THE UTILIZATION OF ALGICIDAL BACTERIA FOR IMPROVED LIPID EXTRACTIONS AND INSIGHTS INTO NEUTRAL LIPID PRODUCTION IN A WAX ESTER ACCUMULATING BACTERIUM

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Abstract

As the limited quantities of fossil fuels on this planet diminishes, along with the increase in our society's demand, the identification and production of next generation fuels and alternatives to petroleum based chemicals become an increasingly important field, from both an economical and environmental stand point. Microorganisms may hold the key to producing these compounds as many algae and bacteria have been found to produce high quantities of lipids and other bioproducts similar to those obtained from fossil fuels. The first part of this thesis evaluates the potential application of two algae degrading bacteria to aid in lipid extractions from the microalgae *Neochloris oleoabundans* and *Dunaliella tertiolecta*. The second and third part of this thesis extends the knowledge of the wax ester biosynthetic pathway in *Marinobacter aquaeolei* VT8 through the analysis of transcriptional levels within wild-type cells and gene deletion of enzymes within this biosynthetic pathway.

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Chapter 1

Potential Application of Algicidal Bacteria for Improved Lipid Recovery with Specific Algae

Summary

The isolation and utilization of specific strains of natural algicidal bacteria isolated from shallow wetland sediments were evaluated against several strains of algae with potential immediate or future commercial value. Two strains of bacteria, *Pseudomonas mendocina* AD6 and *Aeromonas veronii* AD9 were identified and demonstrated to have algicidal activity against the microalgae *Neochloris oleoabundans* and *Dunaliella tertiolecta*. These bacteria were further evaluated for the potential to improve lipid extraction using a mild solvent extraction approach. *A. veronii* AD9 showed nearly a 20 fold increase in lipid extraction with *D. tertiolecta*, while both bacteria showed a 10 fold improvement in lipid extraction with *N. oleoabundans*.

Introduction

The cycling of organic compounds in nature involves both processes that synthesize complex biological compounds and processes that degrade these compounds back to simple molecules. In this manner, photosynthetic organisms utilize carbon dioxide, water and sunlight to produce carbohydrates and more complex biomolecules, which are later used by heterotrophic organisms for their own growth, thus comprising the various components of the carbon cycle. Important in this cycle are organisms involved in the decomposition of decaying matter in aquatic systems, including various microbes that might assist in breaking down larger organisms, including microalgae.

Previous reports of algicidal bacteria have focused on bacteria acting against bloom forming algae or cyanobacteria that are known to produce various toxins that can affect human and animal health (*1-10*). While these reports have identified a range of different bacteria that can be detrimental to the growth of these algae that are undesirable, less information is available about organisms that degrade algae cells that might have value as sources of specific high value compounds and biofuels. Many algae are viewed as an ideal future feedstock for biological oils, though the costs associated with extraction and conversion of these fuels are often a primary barrier to economic feasibility of algal fuels (*11-14*).

The dependence of advancing societies on fossil fuels, the high cost associated with these fuels and the eventual depletion of sources of them has resulted in a strong and recently renewed interest in alternative biofuels. Several next generation biofuels and biomass feedstocks, including algae, show potential benefits versus current seed oil based biodiesel and corn derived ethanol (*11-12, 15*). Further, many algae are already utilized as sources of unique bioproducts important to nutrition, pharmaceuticals and as specialty chemicals (*12, 16-17*).

In this report, we isolated and investigated the potential of two algicidal bacteria, isolated from shallow wetland sediments in Minnesota, to be utilized as a potential means of improving lipid extraction under mild conditions, with a common solvent typically utilized to extract seed oils in agriculture. The potential improvement in quantities of

lipid extracted and the affect these strains had on the cell walls of several strains of algae important in aquaculture or as a source for unique bioproducts including biofuels is presented.

Materials and Methods

Algal and bacterial strains and growth conditions. All algal strains were obtained from the UTEX culture collection (Austin, Texas). *Dunaliella tertiolecta* UTEX LB 999 was grown in artificial seawater media (1 mL trace element solution, 18 g NaCl, 500 mg KCl, 200 mg $MgSO_4 \cdot 7H_2O$, 20 mg $CaCl_2 \cdot 2H_2O$, 200 mg K_2HPO_4, 50 mg $Na_2SiO_3 \cdot 9H_2O$, 600 mg $NaNO_3$, 300 mg Na_2SO_4, 10 mg ferric ammonium citrate, 50 mg silt, and 100 µL vitamin stock solution, all per liter and adjusted to pH 7.8). *Neochloris oleoabundans* UTEX 1185, *Scenedesmus dimorphus* UTEX 417, and *Chlorella sorokiniana* UTEX 1602 where all grown in a freshwater media based on Bristol media (1 mL trace element solution, 300 mg K_2HPO_4, 80 mg $MgSO_4 \cdot 7H_2O$, 20 mg $CaCl_2 \cdot 2H_2O$, 200 mg Na_2SO_4, 25 mg NaCl, 15 mg ferric ammonium citrate, 600 mg $NaNO_3$, all per liter and adjusted to pH 7.6). The stock vitamin solution contained 5 mg/mL thiamine HCl, 0. 1 mg/mL biotin, and 0.1 mg/mL cyanocobalamin. The trace element stock solution contained 1 g boric acid, 1 g sodium ethylenediaminetetraacetic acid (EDTA), 200 mg $MnCl_2 \cdot 4H_2O$, 20 mg $ZnCl_2$, 15 mg $CuCl_2 \cdot 2H_2O$, 15 mg $Na_2MoO_4 \cdot 2H_2O$, 15 mg $CoCl_2 \cdot 6H_2O$, and 10 mg KBr, all per liter. Bacteria were grown on Miller Lysogeny Broth (LB) at 25°C under dark conditions. All reagents were obtained from either Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA) unless otherwise specified.

Isolation of algicidal bacteria. Algal strains were grown using the media described above in 1.4 liter tubes (~ 5 cm x 75 cm) with a 1 mm glass capillary tube to provide constant aeration at a flow rate of 0.3 L air per minute per L of culture media. Air was provided via mass flow controllers providing compressed air combined with 1% CO_2. Cultures were grown for several days following exhaustion of nitrate (limiting reagent) as measured by nitrate indicator strips (Hach Chemical, Colorado). The culture was transferred to a standard media bottle and inoculated with 10 mL of environmental pond sediment samples collected from select sites in Minnesota. Cultures were placed in the dark and mixed daily for several weeks until the algae showed visual signs of stress or deterioration by microscopy. Aliquots were then transferred to fresh culture of the same algal species several additional times to enrich the culture for algal degrading bacteria.

Under this regime, the algal cells provide the only source of carbon or nitrogen to any bacteria growing in the culture. Fresh algal cells were then centrifuged and resuspended at 10X concentration in sterile agar, and bacteria from the enriched cultures were plated on the agar. Plates were visually inspected for zones of clearing surrounding isolated colonies. These colonies were then streaked several times to LB media plates to purify and prepare frozen stocks. Genomic DNA was isolated using the ZR Fungal/Bacterial DNA Kit (Zymo Research, Irvine, CA). Strains were identified by amplifying the 16S rDNA region of the genome using universal primers TPU1 (5' AGAGT TTGAT CMTGG CTCAG 3') and RTU8 (5' AAGGA GGTGA TCCAN CCRCA 3') and sequencing the isolated PCR fragments with RTU8.

Determination of algicidal properties and improvement in lipid extraction. Algal stock cultures were grown in 1.4 liter tubes as described above. Cultures were then divided into 10 mL aliquots and inoculated with purified isolates of potential algicidal bacteria. These co-cultures were incubated at room temperature in the dark and mixed once a day until the degradation of the algae cells were observed as a change in culture color and cell morphology compared to a control culture under the same conditions but devoid of any added bacteria. Free lipids were extracted from the aqueous phase by adding 2 mL hexane to the 10 mL culture and vortexing the solution three times at five second intervals. The hexane phase was then removed and 50 μL of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) was added to 1 mL of the hexane extract to derivatize any free fatty acids. Samples were analyzed by gas chromatography using conditions described previously (*18*).

Scanning electron microscopy. 1 mL of solution was taken from freshly mixed 10 mL algae and bacteria co-cultures. The solution was centrifuged to pellet the cells which were then placed in 2% gluteraldehyde and 0.1 M sodium cacodylate buffer for 2 hours, rinsed in the sodium cacodylate buffer, then placed in 1% osmium tetroxide and 0.1 M sodium cacodylate buffer for 2 hours. Specimens were rinsed in ultrapure water and dehydrated in an ethanol series. Once in 100% ethanol, they were put through two changes of hexamethyldisilazane (HMDS) for 5 min each. Drops of the suspension were

placed on individual round glass coverslips mounted on aluminum stubs and allowed to dry, sputter-coated with gold-palladium, and observed in a scanning electron microscope (S3500N; Hitachi High Technologies America, Inc.; Schaumberg, Illinois) at an accelerating voltage of 10 kV at the University of Minnesota Imaging Center.

Real-time video imaging of bacteria and algae cells. Algal and bacterial cells were grown together in 10 mL co-cultures as described above. After 4 days, several microliters of culture was placed on a microscope slide and viewed using bright field microscopy with an ix70 microscope (Olympus, Center Valley, Pennsylvania) and video was recorded with a SPOT Insight Camera (Diagnostic Instruments, Sterling Heights, Michigan) at the University of Minnesota Imaging Center.

Results and Discussion

Algae are viewed as a potential future feedstock for biofuels based on rapid growth and the production and accumulation of lipids (*11-12*). Many algae are known to accumulate either neutral lipids such as triacylglycerides as a form of energy storage under nutrient depleted conditions or polar lipids that compose the extensive network of membranes in photosynthetic cells (*11-12, 19-21*). Either class of lipid could serve as a feedstock of reduced carbon from which to produce hydrocarbon fuels or other valuable bioproducts, though the polar lipids would be expected to be more difficult to extract. One key hurdle to lipid isolation from algae is the expense involved in a range of extraction approaches (*13-14*). As part of this work, we have investigated the potential of several natural strains of bacteria to assist in the degradation of the algal cell structure and improve the accessibility of the lipids for extraction. These studies, with environmental isolates of specific bacteria, show some promise for developing a natural process to improve the extraction of certain lipids.

Isolation of algicidal bacteria. Several model strains of algae that have specific commercial value or have shown promise for lipid production were selected for these studies, including *Dunaliella tertiolecta*, *Neochloris oleoabundans*, *Scenedesmus dimorphus* and *Chlorella sorokiniana* (*16-17, 19-22*), because of this we chose to use these strains to screen for bacteria which are capable of degrading these algae. Each of these strains were obtained from the UTEX collection of algae and have been maintained in our laboratory for several years as unialgal cultures with no visible signs of contamination and are known to produce high yields of neutral lipids under conditions of nutrient stress.

Bacteria from environmental samples were enriched using an approach that provided algal cells as the sole carbon and nitrogen source as described in the methods. Isolated bacteria were screened for algalytic activity versus a control without any added bacteria. Of the four algal strains screened as part of this work, only *N. oleoabundans* and *D. tertiolecta* were sufficiently prone to degradation using this approach with the isolated strains, while *S. dimorphus* and *C. sorokiniani* showed no visible signs of degradation

within 14 days of inoculation of bacterial communities that resulted in deterioration with the other two strains. The bacterial strains that showed the highest degree or rate of cell deterioration in specific algae included the strains designated AD6 and AD9. These two specific strains of algicidal bacteria resulted in the most significant degradation with *N. oleoabundans* and *D. tertiolecta* and were selected for further study.

Identification of algicidal bacteria. Pure isolates of bacteria obtained from the enrichment process were identified by similarity searches using 16S rDNA sequencing. The two primary strains selected for further study were identified as *Pseudomonas mendocina* AD6 and *Aeromonas veronii* AD9. Additional strains identified included several species of *Citrobacter*, *Pseudomonas putida*, *Stenotrophomonas rhizophila*, *Sphingomonas sp.* and *Azospirillum picis*. The majority of these strains were greater than 99.5% identical over a range of approximately 900 bases. Isolated strains were utilized to screen for their algal cell degradation potential with fresh algal cell cultures. It is interesting that out of approximately 20 bacteria that were isolated or identified, one of the top strains identified in this study was a strain of *P. mendocina*. Another strain, *P. mendocina* DC10 isolated from Lake Dianchi in Yunnan Province, China, has also been implicated in other reports as an algicidal bacterium acting on the cyanobacterium *Aphanizomenon flos-aquae* (*23*).

