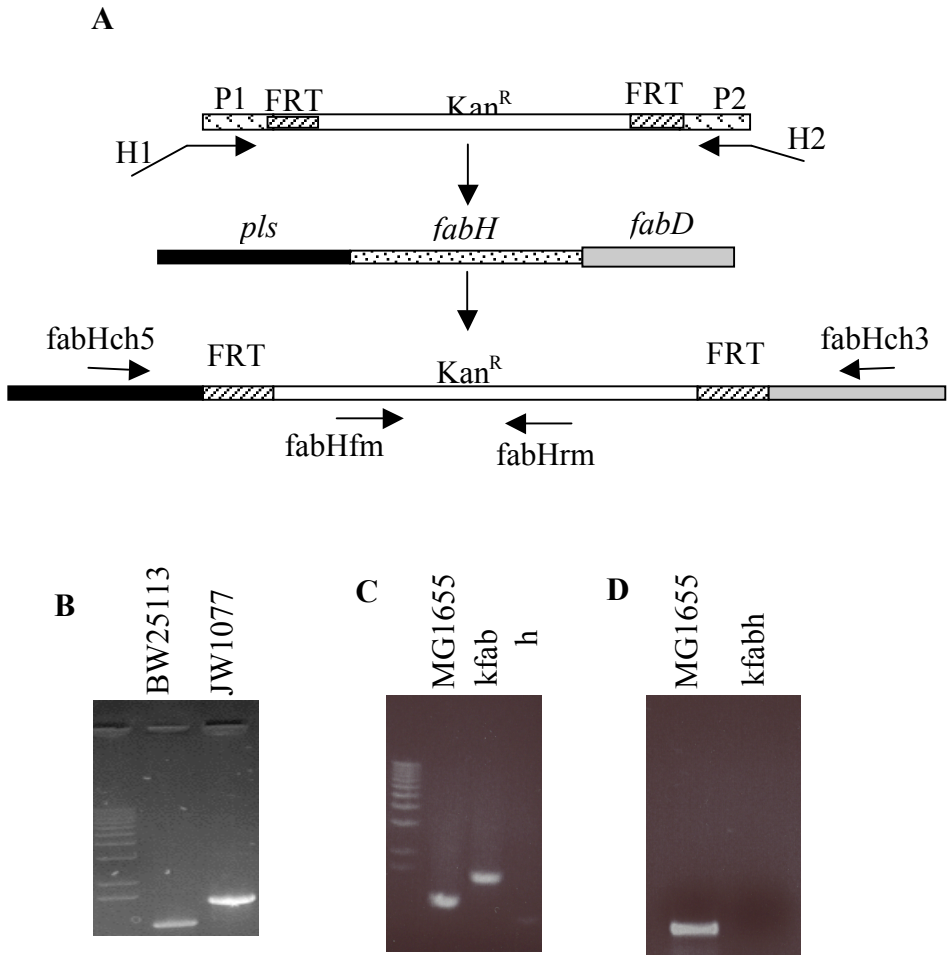
Although the *fabH* gene is not essential for growth, it's clear from our data that there is a growth penalty associated with the absence of this gene function. That this growth phenotype is due to the absence of the KASIII function was evidenced by the complementary effect of ectopically over-expressing the KASIII function from an inducible plasmid system. The effect of these genetic manipulations is coupled with compositional changes in the fatty acids associated with the membrane lipids. However, there isn't a direct relationship between fatty acid composition and the growth capabilities of the resultant strains. Thus, all three strains that grow apparently normally (i.e., those strains carrying a wild type *fabH* allele with or without the ectopically over-expressed *fabH* gene) show different fatty acid compositions, and these are also different from the strain that carries the *fabH::Kan*^{R} mutant allele, which shows a growth deficiency. These finding indicate that these two traits, fatty acid compositional changes and growth reduction, are not directly related. Rather, we speculate that growth reduction may be due to the fact that overcoming the KASIII-deficiency has an energy penalty, thus reducing growth rates. And, the fatty acid compositional changes, which are relatively minor, are due to the effect of having different doses of KASIII activities.

Can *Bacillus* KASIII genes complement *E. coli fabH* gene-functions? In catalyzing the initial condensation reaction of fatty acid biosynthesis, the KASIII enzyme has the sole capability to affect the structure at the ω-end of fatty acids. A structural variation that occurs commonly at this end of fatty acids of bacteria is branching of the alkyl-chain with a methyl group. This branching can occur at the ω-1 (iso-branch) or at the ω-2 (anteiso-branch) positions. In gram-positive bacteria such as *Bacillus* and *Streptomyces* this trait is associated with KASIII enzymes that catalyze a condensation reaction between malonyl-ACP and either isobutyryl-CoA, isovaleryl-CoA or 2-methylbutyryl-CoA to ultimately biosynthesize iso- and anteiso-BCFAs [7, 18]. In this study we generated an *E. coli* strain that is KASIII-null, and we sought to test whether expressing the two KASIII-coding genes of *Bacillus* could complement this deficiency. A partial rationale of this experiment was to clarify the confusion in the past literature [7, 17, 18] on whether these gram-positive KASIII enzymes could instill BCFAs biosynthesis in *E. coli*.

In this study the expression of the *Bacillus yhfB* or *yjaX* genes in either a wild type or *fabH* mutant strain of *E. coli* failed to instill branched chain fatty acid accumulation. However, the expression of the *yhfB* gene did alter the fatty acid composition of the *E. coli* recipient strain. Furthermore, the expression of this *Bacillus* KASIII gene also complemented the growth phenotype of the *E. coli fabH* mutant strain. This contrasts with the effect of expressing the *yjaX* gene, in either the wild type or in the *fabH* mutant *E. coli* background. This latter *Bacillus* KASIII had no effect on fatty acid composition or on the growth phenotype of the *fabH* mutation.

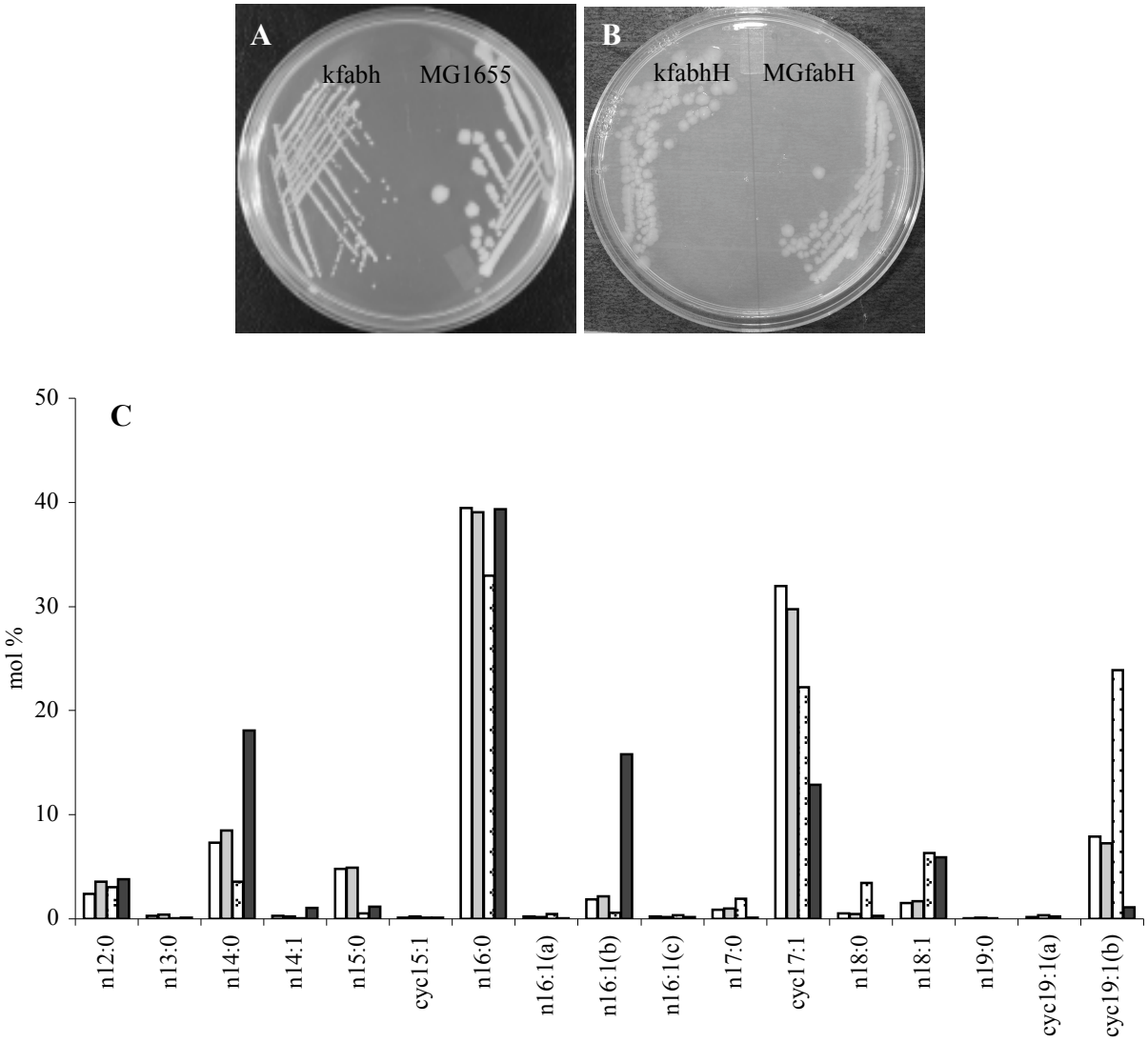
The inability of these *Bacillus* KASIII isozymes to instill BCFA accumulation in *E. coli* contrasts with our own previous studies, in which we showed that these KASIII isozymes could induce BCFA accumulation in *E. coli* strain MH13 [22]. We offer a couple of potential explanations for this variation in results. One possibility is that the ability to produce BCFAs depends on both having the appropriate KASIII enzyme, and the ability to produce the branched chain acyl-CoAs that are needed as substrates for BCFA biosynthesis. Thus, strain MH13 may have a better capacity to produce these precursors than the MG1655 derivative strain used in this study. Another explanation may be the genetic background of the MH13 strain, which carries mutations in fatty acid metabolism genes, specifically *fadR* and *fabA* mutant alleles. *FadR* represses genes required for fatty acid degradation and activates a subset of fatty acid biosynthetic genes (e.g., *fabA*, *fabI*, and *fabB*) [23-26]. Thus, in a *fadR* mutant strain (such as MH13) fatty acid metabolism may be unregulated, which enables BCFAs to accumulate. The consequence of the *fabA* mutation carried in the MH13 strain may be associated with the fact that this mutation affects the ability of the strain to produce unsaturated fatty acids, which are essential for growth. We showed that the ability of this strain to produce BCFAs complements, and rescues the lethal state of not being able to produce unsaturated fatty acids. Therefore, in the MH13 strain the ability to produce BCFAs instills a positive advantage to the strain, which we were able to select for.

Fig. 1. The *fabH::Kan*^{R} mutant allele carried by strain JW1077 (A). Schematic representation of how the *fabH::Kan*^{R} mutant allele was generated; adapted from [20]. A PCR fragment was generated with primers H1 and H2 that contained the gene for Kan^{R}. The primers H1 and H2 are composed of 20-nt priming sequences for the Kan^{R} template, flanked by 36-50-nt, which are homologous to sequences either downstream or upstream of the *fabH* gene. The resulting linear DNA fragment was used to disrupt the *fabH* locus in *E. coli* strain BW25113 [20]. The resulting *E. coli* strain, JW1077, can be selected on media containing kanamycin. Diagnostic PCR assays to confirm the disruption of the *fabH* gene were conducted with primers fabHch and fabHch3 (B and C) or fabHfm and fabHrm (D). Arrows labeled as fabHch5, fabHch3, fabHfm, and fabHrm indicate the position of the PCR primers used in these diagnostic reactions. These diagnostic PCR assays were conducted with templates of genomic DNA isolated from strains that carried either the wild type *fabH* allele (strains BW25113 and MG1655) or the *fabH::Kan*^{R} mutant allele (strains JW1077 and kfabh) as templates. A pair primers used in this PCR reaction, fabHch and fabHch3, which span entire *E. coli fabH* locus; fabHfm and fabHrm, which are inside the *fabH* gene.