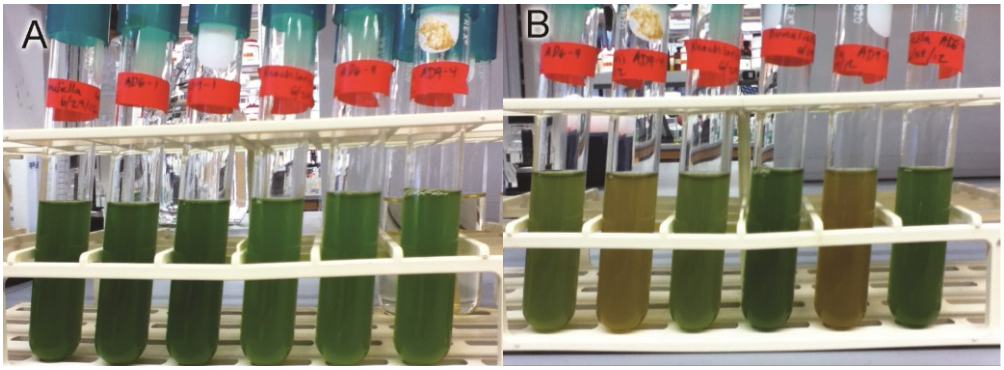

Figure 1. Algicidal Bacteria with Select Algae Strains. The two images above illustrate the visible deterioration of *N. oleoabundans* and *D. tertiolecta* cells exposed to *P. mendocina* AD6 and *A. veronii* AD9. Image A shows the strains immediately after inoculation with the specific bacteria strains. Image B shows the same samples at the time of lipid extraction. Samples in image B are as follows, from left to right, *N. oleoabundans* and *P. mendocina* AD6, *N. oleoabundans* and *A. veronii* AD9, *N. oleoabundans* control, *D. tertiolecta* control, *D. tertiolecta* and *A. veronii* AD9, *D. tertiolecta* and *P. mendocina* AD6.

Screening of algicidal potential. Algal cells showed signs of distress (change in culture color and cell morphology) in cultures containing the selected algicidal bacteria after a few days of exposure. Figure 1 shows an example of the differences in culture appearance for one such experiment using algae cells and specific isolated bacteria. Incubation with *A. veronii* AD9 resulted in rapid deterioration of algal cells for both *N. oleoabundans* and *D. tertiolecta*, while *P. mendocina* AD6 took several additional days. The *D. tertiolecta* co-culture resulted in a more dramatic color change in comparison to the *N. oleoabundans* co-culture. When viewed using bright field microscopy, cells of both algae were withered and deflated with *D. tertiolecta* cells displaying a loss in motility (*N. oleoabundans* is not visibly motile using bright field microscopy). The potential to improve lipid extraction in the treated co-cultures was evaluated by exposing the cultures to a simple solvent extraction protocol, and the lipid recovery was compared to algal cells with no added bacteria. Figure 2 shows the relative quantity of lipids (based

on peak areas for all derivatized fatty acids recovered) obtained from algal cells subjected to each of the primary bacteria versus a control incubated under the same conditions but without any added bacteria. *P. mendocina* AD6 showed only a minor increase in quantities of lipids that were extracted from *D. tertiolecta* (2 fold increase), while *A. veronii* AD9 resulted in the greatest increase in extractable lipids with *D. tertiolecta* (nearly 20 fold). For *N. oleoabundans*, both *P. mendocina* AD6 and *A. veronii* AD9 resulted in a similar increase (approximately 10 fold) in extractable lipids versus the control samples. Lipid profiles (retention times of specific derivatized fatty acids) were similar for the specific algae strain regardless of whether the sample included a specific bacterium or was simply the algal control sample. This indicates that the lipids are primarily derived from the algae, and not related to the specific bacterium in either case.

The comparison of algae culture color verses lipid extractions was investigated by performing lipid extractions with hexane, as described in the methods, on algae samples with and without the algicidal bacteria every day until a change in co-culture color was visible. This time course revealed extraction of lipids only when the color of the co-culture changed, correlating the release of lipids to the culture color.

The broader studies with the two bacterial strains selected revealed a number of interesting features. *P. mendocina* AD6 and *A. veronii* AD9, showed some degree of selectivity and specificity for the algae targeted, as indicated by the concentrations of fatty acids recovered from co-cultures following treatment versus a control (Figure 2). It is possible that lower yields of lipid from *D. tertiolecta* treated with *P. mendocina* AD6 are the result of *P. mendocina* AD6 utilizing the fatty acids as a substrate for growth. Additionally, *Scenedesmus dimorphus* and *Chlorella sorokiniana* seemed far less susceptible to deterioration by these two strains, again indicating a certain degree of specificity for these bacteria toward specific algae. *D. tertiolecta* was especially susceptible to treatment by *A. veronii* AD9, resulting in the highest levels of lipid extracted and a nearly 20 fold improvement in extractable lipids versus the control devoid of any added bacteria. The liquid-liquid partitioning extraction selected here using hexane as a non-polar solvent is a mild technique, and was chosen as it would be representative

of extraction processes that are less energy intensive than using harsh solvents or high energy sonication.

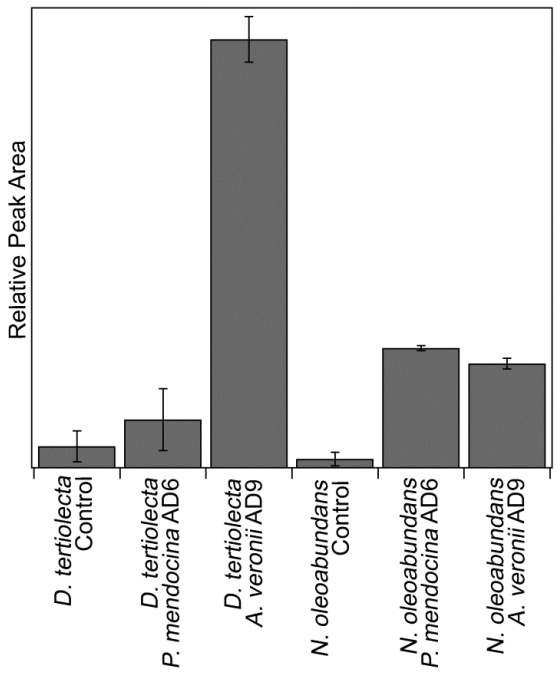

Figure 2. Lipid Extraction of Algae Subjected to Algicidal Bacteria. Shown above are results from lipid extractions of *D. tertiolecta* and *N. oleoabundans* cells exposed to the bacterial strains *P. mendocina* AD6 and *A. veronii* AD9. Lipid quantities are based on relative peak areas of all derivatized fatty acids found in each of the samples. The results presented are based on three separate experiments ($n \geq 3$).

Evaluation of algal cells by electron microscopy. Changes in cell morphology for each algae strain were used as an additional measure of algalytic activity following treatment with specific algicidal bacteria. Scanning electron micrographs of the algae and bacteria co-cultures show higher incidences of specific changes in cell morphology of the two algal strains (Figure 3). *D. tertiolecta* treated with *P. mendocina* AD6 and *A. veronii* AD9 showed similar changes, with the algae cells appearing deteriorated and fragmented. The changes in cell morphology were different for *N. oleoabundans*, as *N. oleoabundans*

cells exposed to *A. veronii* AD9 remained intact but deflated and withered. *D. tertiolecta* and *N. oleoabundans* grow under very different conditions (salt water versus freshwater respectively) and have many other features differentiating them from one another. Certain *Dunaliella* species lack a rigid cell wall, utilizing an elastic plasma membrane that allows the cells to rapidly change in volume in response to changes in osmolarity (*24*). This potential lack of a rigid cell wall may make *D. tertiolecta* more susceptible to biological attack by algicidal bacteria in a different manner than other algae strains.

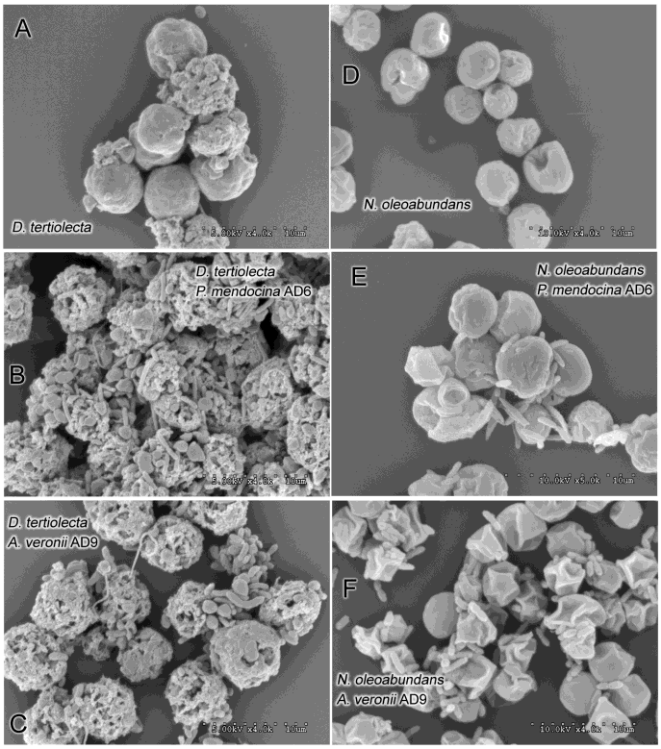

Figure 3. Electron Microscopy of Algae Subjected to Algicidal Bacteria. Shown above are several electron microscopy images of algae control cells and algae inoculated with specific bacterial strains derived from the experiments shown in Figure 1. Images in the left column are of *D. tertiolecta* and those in the right column are of *N. oleoabundans,* with specific sample types (control or with bacteria) denoted on each image.

Real-time video imaging of bacteria and algae cells. The behavior of bacteria and algae were evaluated in real time by visualizing the interaction of *D. tertiolecta* and *A. veronii* AD9 cells. Using bright field microscopy, video was obtained depicting bacterial cells approaching algal cells at high speed, colliding with the algal cells, and then swimming away. There were also a number of events in which the bacteria would collide into the algal cells, back up, then collide again, repeating this a number of times before swimming away. The durations of the interactions between algal cells and bacteria seemed longer than would be expected if these collisions were a result of simple random motions. Previous reports of a bacterium capable of lysing blue-green algae reported a similar feature where close contact between the polar tip of the bacterium and the algae were necessary for the lysis (*10*). This may indicate that the bacteria are using some sort of chemotaxis to find cells and deliver specific algicidal agents, such as a protease or lipase, though this observation is strictly speculative. A video of several interactions is provided as a supplementary file.

Bacterial pathogenicity. The two bacteria, *P. mendocina* AD6 and *A. veronii* AD9, based on this study with specific strains of algae, should be classified as potential plant pathogens. The further potential as pathogens in other organisms was not a focus of this study and has not been thoroughly evaluated, though many strains of *Aeromonas* are generally associated with human disease. *Aeromonas* are ubiquitous in both brackish and fresh water (where all these strains were isolated). Common diseases associated with *Aeromonas* include diarrhea and wound infections (*25*), while specific strains such as *Aeromonas hydrophilia* have been implicated in more serious diseases such as necrotizing fasciitis (*26*), or flesh-eating bacteria syndrome, generally linked to wound infections. *Pseudomonas mendocina* is also a common environmental strain that can cause opportunistic nosocomial infections (*27*). Thus, utilization of either of these strains for large scale application may not be suitable. However, as both of these strains are ubiquitous in nature, it should also be pointed out that this degradation of cells might also be important in the biodegradation of additional materials in the environments where these are found (these strains were isolated from a shallow marsh sediment in

Minnesota). Indeed, *P. mendocina* strains are also highly relevant in the study of biological methods of toluene degradation and in many other biotechnology applications (*28*).

Summary of findings and future works. Future efforts may include genome sequencing and the development of methods to characterize algicidal activity in strict quantitative terms so that the nature of algicidal attack by the strains might be better characterized and tracked. Initial screens of extracellular enzymes unique to these strains revealed several protein bands specific to either strain that could be investigated once a genome sequence were available to utilize techniques such as in gel trypsin digestion and MALDI analysis for identification of potential enzymes. These techniques together with a genome sequence would allow us to clone and characterize specific genes for potential direct applications.

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Chapter 2

Neutral Lipid Production in a Wax Ester Accumulating Bacterium: Insights From Transcriptional Analysis During Lipid Accumulation in Batch Culture

Summary

Organisms which degrade crude oil are important in natural bioremediation of oil spills caused by human efforts to tap valuable resources. The bacterium *Marinobacter aquaeolei* VT8 is one such organism isolated from an oil well, which also produces a long-chain hydrocarbon, the wax ester. Natural neutral lipids such as wax esters are of interest in a variety of commercial applications including cosmetics, lubricants and biofuels. Efforts to replace current fossil fuel resources with potential renewable feedstocks will require a deeper understanding of the metabolic pathways involved in lipid accumulating species. This study utilized reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) to characterize the changes in cellular transcriptional levels of genes involved in a neutral lipid accumulating pathway from a wax ester producing bacterium. Changes in transcription levels of these genes were compared to key genes in fatty acid biosynthesis and fatty acid metabolism, along with several additional genes involved in metabolism or potential housekeeping genes. Results reveal that key regulatory mechanisms are likely associated with upstream processes that produce the building blocks of the final wax ester product, while many of the genes involved in the final wax ester production pathway show only slight elevation in transcriptional levels. The results from the study are discussed in terms of potential regulatory routes and the current model pathway for wax ester production.

Introduction

The global cycle of oil is of interest from both the standpoint of energy and the environment, as efforts by humankind to obtain this valuable resource can result in substantial releases of crude oil through incidents such as the *Deepwater Horizon* oil spill of 2010 in the Gulf of Mexico. While the fate of the released oil in these larger environmental disasters is of immense interest, it is noted that crude oil from natural deposits is routinely released into aqueous environments such as the oceans by natural processes where geological reserves meet surface waters, albeit in far smaller quantities than are seen in these larger disasters. As such, natural populations of organisms such as marine bacteria have evolved to utilize these supplies rich in reduced carbon for use as a biological energy source.

A primary focus related to oil degrading marine bacteria is the oxidation of these oils for energy requirements and potential assimilation of these oils into metabolic building blocks for growth of the living cell. Interestingly, for certain marine species found to degrade oils, these bacteria are also capable of producing natural lipids with economic value, even when grown on simple organic acids or carbohydrates. We have selected the marine bacterium *Marinobacter aquaeolei* VT8, which was isolated from an oil well off the coast of Vietnam (29), as a model species to study metabolic processes in an oil metabolizing and lipid producing bacteria. This bacterium has been grown on long chain hydrocarbons as a sole carbon source, and can produce a natural hydrocarbon, the wax ester, when grown on simple citric acid cycle intermediates such as succinate or citrate as the sole carbon source (18, 29-31).

The purpose of these studies was to develop an understanding of the transcription of genes encoding key enzymes involved in the biosynthesis of wax esters in *M. aquaeolei* VT8 during the stages of wax ester accumulation, and contrasting those to transcription of these genes prior to and following the lipid accumulation stage to understand how the cell mediates transcription of genes for putative wax ester synthesizing enzymes during this process. While a great deal has been elucidated in recent years related to this natural wax ester pathway and key enzymes that may be involved in wax synthesis (Figure 4), a

thorough analysis of the gene transcription during this process is still lacking, and the efforts undertaken here should lay down a framework to develop further studies in the future, while also providing some valuable insights into the changes in transcription for genes coding key enzymes during the lipid accumulation process.

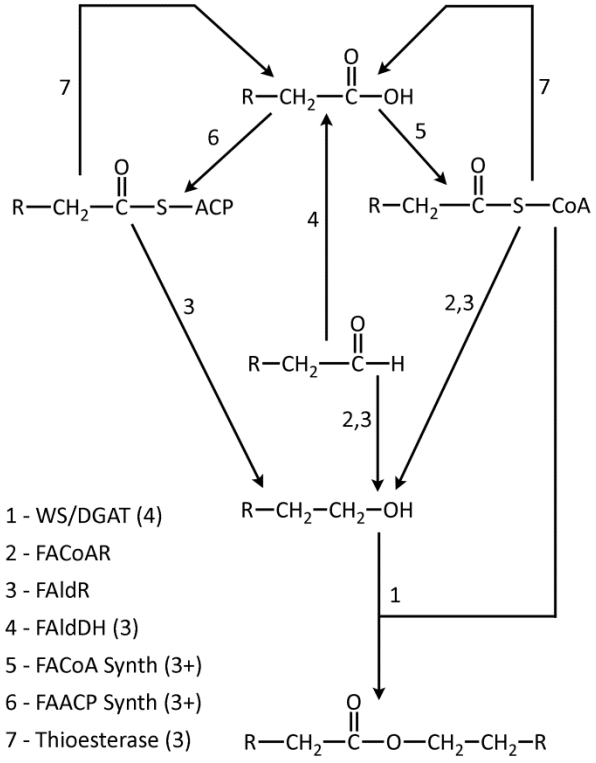

Figure 4. Putative Wax Ester Biosynthetic Pathway. Shown are the proteins that comprise the putative pathway for wax ester synthesis in lipid accumulating bacteria. Various proteins of the pathway are numbered, including the wax synthase (1), fatty acyl-CoA reductase (2), fatty aldehyde reductase (3), fatty aldehyde dehydrogenase (4), fatty acyl-CoA synthetase (5), fatty acyl-ACP synthetase (6) and thioesterase (7). Specific genes that encode these proteins are labeled in Table 1. Values shown following specific enzymes indicate the number of known or putative homologs found in *M. aquaeolei* VT8.

Materials and Methods

Marinobacter growth experiments. *Marinobacter aquaeolei* VT8 was first isolated as a single colony on a Miller Lysogeny Broth (LB) plate. Growth experiments were performed in a nitrogen limited defined media containing the following per liter; 50 g NaCl, 7 grams sodium citrate, 5 g $MgSO_4 \cdot 7H_2O$, 500 mg K_2HPO_4, 200 mg $CaCl_2 \cdot 2H_2O$, 15 mg $FeSO_4 \cdot 7H_2O$ and 640 mg $NaNO_3$, adjusted to pH 7.3 with NaOH and HCl. A loop full of cells (~50 μL total volume) were scraped from an LB plate containing a fresh lawn of cells, and were transferred to a Celstir flask containing 5 L of the nitrogen limited media and 100 μL of polypropylene glycol to minimize foaming during the growth. Aeration was provided by a custom aeration bar with three pinholes, and filtered air (0.2 μm) was provided by a simple aquarium pump. This represented the initial time of the growth experiment, and samples were taken at various time points based on nitrate consumption and cell density. At each time point, a series of samples were drawn, centrifuged and flash frozen for RNA isolation and a separate sample was taken for isolation of cells for quantification of the wax ester fractions. The pH of the culture was adjusted by adding HCl following sampling to maintain the pH below 7.8. Wax esters at each time point were analyzed and quantified versus an external standard as described previously (18), and the lipid quantification and dry cell mass obtained from each sampling period were used to categorize the samples based on three different phases of growth; exponential growth with low wax esters, wax ester production and accumulation stage and wax ester catabolism stage.

RNA isolation and RT-qPCR analysis. RNA was isolated by resuspending frozen cells in 1 mL of TRIzol reagent (Invitrogen, Grand Island, NY), then samples were vortexed several minutes until fully dissolved. Following this, 200 μL of chloroform was added, vortexed, and then centrifuged at 12,000 g for 2 minutes. The upper phase was removed, and further purified using the Direct-zol RNA miniprep kit (Zymo Research, Irvine, CA). RNA was eluted and then treated following the manufacturer directions using the RNase-free DNAse Kit (Qiagen, Hilden, Germany) in a total volume of 100 μL for 10 min at room temp, and then suspended in 300 μL of TRIzol and again isolated

using the Direct-zol miniprep kit. Isolated RNA quantity was measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA), and then 1 μg of total RNA was immediately converted to cDNA using the Improm-II reverse transcriptase kit and random primers (Promega, Madison, WI). Once completed, cDNA was frozen and stored at -20 ˚C. Samples for qPCR were prepared using the SYBR Green Master Mix (Roche, Basel, Switzerland) in a total volume of 400 μL containing 100 ng of cDNA. Samples were prepared in 96 well plates with the addition of specific primer pairs and were analyzed following a standard qPCR protocol on a LightCycler 480 II (Roche, Basel, Switzerland).

Genes were selected in this study based on the desire to better understand the putative pathway of enzymes involved in wax ester biosynthesis in *M. aquaeolei* VT8 (Figure 4). This pathway has been described by others and ourselves (18, 32-36), but details related to the transcription of these genes in *M. aquaeolei* VT8 have not been presented as a complete data set. Table 1 shows the genes selected, which include all of the genes believed to be essential to produce wax esters from the fatty acyl-CoA pool, as has been proposed by others (31, 36). Since the precursors for fatty acyl-CoAs in this study would have to be derived from fatty acid synthesis (as no extraneous lipids were provided in the culture media), we also selected several genes from both fatty acid biosynthesis and fatty acid metabolism pathways, using the KEGG database as a primary template to identify specific genes in *M. aquaeolei* VT8. In addition, several genes were selected from central metabolism that would be expected to participate in the citric acid cycle or additional housekeeping genes, and several genes were selected which we thought would be transcribed at constant levels in the cell, including the gene for recombinase A, which was utilized in this study as the primary reference gene (37). Primers were designed using primer BLAST with a target PCR product size of approximately 200 bp, except for smaller genes (acyl carrier protein) where smaller sizes of approximately 150 bp were selected (Table 2).

All qPCR experiments were performed using cDNA generated from 1.0 μg of isolated RNA based on spectrophotometric quantification. Conditions for qPCR are as

follows; an initial melting cycle of 95°C for 10 minutes followed by the PCR conditions of 95°C for 10 seconds, 58°C for 10 seconds, and 72°C for 15 seconds, repeated 40 times. Data analysis was performed using the crossing point calculation (LightCycler 480 Software Release 1.5.0 SP3, Roche, Basel, Switzerland), and included the reference gene recombinase A (37) in addition to 16S rRNA as reference samples. Data analysis was done by calculating the ΔCp value between each data point and the final time point in the batch growth. Controls were performed for each of the gene targets by comparison of obtained Cp values over a range including a 32 fold decrease in total cDNA using a serial dilution strategy with 6 sample points to confirm a linear relationship based on the exponential function. PCR products were further analyzed by agarose gel electrophoresis to confirm correct sizes of the products (Figure 5).

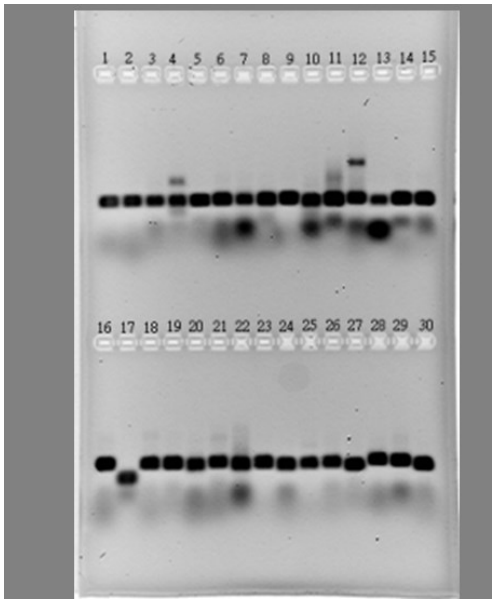

Figure 5. qPCR Primer Fragment Size Verification. Shown is a gel used to verify the correct qPCR primer fragments. Samples were amplified through qPCR and then ran on an agarose gel. All qPCR products should be ~200bp in length with the exception of the acyl carrier protein which is ~150bp (lane 17). Samples with excess bands not at ~200bp were removed from the sample set (lanes 7, 10, 12, 13 for example) and alternative primers were selected.

Table 1. Selected Proteins for mRNA Transcription Analysis.