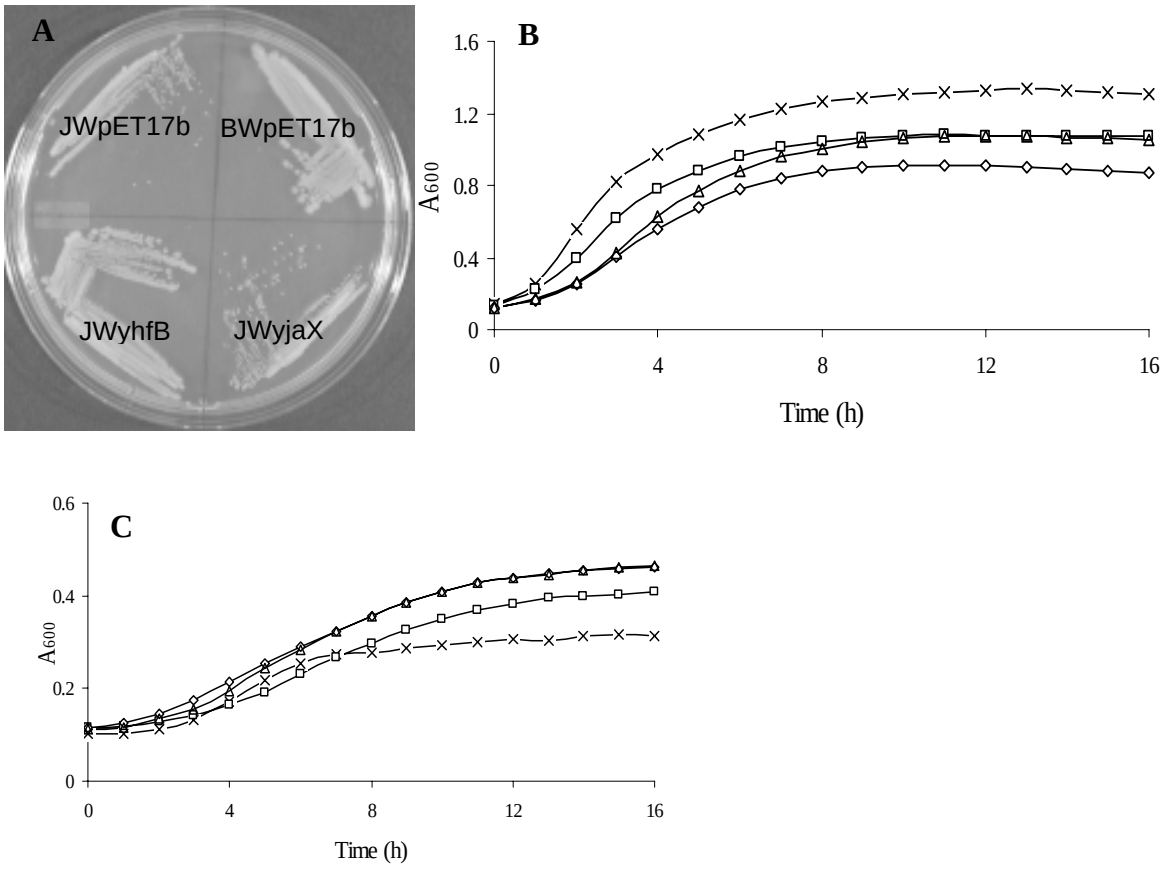
Jin and Nikolau, Figure 1

Fig. 2. Effect of the *fabH::Kan*^{R} mutant allele on growth and fatty acid composition. The indicated *E. coli* strains, defined in Table I, were grown at 37 ^{0}C on solid LB medium either in the absence (A) or presence of IPTG (B). All strains were lysogenized with λDE3, to ensure ectopic expression of the *fabH* gene from a pET-based vector in strains kfabhH and MGfabH. The identical strains were grown at 37 ^{0}C in liquid LB medium with IPTG inducer, and cells were harvested at stationary phase. Lipids were extracted and fatty acids analyzed by GC-MS (C). *E. coli* strains were: MGfabH, ❑; MG1655, ■; kfabh, ⊡; kfabhH, ■.



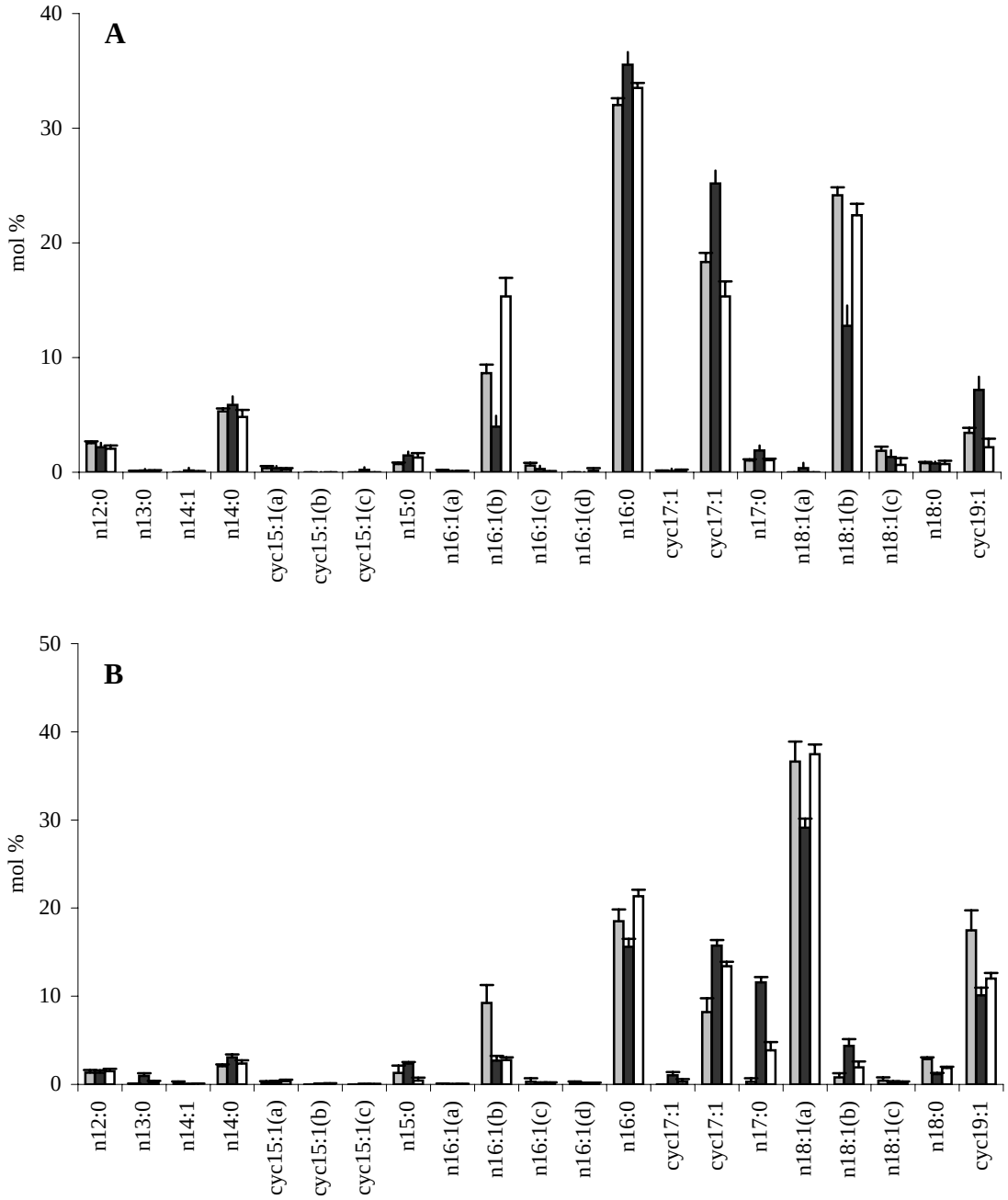
Jin and Nikolau, Figure 2

Fig. 3. Effect of the ectopic expression of the *Bacillus* KASIII genes on growth of *E. coli.* The indicated *E. coli* strains, defined in Table I, were grown at 37 ^{0}C on solid LB medium containing IPTG (A). All strains were lysogenized with λDE3, to ensure ectopic expression of the *Bacillus* yhfB and *yjaX* genes from a pET-based vector in strains JWyhfB and JWyjaX. The identical strains were grown at 37 ^{0}C, with IPTG, either in liquid LB medium (B) or liquid M9-minimal media with glucose as the carbon source (C). Growth was monitored by A_{600}. Strains are: JWyjaX, ◊; JWyhfB, ❑; BWpET17b, ×; JWpET17b, Δ.

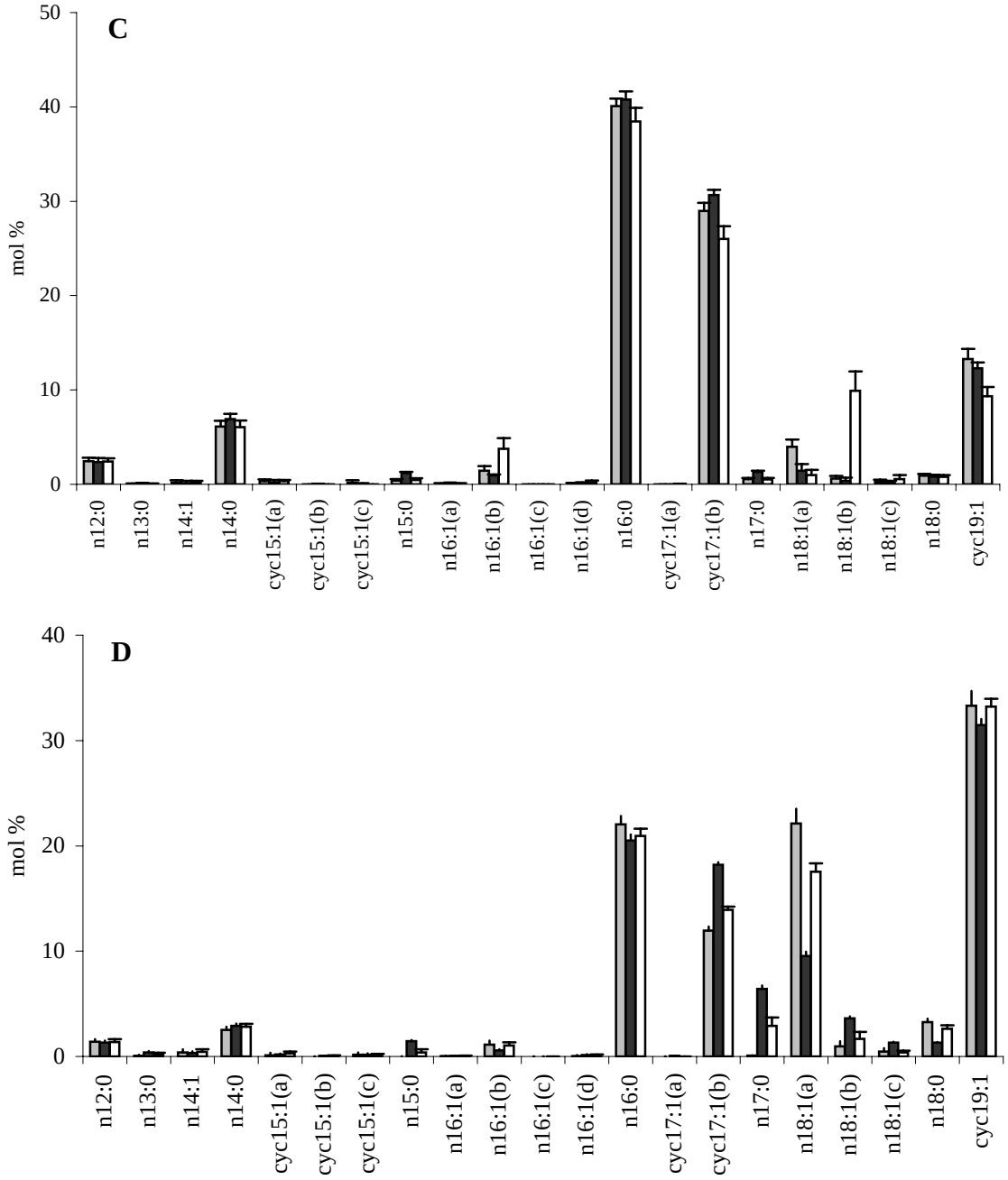


Jin and Nikolau, Figure 3

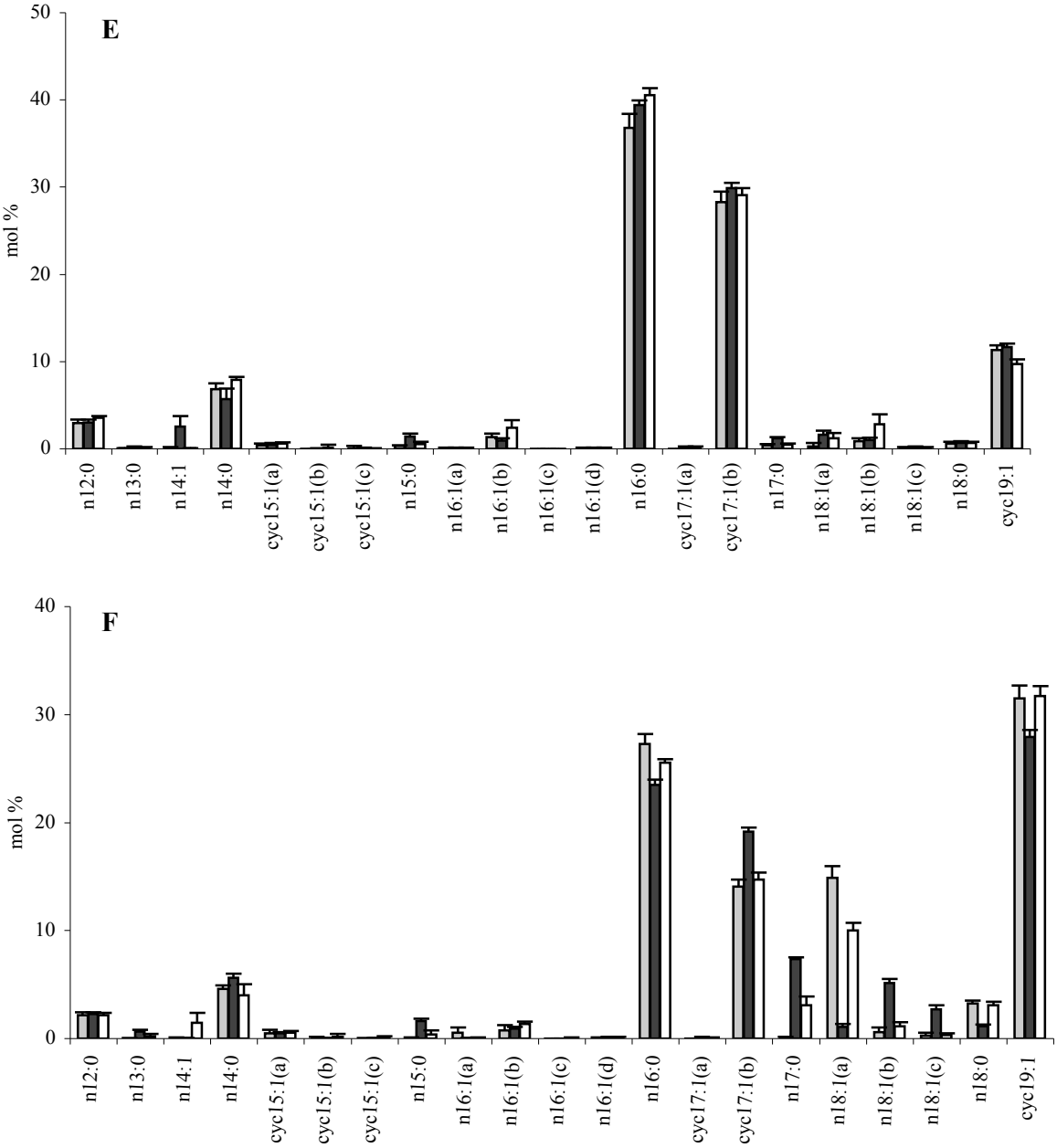
Fig. 4. Effect of the ectopic expression of the *Bacillus* KASIII genes on fatty acid composition of *E. coli.* The *E. coli* strains (all lysogenized with λDE3, to ensure ectopic expression of the *Bacillus* yhfB and *yjaX* genes from a pET-based vector) were grown at 37 ^{0}C, in liquid LB medium containing IPTG. Cell aliquots were collected at 6- (A, B), 24- (C, D) and 48-hours (E, F) post inoculation, and lipids were extracted and fatty acids analyzed by GC-MS. Strains carried either a wild type *fabH* allele (A, C, and E) or the *fabH::Kan*^{R} mutant allele (B, D and F). These strains were transformed with the non-recombinant pET17b vector (▒), or vectors that are expressing the *Bacillus yhfB* (■) or *yjaX* (□) genes.



Jin and Nikolau, Figure 4

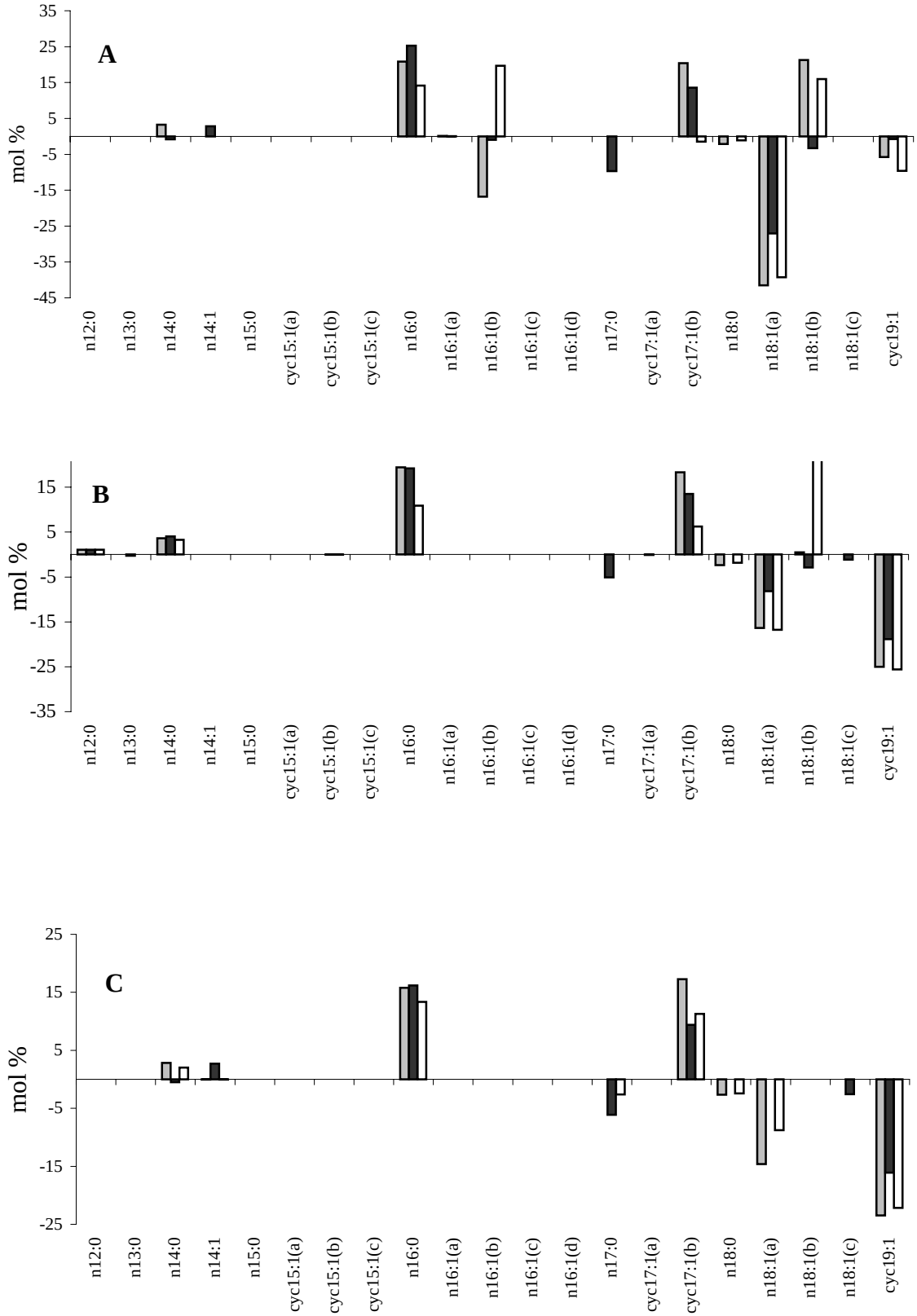


Jin and Nikolau, Figure 4



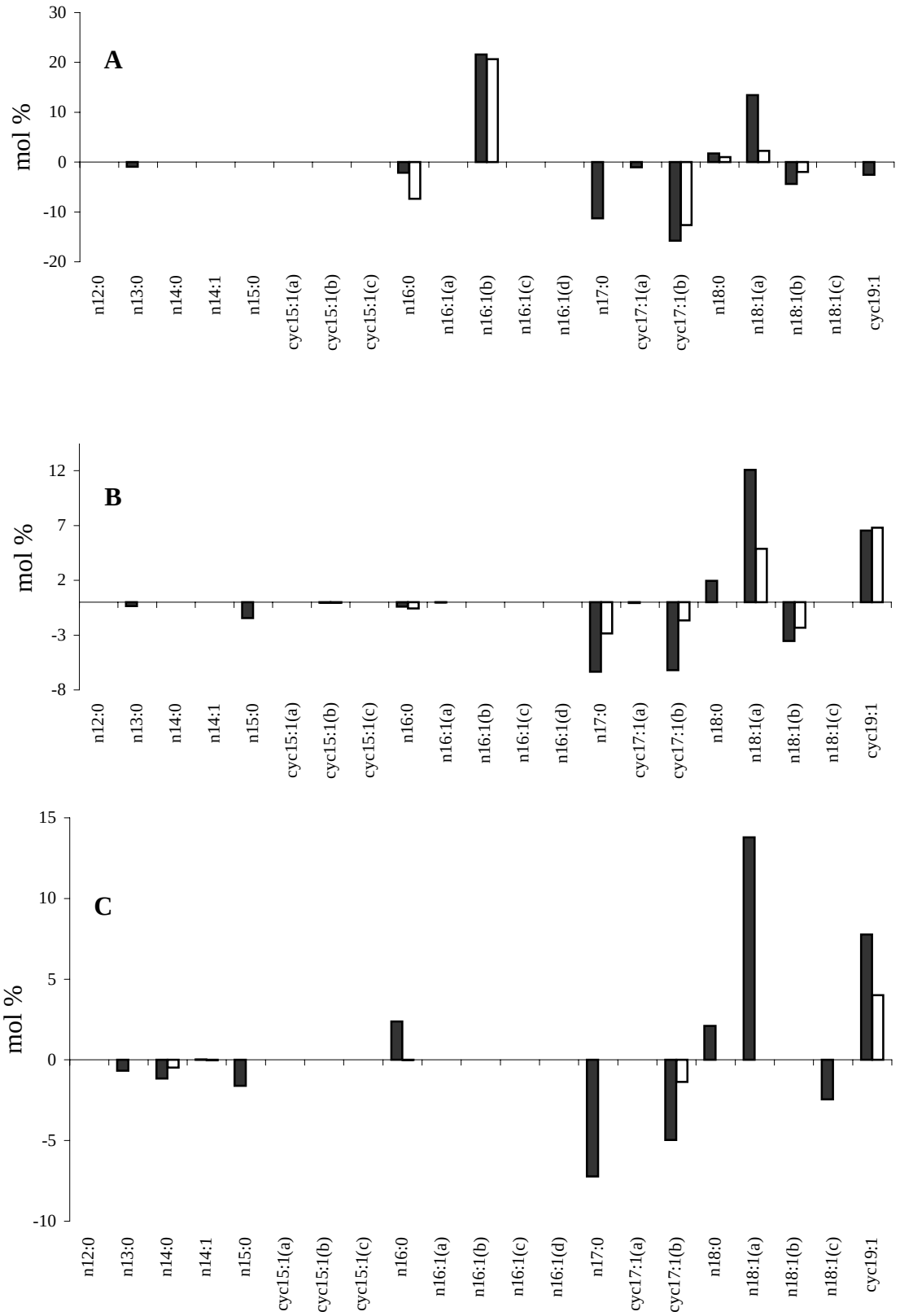
Jin and Nikolau, Figure 4

Fig. 5. Fatty acid compositional changes upon deleting the *fabH* encoded KASIII of *E. coli*. From the data presented in Figure 4, we deduced the differences in the abundances of individual fatty acids of the following pair of strains: BWpET17b – JWpET17b (▒), BWyhfB – JWyhfB (■), and BWyjaX – JWyjaX (□). These strains are isogenic, with the exception of the alleles carried at the *fabH* locus; it's either the wild type allele (labeled with BW as the first 2 letters) or the *fabH::Kan*^{R} mutant allele (labeled with JW as the first 2 letters). These plots are generated from the fatty acid composition data obtained from cells that were collected from cultures at 6-hours (A), 24-hours (B), and 48-hours (C) post inoculation.