Gene Product (Short Reference)	Protein Product (NCBI Reference)[a]	KEGG Pathway (or Putative Pathway)[b]	Accession Number[c]
WS/DGAT #1	Wax Synthase #1	Wax Ester Biosynthesis	YP_957462
WS/DGAT #2	Wax Synthase #2	Wax Ester Biosynthesis	YP_960328
WS/DGAT #3	Wax Synthase #3	Wax Ester Biosynthesis	YP_958134
WS/DGAT #4	Wax Synthase #4	Wax Ester Biosynthesis	YP_960629
FACoA Reductase	Fatty Acyl-CoA Reductase	Wax Ester Biosynthesis	YP_959769
FAld Reductase	Fatty Aldehyde Reductase[d]	Wax Ester Biosynthesis	YP_959486
FAldDH	Fatty Aldehyde Dehydrogenase	Wax Ester Biosynthesis	YP_960668
ACCase	Acetyl CoA Carboxylase β-Subunit	Fatty Acid Biosynthesis	YP_958827
FabB	β-Ketoacyl Synthase	Fatty Acid Biosynthesis	YP_960409
FabD	Malonyl CoA-Acyl Carrier Protein Transacylase	Fatty Acid Biosynthesis	YP_959137
FabH	3-Oxoacyl-ACP Synthase	Fatty Acid Biosynthesis	YP_958649
Acyl Carrier Protein	Acyl Carrier Protein	Fatty Acid Biosynthesis	YP_959135
FACoA Synth #1	Acyl-CoA Synthetase	Fatty Acid Metabolism	YP_958864
FACoA Synth #2	Acyl-CoA Synthetase	Fatty Acid Metabolism	YP_959360
FACoA Synth #3	Acyl-CoA Synthetase	Fatty Acid Metabolism	YP_960125
Ketoacyl Thiolase	3-Ketoacyl-CoA Thiolase	Fatty Acid Metabolism	YP_958424
Acetyl-CoA AcTrans	Acetyl-CoA Acetyltransferase	Fatty Acid Metabolism	YP_960423
FAACP Synth #1	AMP Dependent Synthetase (Acyl-Acyl Carrier Protein Synthetase)		YP_958369
FAACP Synth #2	AMP Dependent Synthetase (Acyl-Acyl Carrier Protein Synthetase)		YP_960150
FAACP Synth #3	AMP Dependent Synthetase (Acyl-Acyl Carrier Protein Synthetase)		YP_957724
TesA	Arylesterase (Thioesterase A)	Biosynthesis of Unsaturated Fatty Acids	YP_958323
TesB	Palmitoyl-CoA hydrolase (Thioesterase B)	Biosynthesis of Unsaturated Fatty Acids	YP_960846
Medium ADH	Medium Alcohol Dehydrogenase		YP_958650
Citrate Synth I	Citrate Synthase I	Citrate Cycle	YP_958427
Aconitase Hyd I	Aconitate Hydratase 1	Citrate Cycle	YP_960930
F0F1 ATPase	F0F1 ATP Synthase β-Subunit	Oxidative Phosphorylation	YP_961130
16S rDNA	16S Ribosomal RNA	Ribosome	NR_027551 (3)
RpoB	RNA Polymerase β-Subunit	RNA Polymerase	YP_957996
RpoD	RpoD family RNA Polymerase Sigma Factor		YP_958213
ppGpp Synth	ppGpp Synthetase I SpoT/RelA	Purine Metabolism	YP_957921
RecA	Recombinase A	Homologous Recombination	YP_959349

[a] Full name of the gene product based on NCBI reference number
[b] Potential pathways that gene products are involved in from KEGG or the pathway shown in Figure 4
[c] NCBI gene or protein accession number
[d] While annotated here as fatty aldehyde reductase, it is noted that this gene product has been reported to have fatty acyl-CoA reductase and fatty acyl-ACP reductase activity (38-39)

Table 2. Primers used in qPCR Experiments.

Gene Product (Short Reference)	Protein / Nucleotide Accession Number	Primer (5' to 3')
WS/DGAT #1	YP_957462	ACCGGTTCCCGTCGTTTCGC
		AGCTGTCGTCCCGGCGTAGT
WS/DGAT #2	YP_960328	GGCCCGGTTTCCGTGCTCAA
		ATACCGGCCGTCAGTGGGGT
WS/DGAT #3	YP_958134	CCGATGCAACTGGCCTCGCT
		TGACGTCCGCCAGAGAGCCT
WS/DGAT #4	YP_960629	TTCAAGCCCTGGCCAGCGAC
		ACGCGCTCGAGCTTGGGTTC
FACoA Reductase	YP_959769	GTTCCGTTCCGCATCTACCG
		CCAGTGCATCGACCACGAAA
FAld Reductase	YP_959486	CCGTCTTCGCGAGGCCGATT
		TGATGGCCAGCGCCTTGTCG
FAldDH	YP_960668	TTTCCGCTGCTGATGGCCGC
		CGCTTGCTGGTCGCCAAAGC
ACCase	YP_958827	GGTGTATCGGCCAGCCTCGC
		AGATGGGCGATGCGCTCACG
FabB	YP_960409	CCGCTCCGGCATTGCCTTCA
		CGGTGGTGTTTGCACGCAGC
FabD	YP_959137	GCCTGCTCTGCTGACCGCAA
		GCCATACGCCCCTGCCCTTC
FabH	YP_958649	GAAACCGCTGCAGACGGCCT
		CTGCCCGGACAAAGCACGGT
Acyl Carrier Protein	YP_959135	TTGTTTGTGAGCAGTTGGGCGTGA
		GTCTCGAATTCCTCTTCCAGGGCCA
FACoA Synth #1	YP_958864	GCCGCGGTGAAGCACCTCAA
		GTTGGCCACCAGGTTGGCGT
FACoA Synth #2	YP_959360	TGCCGTGATGCTGCCGAACAT
		ACCAGCGGCTTGTGCTCCAGAT
FACoA Synth #3	YP_960125	TTCGACGAGAACAGCGTCGCCA
		TGGAACATGGGCGTAACCGGCA
		TCGACGAAATGCCGCTGTTGCG
		TGCATGATGGTGGGCACGCAGT
Ketoacyl Thiolase	YP_958424	GTGGCGCGGCAGATTTCCCT
		GCAGCCGGGTTGTGGTCGAA
Acetyl-CoA AcTrans	YP_960423	CGGCCCGACAGTCTGCCATC
		TGCGGCTGTTGGGCAGTACG
FAACP Synth #1	YP_958369	ACCATGCCGAGTGCGACGTG
		AGCTCTTCCGGGTCCCGCTT
FAACP Synth #2	YP_960150	ACGACACGCCCAAATGGCAGGA
		TTCGCTGGCCGATACGGGGAAT
FAACP Synth #3	YP_957724	TGCGCTGGGTGTGCCTACGATT
		AGCAGTGCGTTGACGGTTCCGA
TesA	YP_958323	TGGAAGCCCACGACCCAACCAT
		TGGTGTAGCGCTGGCCGTAGTT
TesB	YP_960846	TGCGCCCTGGCAATCACAGCAT
		CCAGTCCTGCTCCGACTTGAGCGTA
Medium ADH	YP_958650	TTGTTGGCCGGGTTACCGCC
		TAGCCGCCGTGAGTGACCGA

Table 2. Primers used in qPCR Experiments (continued).

Gene Product (Short Reference)	Protein / Nucleotide Accession Number	Primer (5' to 3')
Citrate Synth I	YP_958427	ACGCCTGTATTGCGTCCGGC
		TGACCTTGGCGCGTGGATCG
Aconitase Hyd I	YP_960930	GACCGCTTCCGCGATCTCGG
		CGCAATTTCGAGGGCCGGGT
F0F1 ATPase	YP_961130	TAGCCCGGTGCTTTGCGGTG
		ACCATCGCCATGGGCAGCAC
16S rDNA	NR_027551 (3 copies)	CCGGCTAACTCCGTGCCAGC
		ACGCATTTCACCGCTACACAGG
RpoB	YP_957996.1	GCGTGTTGAGCGTGCAGTGC
		CCTGGGCCCAACGCGGAAAT
ppGpp Synth	YP_957921.1	CGCGGGGTGATTGCCGAAGT
		GCCGAACCCGGCCGATATGG
RpoD	YP_958213	AGCGCGTTGCCTCCATGGAC
		AGCCCGAAACGGCGGGAAAG
Recombinase	YP_959349	TCCTGCCGTATCCACCGGCT
		AAACCGGGTCCAGAGCGTGC
		GCACGCTCTGGACCCGGTTT
		CCACGTGGCTGTCGCCCATT

Results and Discussion

For these studies, an approach was taken to grow *Marinobacter aquaeolei* VT8 as a batch culture utilizing a media recipe routinely utilized in our laboratory to induce lipid accumulation. The key feature of this defined media is that cells exhaust the source of nitrogen prior to reaching maximum cell density for the specific conditions of growth, and enter into a nitrogen limited state which results in wax ester accumulation. This is believed to occur because the carbon source needed for energy and cellular building blocks remains plentiful, but the nitrogen required for DNA and protein synthesis is not available for further replication (31). The larger batch culture was selected here so that a thorough sampling of the culture could be made through various stages of the wax ester accumulation process. The sampling strategy adopted included multiple samples for total RNA isolation and the harvest of cells for further drying and wax ester quantification. Lipids were extracted using a previously described protocol that isolates primarily wax esters from dried *M. aquaeolei* VT8 cells, which can be analyzed directly using a gas chromatography method with flame ionization detection (GC/FID) to measure specific classes of lipids (18). This method is preferred over indirect methods such as gravimetric approaches, as the specific compound of interest, the wax ester, is separated and specifically quantified using external standards, while polar lipids such as cell membrane components that would contribute to total lipids in certain gravimetric methods are excluded from the measurement by this approach.

Wax production in M. aquaeolei VT8. Figure 6A shows two typical GC/FID chromatograms obtained from extraction of dried *M. aquaeolei* VT8 cells. The waxes produced by *M. aquaeolei* VT8 under similar conditions have been characterized previously (18). *M. aquaeolei* VT8 produces a combination of waxes comprised of primarily C16 and C18 length fatty alcohol and fatty acid derived wax esters, with a primary peak at a retention time of about 26 minutes (in the chromatogram shown in Figure 6A) that corresponds to a C34 wax ester, and secondary waxes that correspond to C32 and C36 wax esters flanking this peak at approximately 25 and 27 minutes respectfully, which has been confirmed previously by GC mass spectrometry (GC/MS).

Smaller peaks result from minor components of C14 fatty acids and fatty alcohols. Figure 6B shows the results from wax ester analysis of three independent batch cultures grown under similar conditions. This analysis shows a typical wax ester accumulation cycle from *M. aquaeolei* VT8. Cells grow exponentially with sufficient amounts of nitrogen to support replication until approximately 40 hours when all the available nitrogen in the media is consumed. Wax ester accumulation begins shortly after that, reaching a maximum quantity at about 100 hours (generally around 10% of dry cell mass). Following this, the culture enters a final stage where wax esters in the cell begin to decline to very low levels after about 150 hours. This increase and decrease in levels of wax esters in the cell during growth is reproducible ($n>5$ independent experiments). Results shown in Figure 6B were arbitrarily fit to a Gaussian curve for the sake of visualization only and are not meant to indicate that the wax ester accumulation cycle behaves precisely in this specific manner. Samples for RT-qPCR were chosen within the various growth phases of *M. aquaeolei* VT8 (exponential growth, wax biosynthesis, and wax catabolism, Figure 6B). To verify reproducibility of the results, samples were tested within an individual growth by quantifying transcriptional levels at all time points (seen in Figure 6B) of one independent growth (Figure 7 and 8). Other samples were also chosen to compare between three independent growths by selection of samples based on the three growth phases (seen in Figure 6B), one sample analyzed per phase of growth (Figure 9). Results in Figures 7, 8, and 9 are plotted as the fold difference in gene transcription levels versus the lipid catabolism time point, based on the assumption that wax ester production at this phase of the growth cycle has halted (as indicated by the wax ester analysis, Figure 6B). The log linearity of the qPCR was confirmed using serial dilution of the cDNA sample as described in the methods. The genes analyzed were categorized into four groups related to their putative roles in the cell, either within the putative wax biosynthetic pathway, fatty acid biosynthesis, fatty acid metabolism, or genes selected controls.

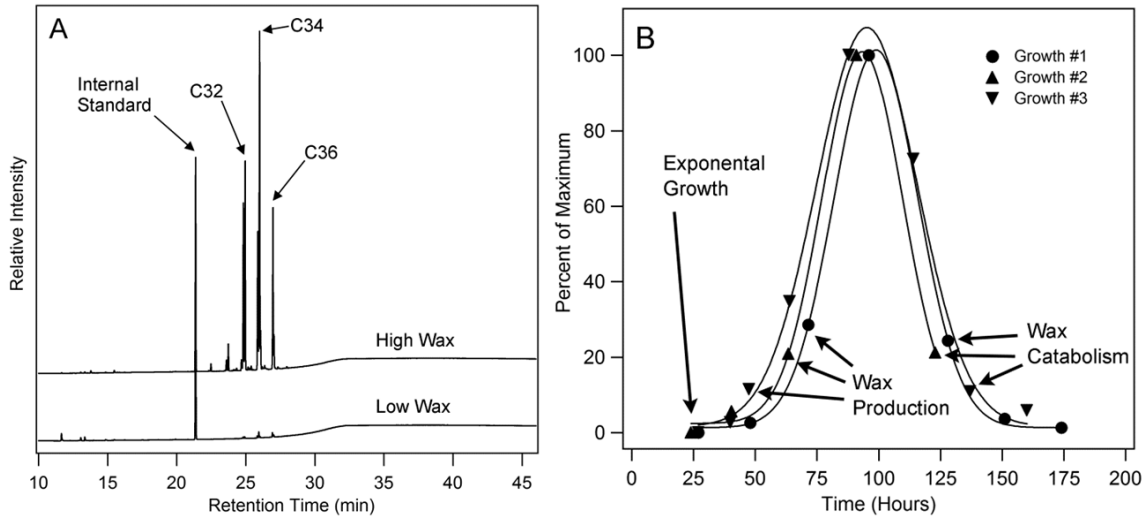

Figure 6. Wax Accumulation During Batch Growth. The graph to the left (A) shows overlaid samples of representative GC/FID chromatograms, of extracted waxes obtained from *M. aquaeolei* VT8 under conditions of low wax and high wax, which were used to calculate peak areas and dry weight percent of wax esters. Shown in the graph to the right (B) are relative areas for wax esters found (normalized to the maximum area found for each individual growth) over each of the batch cultures grown as part of this study. Data is fit to a simple Gaussian curve for clarity.

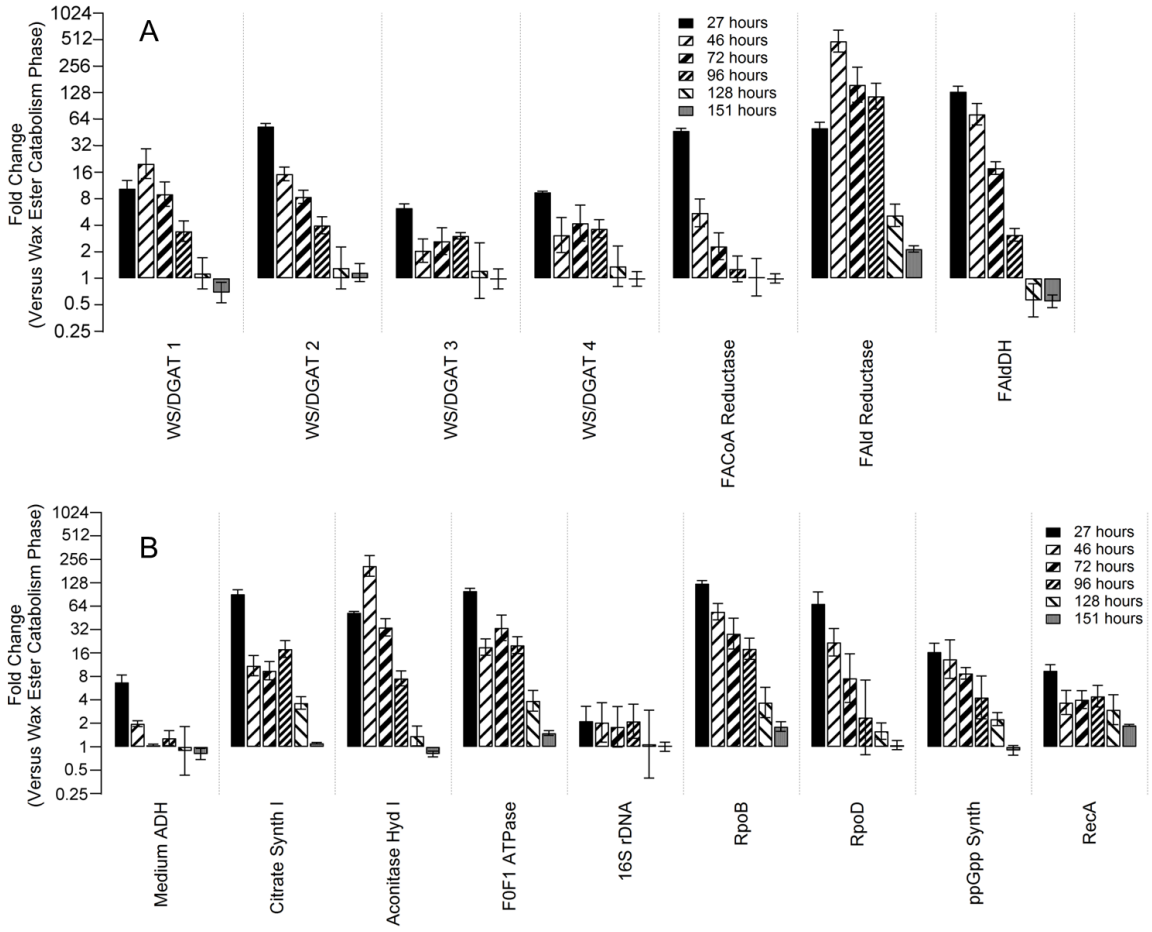

Figure 7. Transcription Levels of Potential Genes Involved in the Wax Ester Biosynthetic Pathway and Select Control Genes for One Independent Growth. The top graph (A) depicts the results from transcriptional analysis of potential genes within the wax ester biosynthetic pathway with the bottom graph (B) depicting transcriptional levels of select control genes. Samples for transcriptional analysis were taken from each time point of one independent growth. Statistics (standard deviations) are based on three separate replicates. Specific details of the genes encoding for these enzymes is found in Table 1.

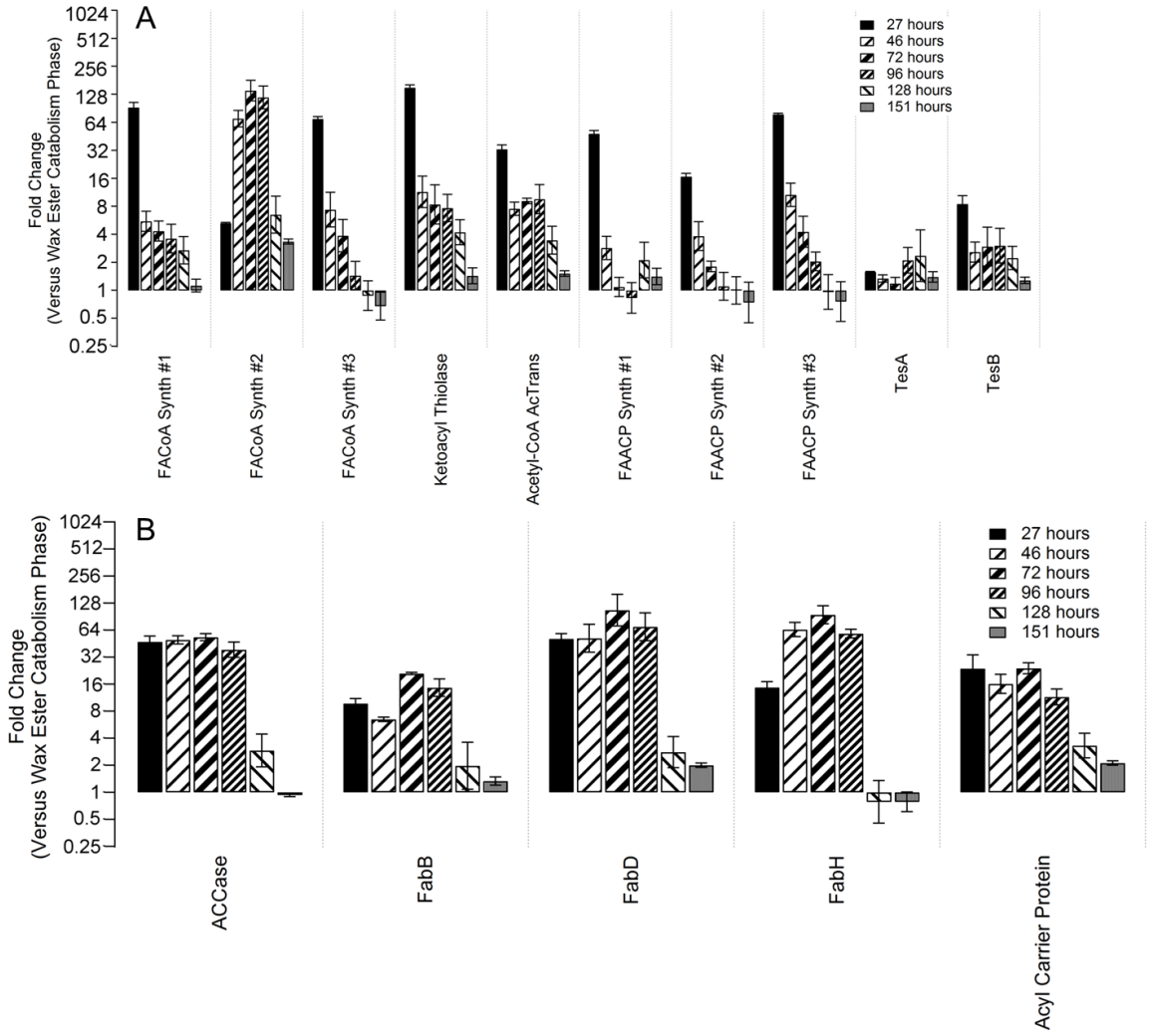

Figure 8. Transcription Levels of Potential Genes Involved in Fatty Acid Biosynthesis and Potential Precursors to the Wax Ester Biosynthetic Pathway for One Independent Growth. Graph A (top) shows results obtained from the transcriptional analysis of potential precursor genes to the wax ester biosynthetic pathway, graph B (bottom) show results of select genes within the fatty acid biosynthetic pathway. Samples for transcriptional analysis were taken from each time point of one independent growth. Statistics (standard deviations) are based on three separate replicates. Specific details of the genes encoding for these enzymes is found in Table 1.

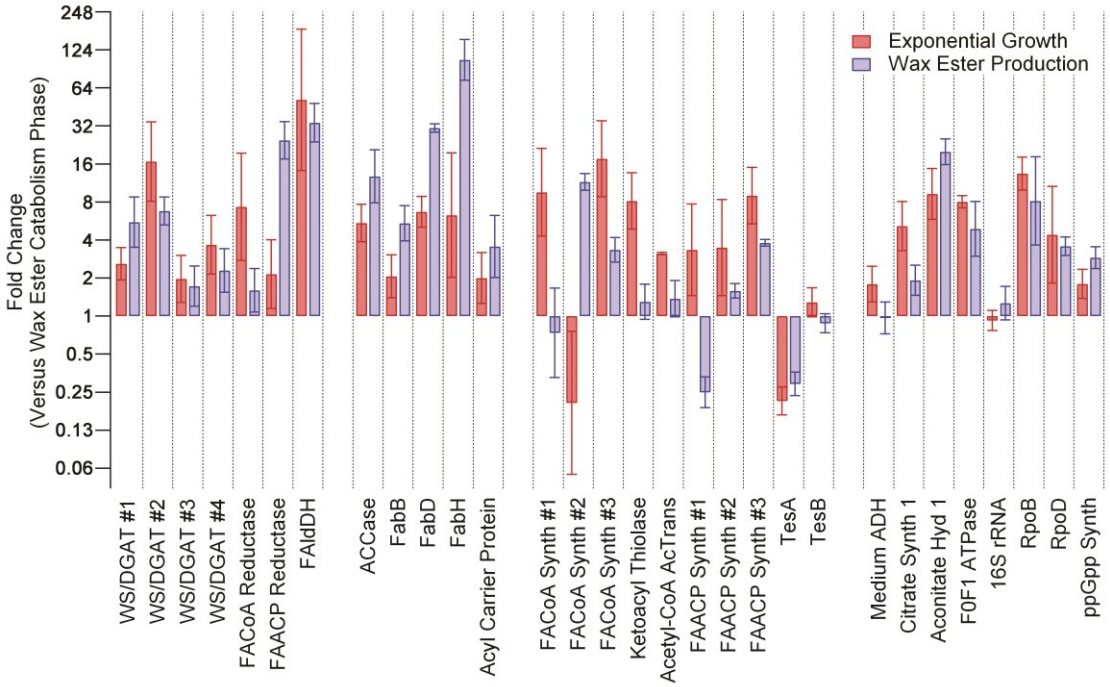

Figure 9. Transcriptional Analysis of Wax Ester Synthesis Genes Compared to Select Genes from Additional Pathways from Three Independent Growths. Shown are the results obtained from RT-qPCR analysis of various genes coding for specific enzymes detailed in Table 1. To illustrate the differences found, the results are plotted as the fold change (on a Log scale) versus the results obtained at the lipid catabolism stage of growth. One sample in each of the growth phases (exponential, wax ester production, and wax ester catabolism) from three independent growths were chosen for analysis. Statistics (standard deviations) are based on three separate replicates.