Jin and Nikolau, Figure 5

Fig. 6. Fatty acid compositional changes upon the ectopic expression of the *Bacillus* KASIII genes in an *E. coli* KASIII-null strain. From the data presented in Figure 4, we deduced the differences in the abundances of individual fatty acids of the following pair of strains: JWpET17b – JWyhfB (■), and JWpET17b – JWyjaX (□). All these strains carried the *fabH::Kan*^{R} mutant allele, and pET-based vectors that where non-recombinant, or expressed either the *Bacillus yhfB* or *yjaX* genes. These plots are generated from the fatty acid composition data obtained from cells that were collected from cultures at 6-hours (A), 24-hours (B), and 48-hours (C) post inoculation.



Jin and Nikolau. Figure 6

Fig. 7. Principle component analysis of fatty acid composition due to the genetic manipulation of KASIII. Data presented in Figure 4 was subjected to PCA analysis. A. PCA analysis of the complete data. B. PCA analysis of the data obtained from manipulations in the strain JW1077, which carries the *fabH::Kan*^{R} mutant allele. C. PCA analysis of the data obtained from manipulations in the strain BW25113, which carries the wild type *fabH* allele. Each data-point is labeled with a five-character code. The key to the code is as follows:

First character: B = strain BW25113, which carries the wild type *fabH* allele, or

J = strain JW1077, which carries the *fabH::Kan*^{R} mutant allele

Second character denotes ectopic expression vector carried by strain:

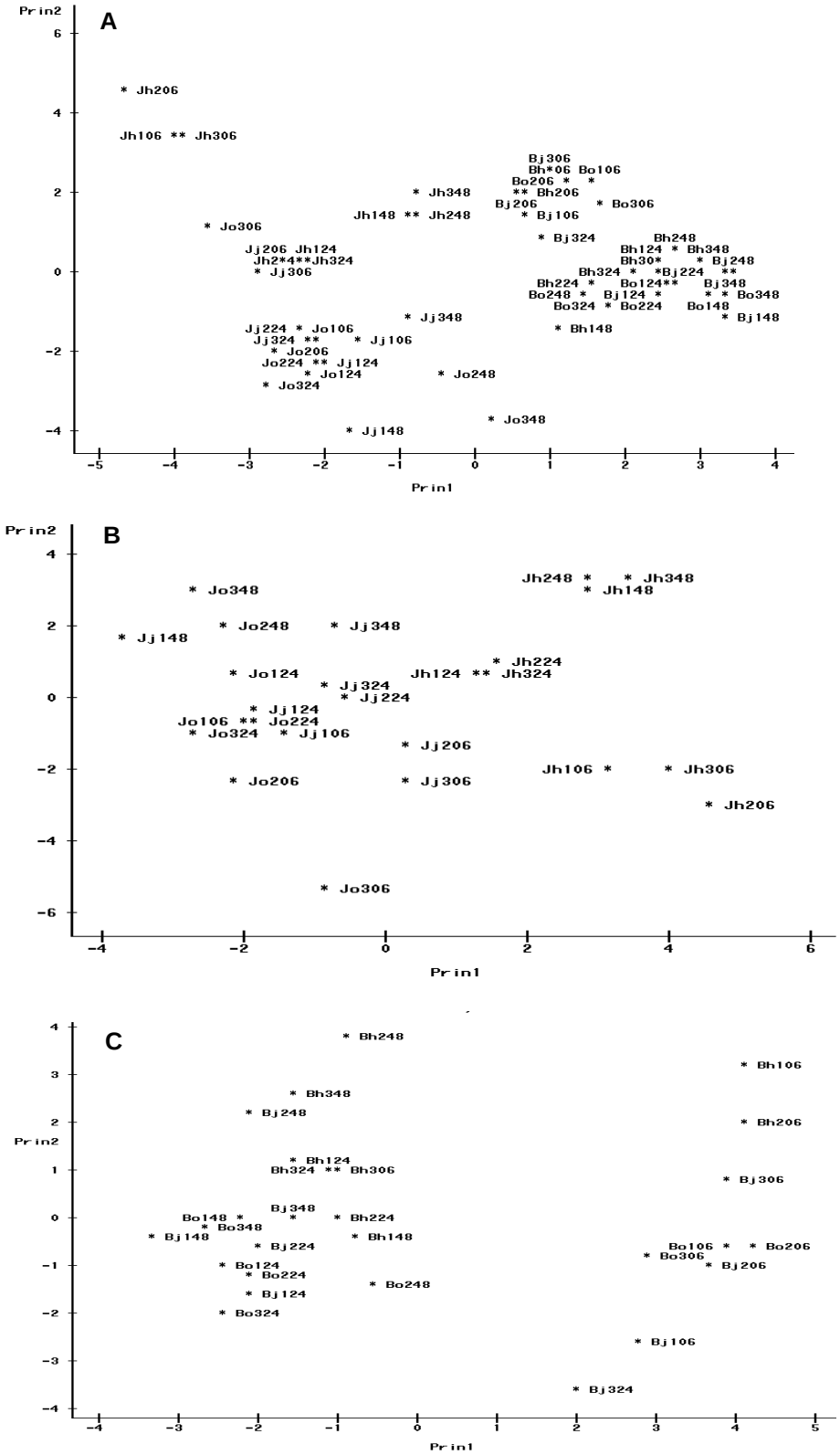
o = the control pET17b vector

h = recombinant pETyhfB vector expressing the *Bacillus yhfB* gene

j = recombinant pETyjaX vector expressing the *Bacillus yjaX* gene

Third character denotes the replicate number of the experiment, 1, 2, or 3

Fourth and fifth character denotes that data came from cells were collected from cultures at 6-hours (06), 24-hours (24), and 48-hours (48) post inoculation.



Jin and Nikolau, Figure 7

Table 1. Bacteria strains and plasmids

Strains or plasmids	Relevant characteristics	Source
Strains		
MG1655	*E. coli* wild type	*E. coli* Genetic Resource center
BW25113	Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), lacIp-4000($lacI^Q$), λ^-, rph-1, Δ(rhaD-rhaB)568, hsdR514	*E. coli* Genetic Resource center
JW1077	*fabh:*:kan^R, Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), lacIp-4000($lacI^Q$), λ^-, rph-1, Δ(rhaD-rhaB)568, hsdR514	Keio Collection
BWyhfB	*E. coli* MG1655 (DE3) expressing the *Bacillus yhfB* gene from pETyhfB	This work
BWyjaX	*E. coli* MG1655 (DE3) expressing the *Bacillus yjaX* gene from pETyjaX.	This work
BWpET17b	*E. coli* MG1655 (DE3) carrying pET17b	This work
JWyhfB	*E. coli* JW1077 (DE3) expressing the *Bacillus yhfB* gene from pETyhfB	This work
JWyjaX	*E. coli* JW1077 (DE3) expressing the *Bacillus yjaX* gene from pETyjaX	This work
JWpET17b	*E. coli* JW1077 (DE3) carrying pET17b	This work
kfabh	MG1655 carrying *fabH*::kan^R	This work
kfabhH	kfabh expressing the *E. coli fabH* gene from pDESTfabH	This work
MGfabH	MG1655 expressing the *E. coli fabH* gene from pDESTfabH	This work
Plasmids		
pETyjaX	pET17b expresses *Bacillus yjaX* gene, Amp^+	PET17b vector from Novagen
PETyhfB	PET11dexpresses *Bacillus yhfB* gene, Amp^+	PET11dvector from Novagen
pDESTfabH	pDEST 17 expresses *E. coli fabH* gene, Amp^+	pDEST17 vector from Invitrogen

Table 2. Primer sequences

Name	Sequence
FabHch5	CTGGTTTTGAGCTGCTGGAC
FabHch3	CAACGGTTTGAGAACCCTGT
fabHfm	GAAAGACCAGATTGGCCTGA
fabHrm	AACCGACGACCAGAGCATAC
forfabh	CACCATGTATACGAAGATTATTGGTACT
Revfabh	CTAGAAACGAACCAGCGCGGA

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Chapter 5. A System for Dissecting the Physiological Role of Mono-acyl Ester Synthase Genes of Arabidopsis

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Abstract

We propose two hypotheses for the physiological function of each of the twelve putative wax synthase genes of *Arabidopsis* may encode isozymes that are involved in the biosynthesis of acyl esters. One is that this gene diversity provides the organism with the capability to synthesize different acyl esters, depending on the substrate specificity of each gene product. The second hypothesis is that this gene diversity provides the organism with the means of regulating the expression of this biosynthetic capability at different developmental stages of the plant. We established a system for assessing these hypotheses using one of the gene members, At5g55380. Promoter::GUS transgenic lines identified the organ and tissue expression pattern of this gene. Analysis of the phenotype and chemotype associated with a T-DNA insertion mutant (allele SALK_060303) identified a number of aberrations in the growth of Arabidopsis that lacks a functional copy of this gene. Specifically, the At5g55380 is highly expressed in roots, and the SALK_060303 mutation affects root growth and fatty acid composition of the roots, particularly of fatty acids associated with the extracellular lipid polymer, suberin. These results suggest that At5g55380 may be involved in generating the acyl esters of suberin.

Introduction

Mono-acyl esters are commercially used in cosmetics, lubricants, polishes, surface coatings, inks, and many other applications [1]. They are mainly obtained from two origins, one is mineral material like brown coat and peat deposits [2]; and the other is living organisms. Mono-acyl esters occur discreetly in various organisms such as animals [3, 4], plants [5], insects, and bacteria [6, 7]. However, the desert bush, jojoba, accumulates a large amount of mono-acyl esters in its seeds (ref). In addition, all plants accumulate mono-acyl esters in their cuticle, which covers the aerial surface of plants, to prevent water evaporation [8, 9], and protect plants from diseases [10] and insects [11]. Similarly, mono-acyl esters occur in the waxes covering the surface of insects, restricting the water loss across insects' cuticle, and preventing desiccation [12]. In most animals, sebaceous glands of the skin produce mono-acyl esters for lubricating and protecting the hair and skin, and preventing drying and irritation of membranes [13]. Diverging from the above functions, mono-acyl esters in marine animals are used for buoyancy, insulation and even echo location [14]. Although mono-acyl esters are not common in the prokaryotes, some bacterial species can produce these molecules. For example *Acinetobacter* sp. generates mono-acyl esters in nitrogen-limited conditions [15, 16].

The mono-acyl esters are generated by the esterification of an alcohol with a fatty acid, derived from acyl-CoA, catalyzed by mono-acyl ester synthase. The first mono-acyl ester synthase was isolated from jojoba embryos. It was expressed in the seed of *Arabidopsis,*

which do not normally accumulate mono-acyl esters. These transgenetic seeds were found to be capable of producing large quantities of mono-acyl esters [17].

Using the jojoba mono-acyl ester synthase cDNA sequence as an entry point into sequence data bases, twelve *Arabidopsis* homologous genes were identified that putatively encode this enzyme. We hypothesize that each of these twelve genes may encode mono-acyl ester synthase isozymes that are involved in the biosynthesis of different mono-acyl ester s from different precursors, i.e. catalyze reaction between different fatty acyl-CoAs and different alcohols, or they are differently expressed during plant development. One of these genes, At5g55380, was chosen for study to begin to address this hypothesis.

Methods

Plant materials and growth conditions. The *Arabidopsis* stock carrying the SALK_060303 allele used in these studies was initially identified by the Salk Institute Genomic Analysis Laboratory (SIGnAL; Alonso et al., 2003). This stock, which is in the Columbia-0 ecotype background, was obtained from the ABRC. A second allele, PST801, was from RIKEN Biorescource Center in Japan and is No-0 ecotype background. Plants were either in grown on soil or Murashige and Skoog solid media supplemented with 0.1% sucrose or in sterile LC1 Sunshine Mix soil (Sun Gro Horticulture, Bellevue, WA). Plants were grown in a controlled environmental room maintained at 26 °C and 37% humidity under continuous illumination with white 40 W Sylvania cold-white florescent bulbs. The white light irradiation was at 150 $\mu mol.m^{-2}.s^{-1}$.

Sterilized seeds were first germinated on the solid Murashige and Skoog media and seedling grown for 14 days. *Arabidopsis* seedlings were then transferred to soil. Seeds were sterilized by soaking sequentially in 0.2 ml of 50 % alcohol for 1 minute, and 0.2 ml 50 % bleach for 10 minutes. Sterilized seeds were then washed three times with sterile water and spread on the Murashige and Skoog solid media.