Transcriptional analysis of potential genes involved in the wax ester biosynthetic pathway. The first group of genes explored were part of the putative wax ester biosynthesis pathway, including the genes for the fatty acyl-CoA reductase and fatty aldehyde reductase that have been characterized previously (33, 35). The fatty aldehyde reductase has also been reported to reduce fatty acyl-CoA or fatty acyl-acyl carrier protein (fatty acyl-ACP) (38-39). These reductase genes were of interest, as the protein

products have been studied kinetically, and indicated a potential role in producing the fatty alcohols required for wax production. As such, their transcript levels were expected to increase during the wax accumulation stage. Transcription of the gene for the fatty acyl-CoA reductase was elevated during exponential growth, but rapidly declined during the initial phase of wax ester accumulation, while the gene for the fatty aldehyde reductase showed elevated transcriptional levels during the wax ester accumulation phase of the culture with approximately 128 fold increase versus wax ester catabolism, dropping only after wax esters had reached their peak concentration during the growth (Figure 10A).

A final gene for a fatty aldehyde dehydrogenase that might participate in oxidizing any free fatty aldehyde present in the cell (40) was expressed at high levels during both exponential growth and wax ester accumulation, emphasizing the potential importance of this gene in maintaining low levels of free fatty aldehyde in the wax ester biosynthetic pathway (Figure 4).

Additionally, we analyzed the genes for the wax ester synthase / acyl-coenzyme A : diacylglycerol acyltransferase (WS/DGAT) enzymes of *M. aquaeolei* VT8. Similar to strains such as *Rhodococcus sp.* RHA1, which contain multiple homologs of the WS/DGAT genes (41), *M. aquaeolei* VT8 contains four putative WS/DGAT enzymes (18, 42), though only two of these have been found to have significant activity with substrates (based on *in vitro* studies) that would result in the natural products found from *M. aquaeolei* VT8 (18, 42). This is in contrast to strains such as *Acinetobacter baylyi* and *Psychrobacter cryohalolentis* K5 that have been studied previously by ourselves and others, and contain only a single copy of the WS/DGAT gene (18, 35). Under batch growth, the first gene coding WS/DGAT #1 is the only gene of the four putative WS/DGAT enzymes that was up-regulated for transcription during lipid accumulation, and the difference was only slight, from 10.5 fold increase to 19.7 fold increase versus wax ester catabolism (Figure 10B). Expression of the gene coding WS/DGAT #2 decreased during lipid accumulation, though the expression level of this gene started significantly higher during exponential growth and was still higher than that of

WS/DGAT #1 (from 53 fold increase down to 15.3 fold increase versus wax ester catabolism). The genes for WS/DGAT #3 and WS/DGAT #4 showed only slight elevation of transcription versus wax ester catabolism.

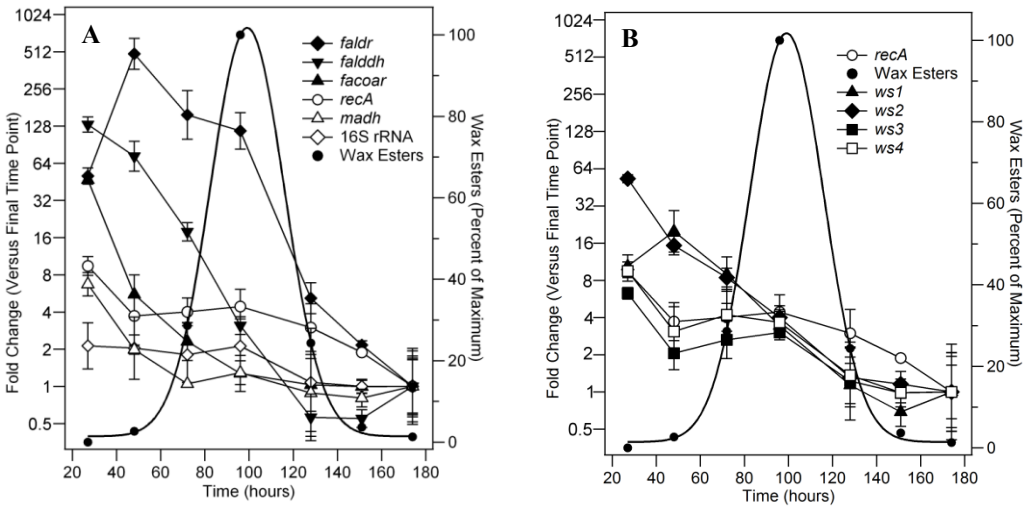

Figure 10. Comparison of Transcriptional Levels of Potential Wax Ester Biosynthetic Genes vs. Lipid Accumulation. Shown are transcriptional levels of the various genes associated with the putative wax ester biosynthetic pathway. Transcriptional levels were compared against the results obtained for the last time point (normalized to 1 fold, left y-axis) and are plotted on a log scale. Results obtained from the wax ester analysis for this culture are fit to a simple Gaussian curve for clarity and shown on the same graph (right y-axis). Where shown, statistics represent the average and standard deviation for three replicates of a single batch growth. The graph to the left (A) show transcription levels for the enzymes involved in the production of the fatty alcohol substrate along with a few genes chosen as controls, and the graph to the right (B) depicts transcription levels of the various wax synthases.

Transcriptional analysis of genes within the fatty acid biosynthetic pathway. A second group of genes targeted in these studies are those involved in fatty acid biosynthesis. Transcript levels of these genes were also expected to increase during wax ester synthesis, as they provide the primary route from the citrate carbon source to the fatty acid primary building blocks required for wax ester production. Four genes from fatty acid biosynthesis were selected along with the gene for fatty acyl carrier protein. Each of these genes showed high levels of transcription during the log phase of growth and increased further during wax ester accumulation, but then dropped dramatically following wax accumulation, and many showed significantly higher changes in transcription levels than were found for the primary wax ester synthesis genes (the transcriptional level of the gene for FabH had more than a 100 fold increase during wax ester accumulation versus wax ester catabolism, Figure 8B). This supports a notion that production of wax esters is heavily influenced by the upstream pathways producing the primary building blocks of the wax esters, while the majority of genes of the wax synthesis pathway are only slightly up-regulated.

Transcriptional analysis of fatty-acyl-ACP synthetase, fatty-acyl-CoA synthetase, and thioesterases. The third set of genes selected were related to fatty acid metabolism, fatty acyl-acyl carrier protein synthetases and thioesterases. The role of these genes in producing enzymes to shuttle building blocks from fatty acid biosynthesis to the fatty acyl-CoA pool is less well understood, and so the genes were chosen to determine if additional insight could be derived from transcriptional levels of these genes. Results revealed the expected decrease in transcriptional levels of the genes coding for ketoacyl thiolase and acetyl-CoA acetyltransferase during lipid accumulation, as this pathway would compete with fatty acid biosynthesis. The three genes for putative fatty acyl-CoA synthetases showed stark differences, with transcription of the genes for the first and third decreasing during wax ester accumulation, while only the second showed a significant increase (Figure 8A). The gene for fatty acyl-CoA synthetase #2 showed a high degree of variance in samples from the exponential phase, and is one of only three genes that were down-regulated during exponential phase, but increased dramatically during lipid

accumulation, showing a 56 fold increase between exponential growth and lipid accumulation (Figure 8A). Three genes selected as they are proposed here to code for potential fatty acyl-acyl carrier protein synthetases (fatty acyl-ACP synthetases) all showed a decrease in transcription during wax ester accumulation. These results must be viewed cautiously, as several additional genes were found by BLAST comparison analysis that were annotated as either fatty acyl-CoA synthetase or fatty acyl-ACP synthetase genes, and specific activities and substrates for each of these have not been fully characterized in *M. aquaeolei* VT8. The genes selected in these studies were based initially on the KEGG annotation and BLAST comparisons, and any further analysis would require parallel characterization studies to determine whether each of the translated genes yield an enzyme that produce fatty acyl-CoA or fatty acyl-ACP as a final product. Finally, we selected the genes coding for two common thioesterases that were found in *M. aquaeolei* VT8. The gene for thioesterase A (TesA) showed slight down-regulation during exponential growth and wax ester accumulation while the gene for thioesterase B (TesB) varied very little over the time points selected (Figure 8A).

Transcriptional analysis of select control genes. Several additional genes in these studies were selected for comparison based on the idea that their expression would be consistent throughout the growth. These included the gene for recombinase A, the 16S rRNA component, genes for ATP synthase, a medium branched chain alcohol dehydrogenase (medium ADH) that is also studied extensively in our laboratory (43), as well as several genes for enzymes involved in central metabolism. Several of these genes showed a relatively high level of transcription during exponential growth with minimal changes in transcription later on in the growth (Figure 7B). These results are what would be expected of these housekeeping genes, further bolstering the confidence in the results found for those genes where transcription either increased or decreased.

Summary of Findings and Future Works. This study revealed several important features related to wax ester accumulation in *M. aquaeolei* VT8. First, expression analysis did not highlight either of the two primary WS/DGAT enzymes (#1 or #2 that have been characterized *in vitro* and shown to have high activity previously (18)) as

being the primary enzyme involved in the final step of the wax ester biosynthesis pathway. This indicates a certain level of redundancy that can only be investigated by studies targeting gene deletion in this strain. Second, *in vitro* studies with the enzymes fatty aldehyde reductase and fatty acyl-CoA reductase previously characterized in our laboratory imply that fatty acyl-CoA reductase is the more active enzyme in yielding fatty alcohols (34, 36). Contrary to that finding, the gene for fatty aldehyde reductase, which was also shown to have fatty acyl-ACP reductase activity (38-39), showed the highest up-regulation in gene expression of all the genes in the direct wax ester biosynthesis pathway. Production of fatty alcohols from fatty acyl-ACP would be favored energetically, as it would require less ATP and only a single enzyme versus a pathway requiring a thioesterase and fatty acyl-CoA reductase. Only one of the genes for a potential fatty acyl-CoA synthetase enzyme (FACoA Synth #2) showed up-regulation, while thioesterases were down-regulated during wax accumulation. This highlights a missing component in the current model to describe how fatty acids derived from fatty acid biosynthesis are directed to the fatty acyl-CoA pool. Third, the greatest changes found in transcription in these studies seemed to be associated with fatty acid biosynthesis genes, supporting a proposal that this upstream pathway has a strong role in regulating wax ester accumulation. The robustness of the results from this analysis and correlations with specific target genes now supports an expansion of these studies to further global analysis of all the genes from *M. aquaeolei* VT8 using RNA-sequencing approaches, which could provide even more insights into the process of wax accumulation and degradation in this model bacterium.

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Chapter 3

Neutral Lipid Production in a Wax Ester Accumulating Bacterium: Insights From *in vivo* Analysis Through Gene Deletion

Summary

The biosynthesis of wax esters in bacteria is accomplished by a unique pathway that combines a fatty alcohol and a fatty acyl-CoA substrate. Previous *in vitro* enzymatic studies indicate that two different enzymes could be involved in the synthesis of the required fatty alcohol in *Marinobacter aquaeolei* VT8. Other *in vitro* enzymatic studies indicate a number of homologous enzymes catalyzing the final step in producing the wax ester. In this study, we demonstrate through a series of gene deletions that *M. aquaeolei VT8* encodes for redundancy in a number of the steps in this biosynthetic pathway as well as the ability of *M. aquaeolei VT8* to produce the fatty alcohol precursor through two different enzymes *in vivo*, but evolution has clearly selected one of these as the preferred enzyme to perform this reaction under wax ester accumulating conditions. These results complement previous *in vitro* studies and provide the first glimpse of the role of each enzyme *in vivo* in the native organism.