Genomic DNA isolation and genotyping PCR. DNA was isolated from a single leaf detached from individual plants of about 3-4 weeks old. Each leaf was homogenized with 0.5 ml of 0.2 M Tris-HCl (pH 7.0), 0.4 M LiCl, 25 mM EDTA, and 1% SDS. The extract was centrifuged for 5 minutes at 15000 g. A 350 μl aliquot of the supernatant was transferred to an Eppendoff tube containing an equal amount of isopropanol. After mixing by inversion, the precipitated DNA was collected by centrifugation for 10 minutes at 15000 g. The supernatant was poured off and the pellet was air-dried for about 60 minutes, and dissolved in 30-50 μl of sterile water.

"Hot-star" PCR reactions were conducted to genotype T-DNA inserted mutants. The 25 μl PCR reaction solution contains 2.5 μl of 10 × PCR buffer, 1 μl of 50 mM $MgCl_2$, 0.5 μl each of 10 mM dNTPs, 6 pmol of each primer and 0.5 unit of Taq DNA polymerase (Invitrogen, Carlsbad, CA). The sequence of PCR primers used in these studies are given in Table 2. PCR reaction proceeded for 32 cycles, including holding at 96 °C for 10 minutes, denaturation at 94 °C for 15 seconds, primer annealing at 60 °C for 30 seconds, and extending at 72 °C for 2 minutes.

RNA isolation, RT-PCR, and 5'-RACE-PCR RNA was isolated from plant leaves using RNeasy Mini Kit (Qiagen, Valencia, CA). For RT-PCR analysis, first-strand cDNA was synthesized using SuperScript™ First-Strand Synthesis System (Invitrogen, Carlsbad, CA). In the first-strand cDNA synthetic reaction, 5 μg of RNA was used with the transcriptase SuperScript™II RT and Oligo dT primer. PCR reaction was executed to amplify first-strand cDNA with a combination of primers, RTF3 and RTR3, which are located within At5g55380 (Table 2).

5'-RACE-PCR was performed with the GeneRace™ Kit (Invitrogen, Carlsbad, CA). RNA isolated from wild type *Arabidopsis* was first treated with calf intestinal phosphatase to remove the 5' phosphate from non-mRNAs or truncated mRNA. The resulting material was then treated with tobacco acid pyrophosphatase to remove the 5' cap structure from intact, full-length mRNA, followed by ligating GeneRace™ RNA Oligo to phosphorylated 5' end of the mRNA (Table 2). The GeneRace™ RNA Oligo will provide a known prime site for GeneRace™ 5'primer after mRNA is transcribed into cDNA (Table 2). The first-strand cDNA was generated using the ligated mRNA as template, oligo dT primer provided by Kit and SuperScript III RT transcriptase. The first-strand cDNA was amplified using the primer pair consisting of 5u, which is specific to the At5g55380 gene, and GeneRace™5'primer (Table 2).

Analysis of fatty acids. The tissue was first subjected to hydrolysis at 110 ^{0}C, in 5% $Ba(OH)_2$ and 50% dioxane). Following acidification, the hydrolyzed fatty acids were recovered by extraction using hexane, methylated and silylated, and analyzed

by GC-MS [18].

Results

Mutant screening and segregation analysis. Genomic-based sequence-comparisons identify twelve *Arabidopsis* genes that are homologous to the mono-acyl ester synthase gene of jojoba [17]. We have identified total of 15 T-DNA insertion or transposon insertion mutants associated with these locus (Table 1). SALK_060303 and PST 80 allele of At5g55380 were selected for detailed analysis.

Out of 37 offsprings from a selfed heterozygous SALK_060303 plant, 9 plants were wild type, 9 plants were homozygous and 19 plants were heterozygous (Table 3). Using χ^2 analysis to test the null hypothesis of Mendilian segregation for a single allele, the p-value is <0.001. Therefore we cannot reject this null hypothesis at the level of 0.05. Namely the SALK_060303 allele segregates at the expected 1:2:1 ratio for a single locus allele. As for PST801allele, 148 offspring from selfed heterozygous plants were genotyped and found to be heterozygous for the mutant allele; neither wild type nor homozygous plants were recovered. This observed segregation is difficult to explain. The failure to recover wild type plants may be due to synthetic lethal or the occurrence of multiple mutations in this stock, some of which maybe lethal or multi-insertion of T-DNA. Because of this complexity, PST801 was not studied further in this research.

Molecular characterization of At5g55380 and structure of the SALK_060303 allele. Comparing the sequence of the cDNA clone that is derived from At5g55380 (clone RAFL99-01-C02; GenBank Accession AF149919) with the *Arabidopsis* genomic sequence, identified the structure of this mono-acyl ester synthase gene. These data established that this gene is not interrupted by introns, and that the 3'-UTR extends 180 nucleotides past the translation stop codon. To identify the 5'-end of this gene, 5'-RACE-PCR was conducted. The resultant sequence established that the 5'-UTR of this gene extended 97 nucleotides upstream of the translation initiation "ATG"-codon. Arabidopsis stocks carrying these mutant alleles were propagated and molecularly characterized. These characterizations accurately identified the positions of the insertions within the either the coding regions of each gene or within the 5'-UTR.

To identify the exact position of the T-DNA insertion that disrupts the At5g55380 gene in allele SALK_060303, PCR products generated with the primer pairs LB and LP, and LB and RP (Fig. 2) were sequenced. The sequence data indicate that the DNA fragment that disrupts this gene is probably a chimeric insertion of the T-DNA element, with outward facing LB termini. In addition these data demonstrate that the T-DNA element was inserted between nucleotide position 1000 nucleotide downstream of ATG start codon and 17 nucleotide upstream of the translation stop codon. Furthermore, this insertion event deleted 42 nucleotides of this gene (Fig. 2). Seventeen of the deleted nucleotides are located within the

computer-predicted 3' non-coding region of the gene and the remaining 25 are located immediately upstream of the stop codon.

The transcription of At5g55380 gene in mutant SALK_060303. T-DNA elements tend to insert as concatemers of 10-kb, thus most T-DNA insertions cause the loss of gene expression. To test if the SALK_060303 T-DNA insertion disrupts the expression of the At5g55380 gene, RT-PCR analyses were performed with RNA template, isolated from either wild type plants or sibling plants homozygous for SALK_060303 allele (Fig. 3). Using PCR primers RTF3 and RTR3, which are located upstream of the T-DNA insertion site, these experiments demonstrate that despite the insertion of the T-DNA, plants homozygous for the SALK_060303 are still capable of transcribing this mono-acyl ester synthase gene. However, bearing in mind that the T-DNA insertion event that disrupted this gene also deleted coding-sequence, the transcript that is expressed from this mutant allele must have an aberrant structure, specifically at the 3'-end of transcript.

Growth phenotypes associated with SALK_060303. To ascertain the affect of the SALK_060303 allele on the growth morphology of Arabidopsis, sterile seeds homozygous for SALK_060303 and their wild type siblings were plated together on Murashige and Skoog solid media, and the growth of the two genotypes were compared. Eight days after germination, the secondary roots of the mutant plants were less dense than those of the wild type plants (Fig. 4A and B). At 14 days after germination, the seedlings were transferred to soil. Two weeks after this transfer, mutant plants appeared smaller than their wild type siblings and the leaves of the mutant were narrower and shorter than the wild type (Fig. 4C).

We noted that this phenotype became more exaggerated when water was withheld from these plants, which may indicate that the At5g55380-function may be involved in maintaining water status of the plant.

Upon flowering the homozygous mutant plants were shorter than their wild type sibling (Fig. 4D) and this trait was maintained until maturity of the flower bolt (Fig. 5A). This mutation also affected the size of the propaqule units. Specifically, siliques on mutant plants are shorter than on wild-type plants (Fig. 5C and D), and seed yields per plant are considerably reduced (Fig. 5B). However, the individual seeds that are recovered from the mutant plants are heavier (by ~20%) (Fig. 5E), and longer (Fig. 5F).

Paralog-specific expression of At5g55380. The organ and tissue specific expression pattern of the At5g55380 gene was assessed using transgenic plants that carried a reporter genes (GFP-GUS) fused to different promoter elements from At5g55380. Three different constructs were generated (Fig. 6A) in which different promoter fragments were fused to a chimeric GFP-GUS gene in the expression transformation vector, pBGWFS7 [19]. Two of these constructs fused 1.55-kb or 0.28-kb of genomic DNA immediately upstream of the "ATG" translational start codon of the At5g55380 gene to the GFP-GUS chimeric gene, to generate WaxSp1, and WasSp2 constructs (Fig. 6A). In the third construct (WaxSp1tar), the GFP-GUS chimeric gene was fused to the 3'-end of the At5g55380 gene (Fig. 6A).

The expression patterns obtained from the resulting three sets of transgenic plants were near identical, thus only the observations from the waxSp2 construct are shown in Figure 7. In

young seedlings, GUS expression, driven by the promoter of At5g55380 gene was expressed in cotyledons and leaves, and although expression is detectable in mesophyll cells of these organs, expression appears to be concentrated in the vasculature (Fig. 6B-E). Just before bolting, expression was stronger in the older leaves than in young expanding leaves, but it was not expressed in the shoot meristem (Fig. 6C). Strong expression was seen in the opened flower (Fig. 6G), and within these flowers it was concentrated in the sepals and pedicles (Fig. 6G and H). Strong expression was also seen in the roots of young seedlings (10-day old plants); particularly in the primary roots (Fig. 6I). Within the root system expression was particularly enhanced at the initiation of the lateral roots (Fig. 6J), and in the root vasculature, but not in root tip (Fig. 6K).

Effect of mutation in At5g55380 on fatty acid composition. Based upon the sequence homology between At5g55380 and the jojoba wax synthase, we assume that the former gene product probably catalyzes the reaction between a fatty acyl-CoA and an unknown alcohol to form a mono-acyl ester. Therefore, mutations in At5g55380 may alter fatty acid profiles from the affected tissues. We therefore compared the fatty acid profiles between sibling plants that carried either the wild type alleles or the SALK_060303 mutant alleles. For this experiment we rationalized that the greatest biochemical effect of this mutation will likely occur in the tissues and organs where the At5g55380 gene is most active. Based upon the fact that At5g55380 is expressed at relatively high levels in roots (Fig. 6), we assessed the root fatty acid profiles.

These analyses identified 38 distinct fatty acids, the relative abundance of 17 of these is significantly altered between the wild type and mutant roots (Fig. 7). Of these altered fatty acids, 11 are less abundant, and 6 are more abundant in the mutant relative to the wild type. Those whose abundances are enhanced in the mutant are generally associated with membrane lipids of the root cells (e.g., 16:0, 18:1(9), 18:2(9,12) and 18:3(9,12,15)). In contrast, the fatty acids whose abundance is reduced in the mutant are usually associated with extracellular polymeric lipid, suberin; these are ω-hydroxy-fatty acids, 2-hydroxyfatty acids and fatty acids of 22-carbons and longer. Suberin is an extracellular polymer that is deposited at distinct locations during plant growth, for example, in several underground tissues, e.g. epidermis, hypodermis, peridermis and the Casparian strips of the root endodermis [20]. Its function is to seal off the entire plant or one of its tissues against loss of water and solutes. Therefore, these results would suggest that At5g55380 might be involved

in the formation of the ester bonds that enables the polymerization of suberin. Thus, mutations that affect this polymerization reaction would be expected to reduce the accumulation of the fatty acids that are components of suberin. However, additional research is required to test this hypothesis directly.

Discussion

The cloning of the jojoba mono-acyl ester synthase gene [21] identified a new class of enzymes that catalyze the formation of mono-acyl esters via a reaction between an acyl-CoA and a primary alcohol. This achievement subsequently lead to the identification of mono-acyl ester synthase genes in bacteria [22], humans [23], and other plant systems. In Arabidopsis, we find 12 genes that share significant sequence homology to the jojoba mono-acyl ester synthase. The studies reported herein were designed to establish a system for testing the hypothesis that these genes catalyze the synthesis of acyl esters. Moreover, the system we established has the additional capability to address the physiological function of the gene redundancy present within this gene family. Thus, utilizing this system we can test the hypotheses that the Arabidopsis mono-acyl ester synthase gene redundancy provides the organism the ability to produce different acyl esters, depending upon the substrate specificity of each gene product. Alternatively, this redundancy may provide the organism the means of regulating the expression of this biosynthetic capability at different developmental stages of the plant; this would manifest different organ and tissue specific gene expression pattern for each mono-acyl ester synthase gene. We established a system for addressing each of these hypotheses, using one of the 12 mono-acyl ester synthase genes (At5g55380) as the test-bed for these experiments.

Initially we characterized the structure of this gene by sequencing the corresponding cDNA. Comparing this gene sequence to the genomic sequence of the At5g55380 gene, established the 3'-end of the gene, and that this gene (as with all the mono-acyl ester synthase genes) is not interrupted by introns. The 5'-end of the gene was identified by 5'-RACE experiments.

To assess the biochemical properties of the gene product, and begin the characterization of the substrate specificity of the gene, the complete cDNA sequence was expressed in *E. coli*, using standard expression systems (see Appendix). Unfortunately due to the unstable nature of the recombinant gene product, we were unable to continue this line of experimentation.

GUS-reporter gene constructs were used to assess the organ and tissue expression pattern of the At5g55380 gene. These experiments identified a pattern of expression that is highly induced in the roots of Arabidopsis. Based upon this observation we rationalized the experiments for characterizing the phenotype and chemotype of plants carrying a disruption mutation in this gene. Specifically, we characterized two mutant alleles, SALK_060303 and PST801. Unfortunately, we were unable to recover the PST801 allele from the stock obtained from the Riken Seed Stock Center. However, the SALK_060303 allele, which carries a T-DNA insertion near the 3'end of the gene ORF that also deletes a portion of the gene sequence provided biological insights as to the consequence of the lack of At5g55380 gene-function.

The SALK_060303 mutation altered a number of growth characteristics of the plant, including its growth stature, root morphology, and seed yields. Biochemical characterization of the fatty acids alterations in this mutant suggests that lipids associated with the extracellular matrix of the roots, possibly suberin, may be altered by this mutation. Such an alteration is consistent with the fact that At5g55380 is highly expressed in roots, and that root morphology is affected by this gene mutation.

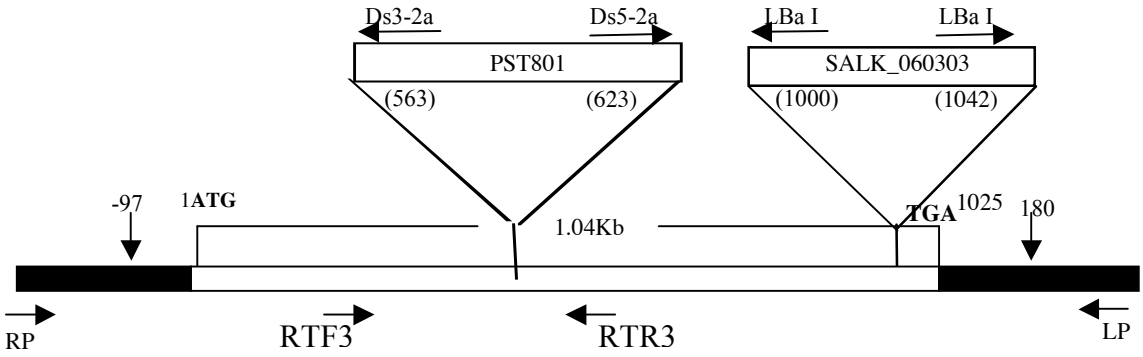
Additional experimentation will be required to evaluate these findings. Specifically, either a genetic complementation experiment or the characterization of a second allele is needed to authenticate that the observed phenotypes and chemotypes associate with the SALK_060303 stock is a consequence of the disruption of the At5g55380 gene. Additional biochemical studies are needed to assess that the altered fatty acid profiles in the SALK_060303 are due to altered suberin structure. Should this mutant affect suberin deposition, it will prove invaluable in characterizing suberin structure and biosynthesis, because to date there are no known mutants that affect this poorly characterized molecule.

Fig. 1. Homology between jojoba mono-acyl ester synthase and Arabidopsis At5g55380. Comparison of the amino acid sequence of the jojoba mono-acyl ester synthase (top sequence) with the protein product of the Arabidopsis At5g55380 gene (bottom sequence). Identical residues are identified between the two sequences, and conserved changes are marked with a "+" symbol. The two sequences share 45% sequence identity.

```
jojoba 3     VEKELKTFSEVWISAIAAACYCRFVPAVAPHGGAXXXXXXXXXXXXXXXXXXXXSSFHLG  62
             +E++ +   EVWISA+ +  YC ++ +    G  LRLL +LPV +LF+ LPL LS  H
55380  1     MEEKFRNLIEVWISALISLSYCYYISSKLSKG-VLRLLSILPVCILFLVLPLFLSCVHFC  59

jojoba 63    GPTALYLVWLANFKLLLFAFHXXXXXXXXXXXXHFISTTLLPIKFRDDPSNDHEKNKRTL  122
               + L+L WLANFKLLLFAF  GPL      L  FI    LPIK R DPS +   N
55380  60    AISVLFLSWLANFKLLLFAFDEGPLFPLPPKLSRFICFACLPIKIRQDPSPNAIPNLHPK  119

jojoba 123   SF-EWRXXXXXXXXXXXXGILKIYEFRKDLPHFVISVLYCFHFYLGTEITLAASAVIAR  181
                +W   V  +  V   +L +YE+R  LP FV+  LYC H YL  E+ L     +
S55380 120   PMPKWVLAVKILVLGV----LLHVYEYRDGLPRFVVLALYCLHIYLEVELVLVFVGAVVS  175

jojoba 182   ATLGLDLYPQFNEPYLATSLQDFWGRRWNLMVSDILGLTTYQPXXXXXXXXXXXXXXAG  241
               LG ++ P FNEPYLATSLQDFW RRWNLMVS +L  T + PV+R   R +     +
55380  176   TLLGCNIEPVFNEPYLATSLQDFWSRRWNLMVSAVLRSTVHIPVQRFFKRILSPDGAMFA  235

jojoba 242   AMLVAFTVSGLMHEVFFFYLTRARPSWEVTGFFVLHGVCTAVEMVVKKAVSGKVRLRREV  301
              ++ +F VSGLMHE+ +FY+R  P+WEVT FFVLHG  TA E+VK+    +    R V
55380  236   GVMASFFVSGLMHELLYFYMIRKPPTWEVTCFFVLHGAATATEIAVKRTQWLRPP-HRAV  294

jojoba 302   SGALTVGFVMVTGGWLFLPQLVRHGVDLKTIDE  334
              SG + + FV VTG WLFL Q++R+ V  K I E
55380  295   SGLVVLTFVSVTGVWLFLAQVLRNNVHEKAIGE  327
```

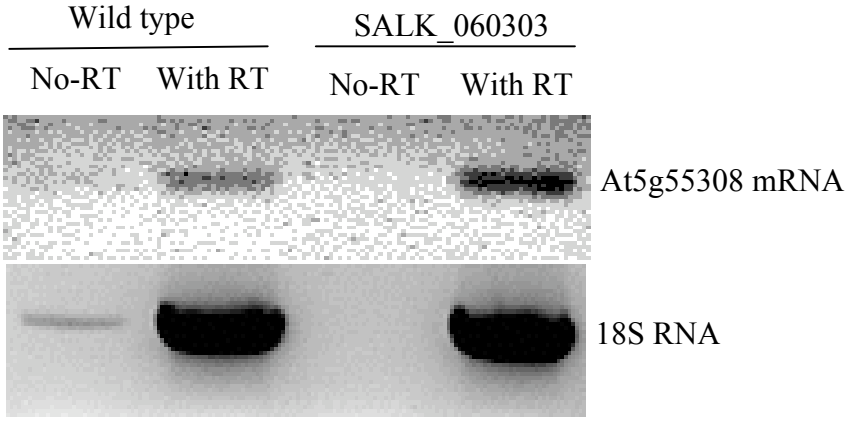
Jin and et al., Figure 1

Fig. 2. A schematic representation of structure of the Arabidopsis At5g55380 gene. The gene is 1,025-nt in length, starting from the translation start codon ^{1}ATG and finishing at the translational stop codon, TGA^{1025}. The ends of the 5'-UTR and 3'-UTR are indicated by vertical arrows, and are located 97-nt upstream and 180-nt downstream of the ORF, respectively. The position of the T-DNA insertion in the SALK_060303 allele is indicated, which was deduced from DNA sequences of PCR products generated with primers pairs, LBa1 and LP, and Lba1 and RP. The position of the Ds transposon in the PST801 allele is indicated as catalogued at the Riken database (http://rarge.gsc.riken.jp/dsmutant/index.pl). Horizontal arrows labeled, RP, LP, LBa1, Ds5-2a, Ds3-2a, RTF3 and RTR3 indicate the location and orientation of primers used in genotyping PCR reactions and in RT-PCR reactions. Sequences of these primers are given in Table 2.



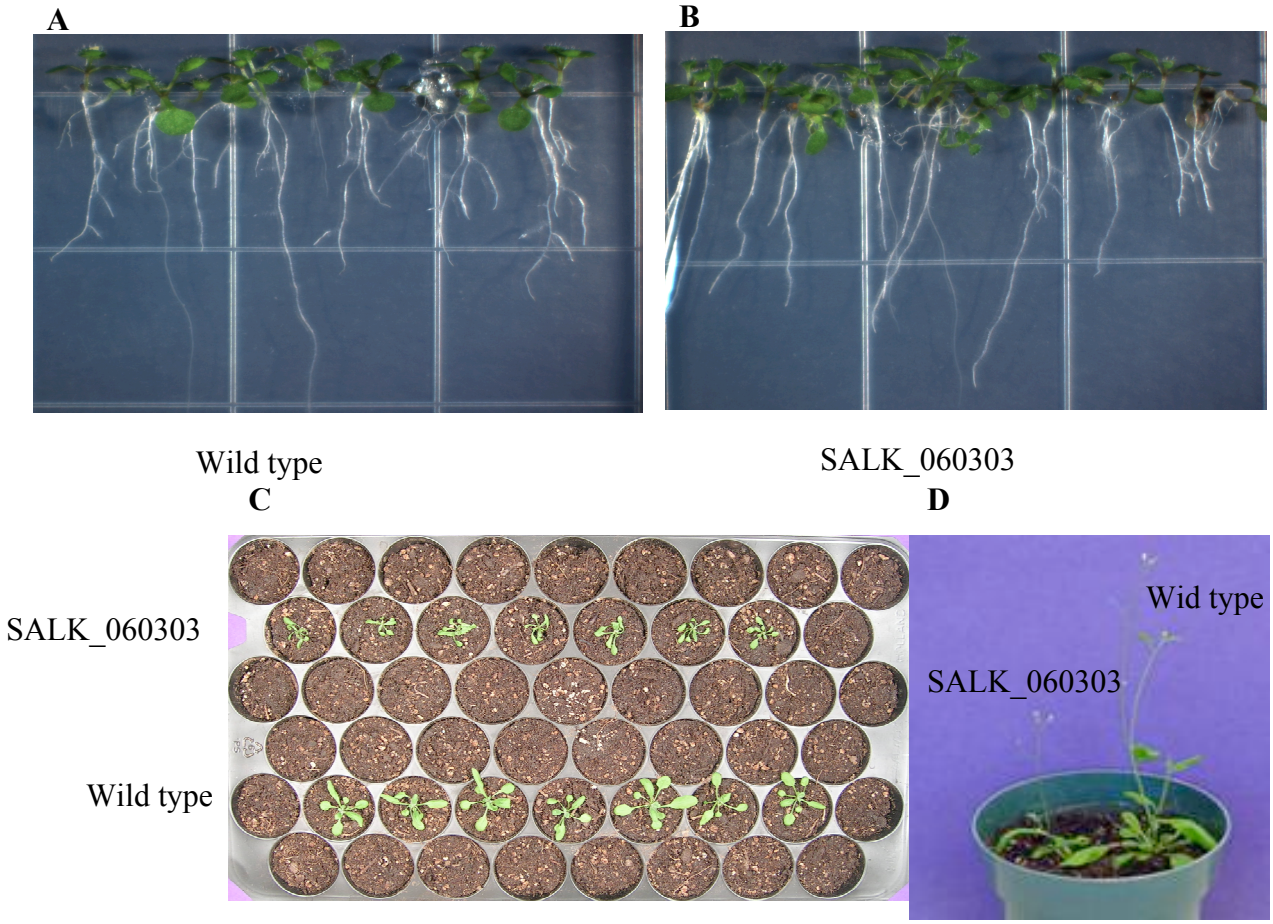
Jin and et al., Figure 2

Fig. 3. RT-PCR assay of the expression of the At5g55308 gene. RNA was extracted from sibling plants that carried either the wild type allele or the SALK_060303 allele in the homozygous state. RT-PCR was conducted using the primer pair RTF3 and RTR3, as described in the Methods. Control experiments were conducted without the reverse transcriptase reaction. For loading uniformity RT-PCR was conducted for the 18S RNA.