Introduction

Wax esters are of interest in the biotechnology field due to their ability to be used as alternatives to petroleum based compounds found in cosmetics, lubricants, and plastics, as well as their potential to be used as an alternative fuel. A number of organisms have been found to produce wax esters by forming an ester bond between a fatty alcohol and an activated fatty acid. In the cell wax esters serve as storage molecules during nutrient limited conditions. *Marinobacter aquaeolei* VT8 is one such organism that has the capability of producing a high concentration of wax esters, yet little is known about the biosynthetic pathway which produces these wax esters. A number of genes have been identified as potentially playing a role in wax formation in *M. aquaeolei* VT8 through *in vitro* enzymatic analysis (18, 34, 36, 38-39, 42).

Biosynthesis of wax esters is accomplished by the combination of several different enzymes. The wax ester synthase / acyl-coenzyme A:diacylglycerol acyltransferase (WS/DGAT) enzyme catalyzes the esterification of a fatty acyl-CoA substrate with a fatty alcohol (Figure 4 in Chapter 2). *M. aquaeolei VT8* contains four putative WS/DGAT enzymes (18, 42), though only two of these have been found to have significant activity based on *in vitro* enzymatic studies. This differs from other wax ester producing bacteria such as *Acinetobacter baylyi* and *Psychrobacter cryohalolentis* K5, which contain only one WS/DGAT gene (18, 35). Because of this *M. aquaeolei* VT8 provides a unique opportunity to study the selectivity of enzymes which catalyze similar reactions *in vivo*.

While the fatty acyl-CoA utilized by the WS/DGAT is proposed to come directly from the fatty acyl-CoA pool, the fatty alcohol is believed to be produced through the action of several reductase enzymes acting on activated fatty acids or fatty aldehydes. *M. aquaeolei* VT8 contains at least two enzymes that have been found to produce fatty alcohols from several different substrates *in vitro*, including fatty aldehydes, fatty acyl-CoAs and fatty acyl-ACPs (34, 36, 38-39). Additionally, both of the enzymes from *M. aquaeolei* VT8 have shown significantly higher activity than was reported for other enzymes when tested using *in vitro* assays (34, 36). Thus, it was of interest to determine which of the two enzymes found in *M. aquaeolei* VT8 is responsible for the production of

the fatty alcohol as well as which of the wax synthases are responsible for the final reaction in this species under wax ester accumulation conditions. To determine the roles of these different enzymes, efforts were undertaken to delete each of the genes using homologous recombination.

Materials and Methods

Strains and reagents. *Marinobacter aquaeolei* VT8 was obtained from the American Type Culture Collection (ATCC) and cultured aerobically on Miller Lysogeny Broth (LB) media at 30˚C. *Escherichia coli* WM3064 (44) was grown on LB supplemented with 20 μg/mL diaminopimelic acid (DAP) at 37 ˚C. For wax ester production, *M. aquaeolei* VT8 was grown on a minimal media (18). When appropriate, media was supplemented with kanamycin at 50 μg/mL. All reagents were obtained from Sigma Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA) unless otherwise specified.

Conjugation of M. aquaeolei VT8. The *M. aquaeolei* VT8 conjugation procedure was derived from methods for the conjugation of *Psychrobacter arcticus* 273-4 and *Marinobacter adhaerens* (45, 46). Briefly, cultures of the donor cells, *E. coli* WM3064 which contain the specific plasmid, and recipient cells, *M. aquaeolei* VT8, were grown separately on LB plates, then mixed at a ratio of 1:3 donor to recipient cells and spotted onto an LB plate containing DAP, then incubated at 30°C for approximately 24 h. Cells were collected from two spots, washed with LB, and resuspended in 100 μL LB, and spread onto LB agar plates devoid of DAP but containing kanamycin for selection. These plates were then incubated at 30°C for 2 – 4 days at which time colonies were selected and streaked several times to fresh plates prior to PCR verification of deletions using primers flanking the regions of DNA that were manipulated.

Single gene deletion experiments in M. aquaeolei VT8 – Single gene deletions were accomplished by constructing a plasmid vector containing the mobilization element from pBBR1MCS-2 vector, incorporated into a pUC19 derivative vector pBB053. The regions flanking the genes of interest were amplified by PCR and cloned into a separate pUC19 derivative vector (pBB053 or pBBTET3) with a different antibiotic marker, and then shuffled to the deletion vector with the mobilization element. Finally, the kanamycin resistance cassette from pBBR1MCS-2 was placed between the two flanking regions to replace the target gene upon double homologous recombination. Specific details of the construction of these vectors are outlined in Table 3 and a list of the primers used to construct these vectors and verify gene knockouts are shown in Table 4. A map of typical

plasmids used for these deletion studies is shown in Figure 11. A list of reference names for genes and gene products is provided in Table 5 for reference.

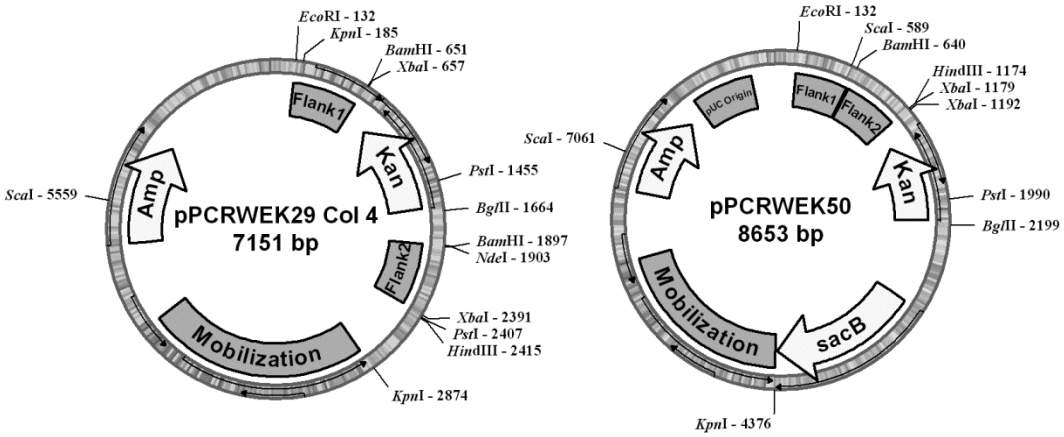

Figure 11. Key Plasmids for Gene Deletion Studies. Shown are representations of two of the final plasmid constructs utilized for gene deletion studies. Plasmid pPCRWEK29 was used to perform double homologous recombination by replacing the gene of interest (*facoar* in this construct) with the antibiotic marker for kanamycin All other deletions utilizing the double homologous recombination method use similarly constructed plasmids, only differing by the specific flanking regions. Plasmid pPCRWEK50 was used to perform a single homologous recombination that was selected using the kanamycin marker. A counter selection following a second recombination event using the toxicity of the *sacB* gene product resulted in markerless deletion of the *faldr* gene. Images produced using the program pDRAW32 (Acaclone Software).

Table 3. Key Parent Plasmids and Relevant Derivatives used for the Construction of *Marinobacter aquaeolei* VT8 Manipulated Strains. [a]

Plasmid[b]	Relevant Gene(s) Cloned or Plasmid Manipulation	Vector	Source
pBBR1MCS-2	Plasmid containing mobilization element		(47)
pSMV3	Plasmid containing *sacB* gene		(44)
pBB053	Removed *Nde*I site from pUC19 by silent mutation	pUC19	This Study
pBB114	Replaced pUC19 Amp resistance with Kan resistance cassette from pUC4K, then removed NsiI and HindIII sites from cassette by silent mutation	pUC19	pUC4K (48)
pBBTET3	Replaced pUC19 Amp resistance with Tet resistance cassette from pRK415	pUC19	pRK415
pPCRKAN4	Cloned Kan cassette from pBBR1MCS-2 into pBBTET3	pBBTET3	This Study
pPCRMOB4	Moved mobilization element from pBBR1MCS-2 into pUC19	pUC19	This Study
pPCRSACB6	The *sacB* gene from pSMV3 was cloned into pBB053 and then EcoRI, HindIII, XbaI and KpnI sites were removed by site-specific mutagenesis with silent mutations	pBB053	This Study
pPCRSACB7	Moved *sacB* gene cassette from pPCRSACB6 to pBBTET3	pBBTET3	This Study
pPCRWEK4	Derivative of pBB053 for gene insertions	pBB053	This Study
pPCRWEK5	Moved mobilization element from pPCRMOB4 into pPCRWEK4	pBB053	This Study
pPCRWEK12	Cloned gene *facoar* and flanking regions from *M. aquaeolei* VT8 genome with primers BBP1477 and BBP1478 into pBBTET3 EcoRI and XbaI sites	pBBTET3	This Study
pPCRWEK14	Performed PCR with primers to remove gene for *facoar* from pPCRWEK12 leaving flanking regions and adding BamHI site	pBBTET3	This Study
pPCRWEK20	Cloned gene *faldr* and flanking regions from *M. aquaeolei* VT8 genome with primers BBP1522 and BBP1523 into pBBTET3 EcoRI and XbaI sites	pBBTET3	This Study
pPCRWEK26	Moved *facoar* flanking segments fragment from pPCRWEK14 into pPCRWEK5	pBB053	This Study
pPCRWEK27	Performed PCR with primers to remove gene *faldr* from pPCRWEK20 leaving flanking regions and adding BamHI site	pBBTET3	This Study
pPCRWEK29[c]	**Moved Kan cassette from pPCRKAN4 into BamHI cut pPCRWEK26**	**pBB053**	**This Study**
pPCRWEK32	Moved *faldr* flanking segments fragment from pPCRWEK27 into pPCRWEK5	pBB053	This Study
pPCRWEK33	**Moved Kan cassette from pPCRKAN4 into BamHI cut pPCRWEK32**	**pBB053**	**This Study**
pPCRWEK48	Moved *sacB* gene cassette from pPCRSACB7 into KpnI and HindIII cut pPCRWEK32	pBB053	This Study
pPCRWEK50[c]	**Moved Kan cassette cut with BamHI from pPCRKAN4 into BglII cut pPCRWEK48**	**pBB053**	**This Study**

[a] Construction of the various plasmids used for gene deletions where constructed using the same approach. Shown here are the plasmids used for deletions of the genes *facoar* and *faldr*

[b] Sequences of all plasmids in this study are available upon request

[c] Plasmid maps are also provided in Figure 8

Table 4. Primers Used for Plasmid Construction and Knockout Verification.

Primer Designation	Primer Sequence [a]	Purpose
BBP1318	5'GACTAAGC TTGGAGATTT ACGAAGCCGC TTTCGAC3'	Cloning *ws1* gene and flanking region
BBP1319	5'GACAGAAT TCCAACTGTG AACAGGAAGA ACGC3'	Cloning *ws1* gene and flanking region
BBP1320	5'GACAGGAT CCTGTAATC AGGCCAGCTG ATCGGCTC3'	*ws1* gene removal
BBP1321	5'GACAGGAT CCAGAGAAAG AGCTGGTCAG TGGG3'	*ws1* gene removal
BBP1473	5'GACAGAAT TCGAAGCTGA TGAACGCAGC GCTGAC3'	Cloning *ws2* gene and flanking region
BBP1474	5'GACTAAGC TTGCAGGGTA AGAATGGTGAT CAGGCTG3'	Cloning *ws2* gene and flanking region
BBP1475	5'GTCAGGAT CCATATGTTG TCATTCTCCG CCAGTCATTT GTG3'	*ws2* gene removal
BBP1476	5'GTCAGGAT CCCCCAGGAC GGGGTCAGTCGC TTTGC3'	*ws2* gene removal
BBP1477	5'GACATCTA GACTGGATCT TGTCTTCCCG GGAACCAC3'	Cloning *facoar* gene and flanking region
BBP1478	5'GACAGAAT TCTGGATTTC ACCGGCATCG ATCC3'	Cloning *facoar* gene and flanking region
BBP1479	5'GACAGGAT CCATATGTAC TCCATTCTGC CTGTTGTGTT TTTG3'	*facoar* gene removal
BBP1480	5'GACAGGAT CCGATATACT GGTAATCGTC GTTATAAACC AAG3'	*facoar* gene removal
BBP1522	5'GNNNGAAT TCGATCGCGC CAGTCTTGCT CGTCATTTG3'	Cloning *faldr* gene and flanking region
BBP1523	5'GNNNTCTA GAAGCTTCGA AGCGTTCAGG ACACCGTCCT CGAAC3'	Cloning *faldr* gene and flanking region
BBP1524	5'GNNNGGAT CCCTTCTCCG GGGCAGGAAA GCGTTTCTG3'	*faldr* gene removal
BBP1525	5'GNNNGGAT CCGATAGAAC TCCTTCTCTG AGATCACTAA TGCCG3'	*faldr* gene removal
BBP1441	5'CACGTTGA TTCTGCTCAC TGAACAACTACTC3'	*ws1* deletion confirmation
BBP1442	5'CCTGAGTT TCTTCGGCAT CGATCTGTTCTTC3'	*ws1* deletion confirmation
BBP1546	5'GTGACCAT CAAACCAATG GTGCCGTTCTTC3'	*ws2* deletion confirmation
BBP1547	5'CACACCAT CAATATCTAC ACAGATGTTCAC3'	*ws2* deletion confirmation
BBP1558	5'CGAGATGC TGAACGTTCA TGTTGGC3'	*faldr* deletion confirmation
BBP1559	5'CACAGAGT GGATCGCACC AATACG3'	*faldr* deletion confirmation
BBP1548	5'GTATTCGC CTGCCTCCGG GTACTTC3'	*facoar* deletion confirmation
BBP1549	5'CACACGCG AAAGACAAGA AGGAAGC3'	*facoar* deletion confirmation

[a] Specific restriction enzyme sites added to primers are underlined for clarity

Table 5. Selected Gene Targets for Deletion.