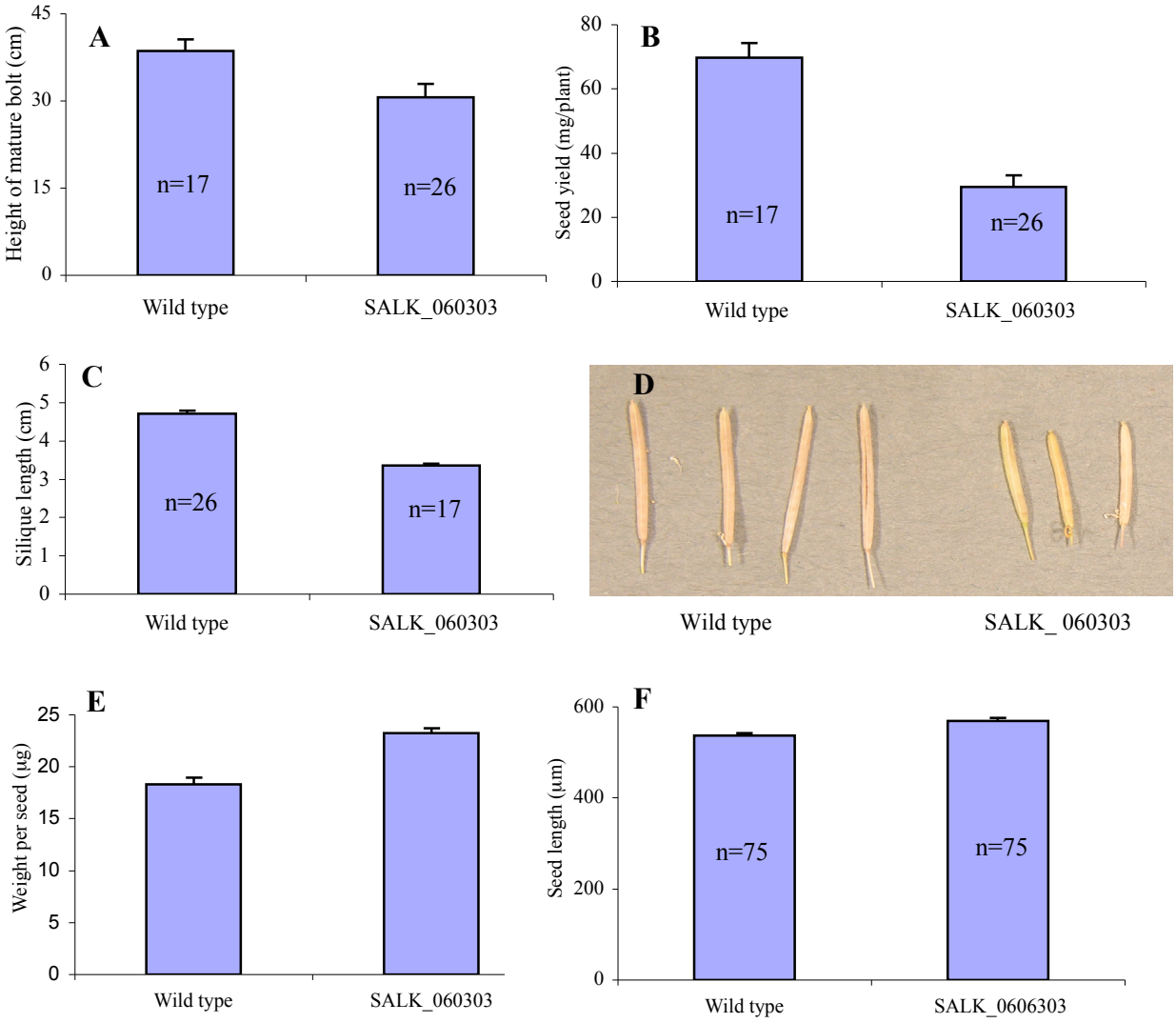
Jin and et al., Figure 3

Fig. 4. Effect of mutation in At5g55308 on growth morphology. The growth morphology of sibling plants that were homozygous for the wild type or SALK_060303 allele was compared at different stages of development. A and B. Eight days old seedlings grown on Murashige and Skoog solid media. C. Seedlings from panels A and B, were transferred to soil and observed two weeks later. D. Plants at about 7-days after flowering.



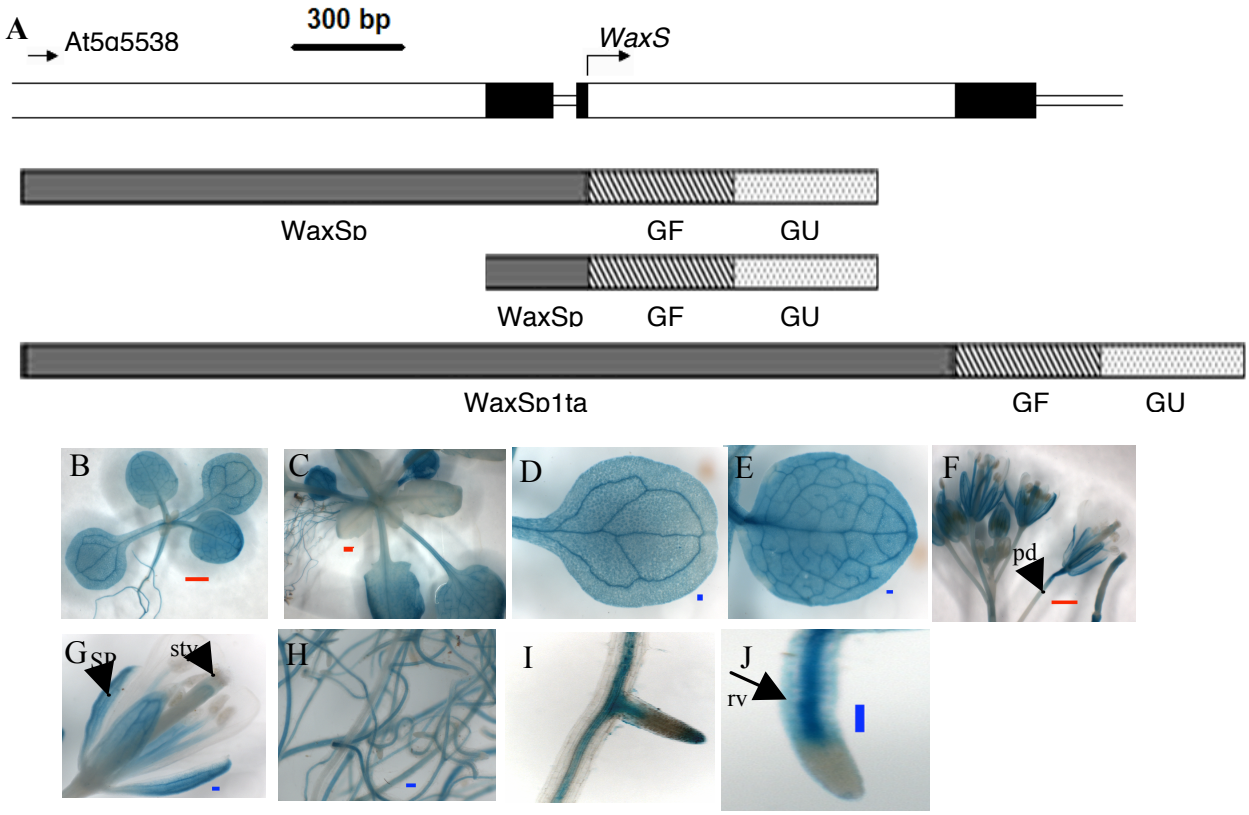
Jin and et al., Figure 4

Fig. 5. Growth phenotypes of SALK_060303 allele. Quantitative comparisons of a number of growth characteristics of sibling plants that were homozygous for either the wild type or SALK_060303 allele. Height of mature bolts (A); seed yield per plant (B); lengths of the youngest 5 siliques on a bolt at maturity (C), which are illustrated in panel D; weight per seed (E); length of mature seeds was determined (F). Data are averages of n determinations, and statistical analysis show that the differences in phenotype between mutants and wild type plants were demonstrated at a significance level of 95%.



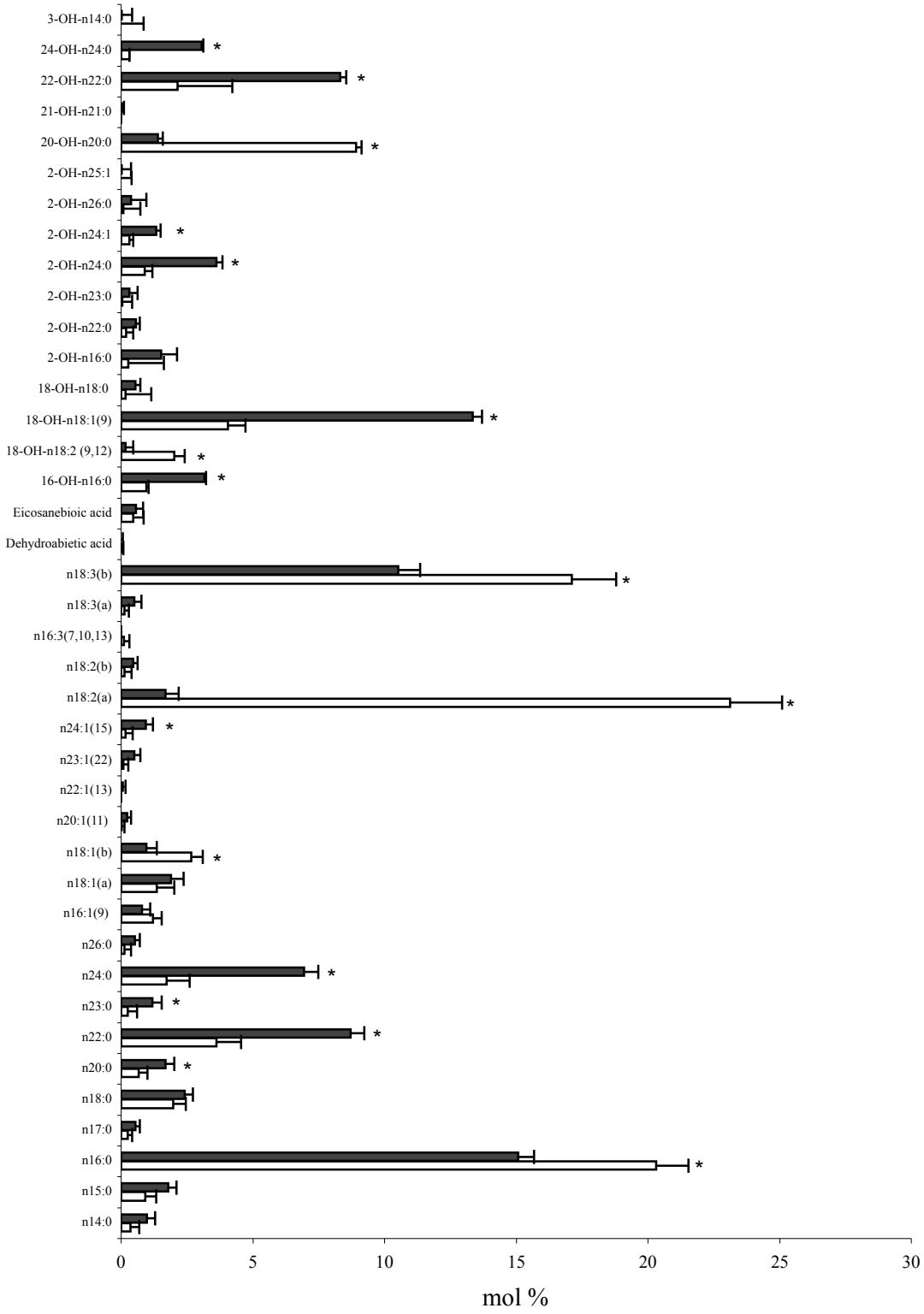
Jin and et al., Figure 5

Fig. 6. Expression patterns of At5g55380::GUS transgene. A. Three sets of transgenic lines were generated (WaxSp1, WaxSp2, and WaxSp1tar), which fused a GFP-GUS chimeric gene to different portions of the At5g55380 gene. The lengths and position of the fragments cloned into the constructs were: WaxSp1, 1551-bp upstream from position -1; WaxSp2, 280-bp upstream from position -1. WaxSp1tar, the promoter region plus the At5g55380 ORF. GUS activity assays for all three were very similar, and only data from the WaxSp2 transgenic lines are shown here. The expression of the At5g55380 gene visualized by GUS activity stains in young seedlings (B), in plants just before bolting (C), isolated cotyledon (D), isolated young leaf (E), flowers and buds (F and G), and roots (H, I, J). pd = pedicle, sp = sepal, sty = style, rv = root vasculature.



Jin and et al., Figure 6

Fig. 7. Effect of mutation in At5g55308 on fatty acid composition of roots. Fatty acids were extracted and analyzed from roots of 18-day old seedlings grown on Murashige and Skoog solid media. Plants were sibling and were homozygous for either the wild type (■) or SALK_060303 (□) allele. The asterisks indicate the fatty acids have a significant difference between the wild type and mutant.



Jin and et al, Figure 7

Table 1. Mutants

TAIR gene number	Number of mutants	Name of mutants	Position of insertion
At5g55320	3	SAlK_139311	5’ UTR
		SALK_044738	5’ UTR
		SALK_117259	Gene
At5g55330	2	SALK_052519	5’ UTR
		SALK_051439	5’ UTR
At5g55340	1	N_140746	Gene
At5g55350	2	SALK_054650	5’UTR
		SALK_013575	Gene
At5g55360	1	SALK_044276	Gene
At5g55370	1	SALK_027674	Gene
At5g55380	2	SALK_060303	Gene
		PST801	Gene
At5g51420	2	SALK_018462	Gene
		SALK_078710	Gene
At3g51970	0		
At1g34490	1	SALK_062831	Intron
At1g34500	0		
At1g34520	0		

Table 2. Primer sequences to characterize At5g55380.

Name	Purpose of primers	Primer sequence (5'-3')
LP	Genotyping PCR	AAACGACGCGGCATGATTAAA
RP	Genotyping PCR	TGGTTTGCCTCGGTTTGTTGT
LbaI	Genotyping PCR	TGGTTCACGTAGTGGGCCATCG
Ds3-2a	Genotyping PCR	CCGGATCGTATCGGTTTTCG
Ds5-2a	Genotyping PCR	TCCGTTCCGTTTTCGTTTTTTAC
RTF3	RT-PCR	GGCAAACCATCCCTGTATTC
RTR3	RT-PCR	TCCAAAGGTGTTCTTCGTCTC
GeneRace™5'primer	RACE-PCR	CGACTGGAGCACGAGGACACTGA
5u	RACE-PCR	CAAACTCTGCTGCTTCCACA
GeneRace™ RNA Oligo	RACE-PCR	CGACUGGAGCACGAGGACACUGAC AUGGACUGAAGGAGUAGAAA

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Chapter 6. General Conclusion

The goals of the research in this dissertation are to generate the fundamental technologies that can be used to improve the production and quality of vegetable-oil based biodiesel and biolubricants. Specifically, we sought to test the hypothesis that BCFAs can substitute for unsaturated fatty acids in maintaining fluidity of such oil-based products. Toward this goal, we sought to test the utility of two *Bacillus* KASIII-coding genes (*yhfB* and *yjaX*) in producing BCFAs.In conducting this research it was necessary to examine if the *E. coli* KASIII gene (*fabH*) was essential for initiating fatty acid biosynthesis and whether the *Bacillus* KASIII genes are interchangeable with that of *E. coli*.

Another part of the dissertation was to study the biochemical and physiological functions of putative mono-acyl ester synthase genes, which can catalyze the reaction between acyl-CoA and alcohol to generate mono-acyl ester in *Arabidopsis*.

BCFAs project

In term of explanation for the redundancy in genes that code for KASIII in the *Bacillus* genome, we demonstrate that while the *yjaX*-encoding KASIII is expressed in the wild type state, the expression of *yhfB*-encoding KASIII was detected only when the *yjaX* gene-function was deleted.

The relationship between the growth phenotype and fatty acid chemotype of each KASIII mutant (strains *yjaX-m* and *yhfB-m*) grown at a normal temperature (37 ^{0}C) and at a lower

temperature (16 ^{0}C) was defined by this research. At lower temperature, more anteiso BCFAs and shorter chain fatty acids accumulated. By disrupting *yjaX* gene, it was found the *yhfB* gene can generate more anteiso and shorter chain fatty acids. This finding is consistent with the growth phenotype observed at 16 °C of the wild type strain and two mutant strains.

In this research, we successfully expressed the *Bacillus yhfB* and *yjaX* genes in an *E. coli* strain that cannot synthesize unsaturated fatty acids, and demonstrated that these transgenic manipulations confer BCFA biosynthesis in a host that normally does not synthesize these molecules. BCFAs in this *E. coli* strain complemented unsaturated fatty acid auxotroph. This finding indicates that different mechanisms for altering membrane fluidity, that of unsaturation and branching of alkyl chains, are interchangeable. Specifically, BCFAs can substitute for unsaturated fatty acids to maintain membrane fluidity. We also found the BCFAs, accumulated in recombinant *E. coli* harboring *Bacillus yhfB* gene, can be incorporated into membrane lipids. The significance of these findings is that it indicates it may be possible to create viable transgenic plants, which can accumulate only saturated but branched fatty acids, which would improve the quality of biodiesel and biolubricant applications.

The experiments conducted in this study have shown that in *E. coli* the KASIII enzyme (encoded by *fabH*) is not essential for growth. This is in contrast to a previous report [1], but consistent with the findings from a systematic genome–wide deletion mutant strains [2] developed by a homologous recombination method [3].

Although the *fabH* gene is not essential for growth, it's clear from our data that there is a growth deficiency associated with this mutation. That this growth phenotype is due to the absence of the KASIII function was evidenced by the complementary effect of ectopically over-expressing the KASIII function from an inducible plasmid system. Although there is coupling between genetic manipulations of KASIII and compositional changes in the fatty acids associated with the membrane lipids. However, there isn't a direct relationship between fatty acid composition and the growth capabilities of the resultant strains.

Unfortunately, the expression of the *Bacillus yhfB* or *yjaX* genes in either a wild type or *fabH* mutant strain of *E. coli* failed to instill BCFA biosynthesis in this *E. coli* strain. This is contrast to our original assumption of potential competition between *E. coli* KASIII and *Bacillus* KASIII prevents the *Bacillus* KAS III from accumulating a large quantity of BCFAs in recombinant *E. coli*. The failure to produce BCFAs in some strains of *E. coli* suggests that the supply of branched chain acyl-CoA substrates may be important in generating BCFAs in transgenic organisms.

Mono-acyl ester synthase

In Arabidopsis, we find 12 genes that share significant sequence homology to the jojoba mono-acyl ester synthase. The studies reported herein were designed to establish a system for testing the hypothesis that these genes catalyze the synthesis of acyl esters with different substrate specificity or they are expressed at different stage of the development of the plant. The putative mono-acyl ester synthase genes At5g55380, was used to establish this system.

We characterized the structure of the At5g55380 gene by sequencing the corresponding cDNA and by performing 5'–RACE-PCR. The 3' and 5'-end of the gene was thereby established. GUS-reporter gene constructs were used to assess the organ and tissue expression pattern of the At5g55380 gene. These experiments identified that the At5g55380 gene is highly expressed in the roots of Arabidopsis. The mutation in the At5g55380 gene carried by the SALK_060303 mutation altered a number of growth characteristics of the plant, including its growth stature, root morphology, and seed yields. Biochemical characterization of the fatty acids in roots of this mutant suggests that the mutation in the At5g5380 gene might alter composition of suberin. The alteration is consistent with the fact that the At5g55380 gene is highly expressed in roots, and that root morphology is affected by this gene mutation.

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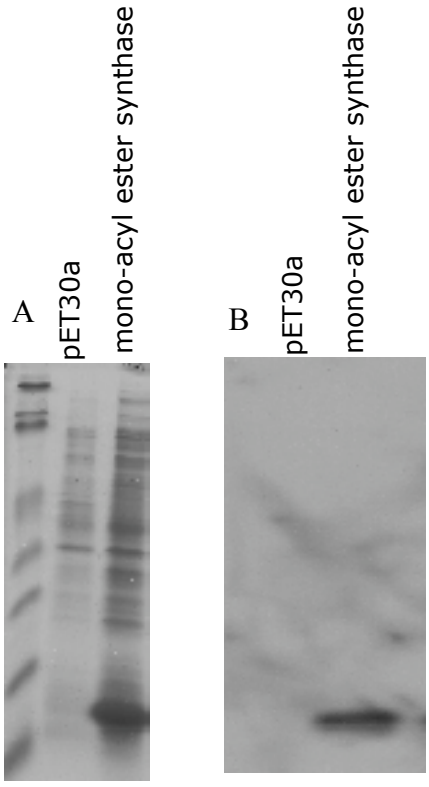
Appendix

Expression of At5g55380 in *E. coli* to make antibody. To easily detect the expression of At5g33380 in *Arabidopsis*, the antibody directed against the At5g55380 protein was made. The PCR fragment amplified from cDNA (, which is derived from At5g55380, was cloned into the pET30a (Table 1). The recombinant pET30a vector was then transformed into *E. coli* BL21 (DE3). Intensive band in the Coomassie blue stain indicated the expression of the At5g55380 protein taged with his and s protein, but its size,19- kDa, was smaller than 27.8- kDa as expected (Fig. 1A). Since we had observed intensive band with the expected size in coomasie blue stain gel before, we think that smaller-size intensive band was resulted from degraded At5g55380 protein. The degraded At5g55380 protein was purified by affinity chromatography and used to generate antibody in rabbit. Fig.1B showed the reaction of the resulting antibodies against the recombinant at5g55380 gene carried by pET30a.

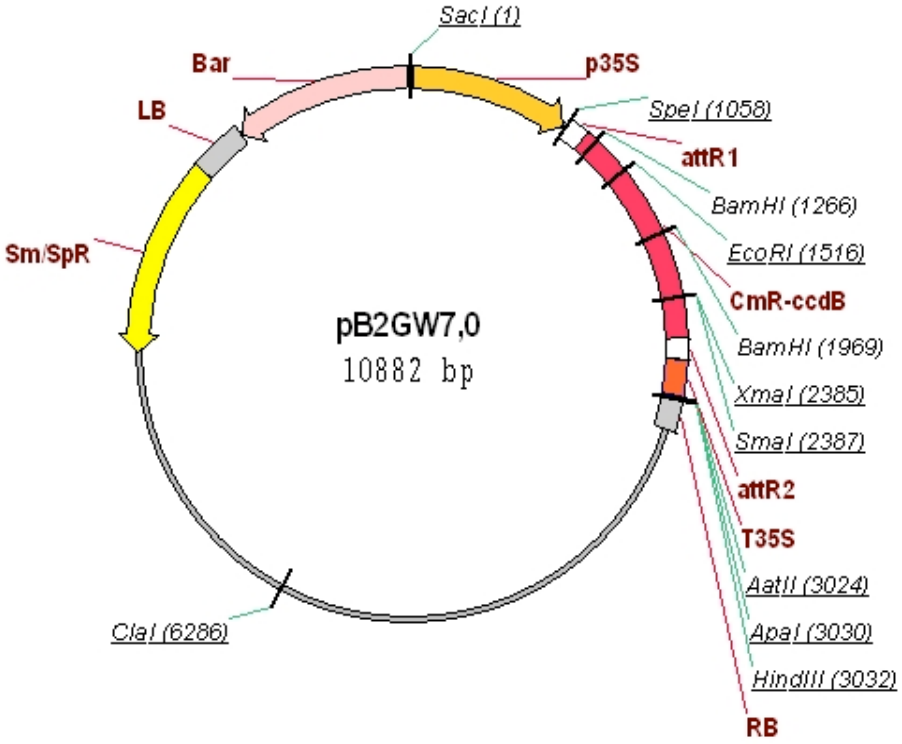
Cloning and agrobacterium-mediated transformation. At5g55380 was amplified using genomic DNA isolated from wild type Columbia ecotype with the pair primers 55380PenF and 55380PenR (Table 1). The PCR products were cloned into pENTR^{TM}/D-TOPO pENTR^{TM} using Direstional TOPO Cloning Kits (Invitrogen, Carlsbad, CA), giving rising At5g55380 entry clone. The expression construct pBWS8 was made by performing an LR recombination reaction between the At5g55380 entry clone and destination vector pB2GW7,0.

Fig.1. Expression of At5g55380 from cDNA to make antisera. SDS-PAGE (A) and western blot analysis (B) of *E. coli* strain BL21 (DE3) transformed with non-recombinant pET30a vector, and recombinant vector containing At5g55380.



Jin and et al, Figure 1

Fig. 2. Expression construct used in Agerobacterium – mediated plant transformation. At5g55380 entry clone was inserted into expression vector of pB2GW7,0 between AttR1 and AttR2 by LR recombination.



Jin and etc., Figure 2

Table 1. Primer sequences.

Name	Purpose of primers	Primer sequence (5'-3')
wax-syn-f	Amplify PCR from cDNA	CCATGGAAGAAAAGTTTAGAAACTT
wax-syn-b	Amplify PCR from cDNA	TACGCCAAGCTCGAAATTAA
55380PenF	Cloning to pentry	CACCCTGGCGGTTGGATTAATGAC
55380PenR	Cloning to pentry	TTCGTATGACAAATCCAGACAAA