Gene Product (Short Reference)[a]	Protein Product (NCBI Reference)[b]	KEGG Pathway (or Putative Pathway)	Accession Number[c]
WS/DGAT #1	Wax Synthase #1	Wax Ester Biosynthesis	YP_957462
WS/DGAT #2	Wax Synthase #2	Wax Ester Biosynthesis	YP_960328
WS/DGAT #3	Wax Synthase #3	Wax Ester Biosynthesis	YP_958134
WS/DGAT #4	Wax Synthase #4	Wax Ester Biosynthesis	YP_960629
FACoA Reductase	Fatty Acyl-CoA Reductase	Wax Ester Biosynthesis	YP_959769
FAld Reductase	Fatty Aldehyde Reductase[d]	Wax Ester Biosynthesis	YP_959486

[a] Simple reference for gene product
[b] Full name of the gene product based on NCBI reference number
[c] NCBI gene or protein accession number
[d]While annotated here as fatty aldehyde reductase, it is noted that this gene product has been reported to have fatty acyl-CoA reductase and fatty acyl-ACP reductase activity (38-39)

Single gene deletion with markerless counter selection – Single gene deletions with markerless selection were constructed as above for double homologous recombinations, with the following exceptions. For counter selections, the *sacB* gene and promoter from the pSMV3 plasmid (49) was cloned into a separate plasmid and multiple restriction sites were removed by site-specific mutagenesis to optimize the gene construct for future use. A vector was then constructed containing the *sacB* gene, the mobilization element described above, and the kanamycin cassette described above, except that all fragments were inserted into the vector outside of the segment containing the flanking region fragments. This new plasmid is called pPCRWEK50, and a map of the plasmid is shown in Figure 11. Following the conjugation protocol described above, single homologous recombination was used to integrate the entire plasmid into the genome. Once isolated, a counter selection protocol was used by growing *M. aquaeolei* VT8 in the presence of sucrose. While this procedure has been used successfully in other strains, selection of the markerless gene deletion following a second recombination event was not very efficient, and took several transfers in sucrose containing media before a successful gene deletion was obtained by screening multiple colonies grown on LB plates and then identifying those which no longer grew on LB plates supplemented with kanamycin.

Wax ester production in gene deletion strains – Once gene deletion strains were confirmed, cultures were grown on a shaker flask in wax ester producing media, harvested, lyophilized and extracted for wax ester analysis as described previously (18). Each gene deletion was grown as three independent cultures and harvested along with a control of the wild-type strain.

Results and Discussion

The marine bacterium *Marinobacter aquaeolei* VT8 produces wax esters under nutrient limited conditions when grown in the presence of simple carbons sources (such as acetate, citrate or succinate) in a minimal media (18). Previous studies in our laboratory have characterized several key enzymes that could participate in the wax ester biosynthetic pathway (18, 34, 36). One feature associated with wax ester production in *M. aquaeolei* VT8 is redundancy of several of the enzymes involved in this pathway (Figure 4 Chapter 2). This includes multiple copies of homologs for the wax ester synthase enzyme (18), and two alternative enzymes that have been found to reduce more oxidized pathway intermediates (such as fatty aldehydes or activated fatty acids) to fatty alcohols (18, 34, 36, 38). The reasons why *M. aquaeolei* VT8 has enzyme redundancy within this pathway is unclear, as are the roles that different enzymes play *in vivo* during wax ester production. This feature of enzyme redundancy differentiates *M. aquaeolei* VT8 from other model wax ester accumulating organisms such as *Acinetobacter baylii* that are reported to have only a single enzyme for each of these roles (18, 34, 36, 38, 50-51).

The goal of these experiments was to determine the roles, under wax ester accumulating conditions, of the different enzymes in the wax ester biosynthetic pathway of the model organism *M. aquaeolei* VT8. To address the question of what role these enzymes play and the function of the enzymes *in vivo*, gene deletions followed by wax ester production analysis was performed. To produce single gene deletions, we selected an approach utilizing double homologous recombination and conjugation strategies. A similar approach to what we have taken was recently reported for an alternative strain of *Marinobacter* (45). We found that pUC19 derived plasmids did not replicate in *M. aquaeolei* VT8, and could thus serve as a suicide vector for genome integration studies if they contained the proper mobilization element (47), and found the kanamycin cassette from pBBR1MCS-2 (47) to be an ideal selection marker with *M. aquaeolei* VT8.

Single gene deletions for facoar, faldr, ws1, and ws2. The double homologous recombination strategy described above was successful in isolating strain Δ*facoar*

containing a single gene deletion of the gene coding for FACoAR and separately strain Δ*faldr* containing a single gene deletion of the gene coding for FAldR. We will utilize our previous naming scheme of fatty aldehyde reductase (FAldR) for the latter gene product for clarity, though we acknowledge others have reported additional activities for this gene product (38-39). Knockouts were verified via PCR by choosing primers which annealed outside of the region being manipulated and then performing a restriction digest of the fragment looking for correct number of fragments and sizes. Figure 12 shows a representation of this verification scheme using the *faldr* gene and knockout as well as gels showing the resulting fragments.

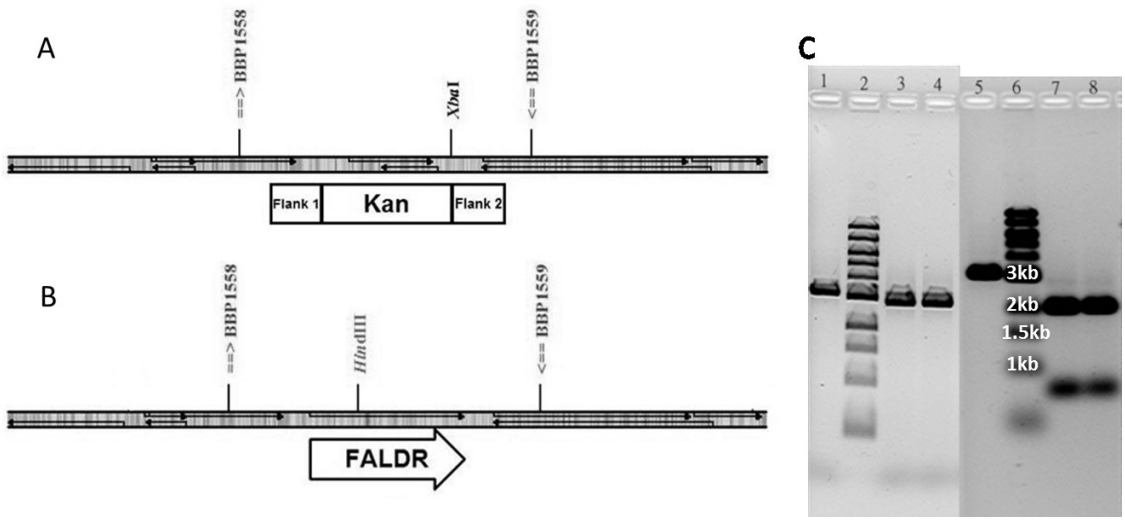

Figure 12: Gene Knockout Verification Utilizing PCR and Restriction Digest. Displayed is the scheme for verifying gene deletions within *M. aquaeolei* VT8. Primers were chosen outside of the manipulated region (in this case primers BBP1558 and BBP1559 seen in panel A and B). Products are then digested with XbaI and fragment number and sizes obtained from the knockout strain are compared with that of wild-type. In panel C we see these products for the *Δfaldr* strain, with lanes 1, 3-4 amplified with BBP1558 and BBP1559 and lanes 5, 7-8 are those fragments digested with XbaI. Lane 1: wild-type (3102bp); 2: 1kb ladder; 3: *Δfaldr* colony 1 (2778bp); 4: *Δfaldr* colony 2 (2778bp); 5: wild-type (3102bp); 6: 1kb ladder; 7: *Δfaldr* colony 1 (2019 and 759bp); 8: *Δfaldr* colony 2 (2019 and 759bp).

Knockouts were also verified by analyzing the gene copy number through qPCR using the methods described in Chapter 2 of this thesis. Primers which annealed to the genes of WS1, WS2, FAldR, FACoAR, the kanamycin gene, recombinase A, and 16S rRNA were designed and utilized to determine copy number within each strain. Crossing point values were obtained and normalized to the reference gene recombinase A (37), as only one copy of *recA* exists in *M. aquaeolei* VT8. Figure 13 depicts an example of one of the knockout strains compared to wild-type. Table 6 lists the primers used in to determine copy number of the various genes within the knockout strains.

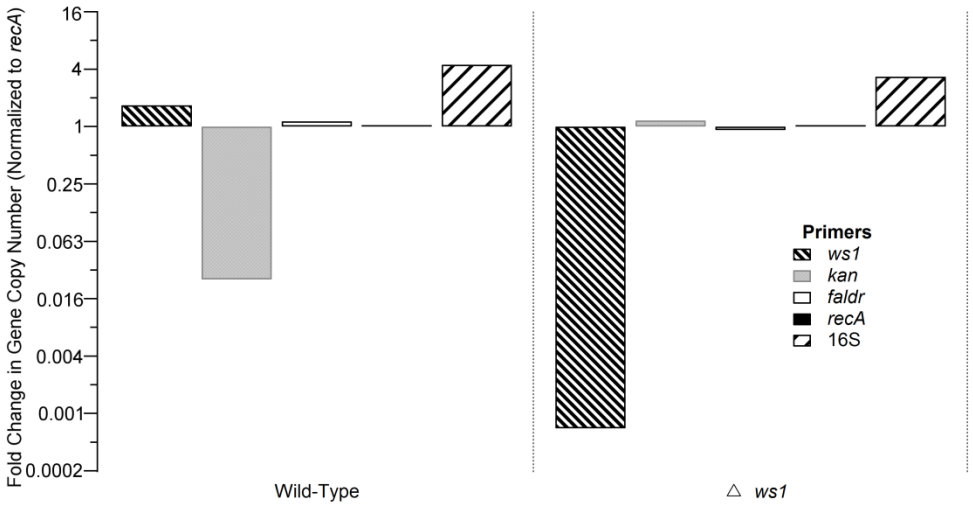

Figure 13. Gene Knockout Verification via Gene Copy Number Analysis. Shown above is a graph depicting gene copy number of select genes of the *Δws1* strain compared to wild-type. Here, gene copy number of ws1, kanamycin, faldr, recA, and 16S are compared within wild-type *M. aquaeolei* VT8 and *Δws1* strain. The lack of the kanamycin gene within wild-type can be seen and within the *Δws1* strain we see the deletion of the *ws1* gene as well as the presence of the kanamycin gene, confirming the knockout and supplementation with the kanamycin gene. Primer sequences used for amplification can be found in Table 6.

Table 6. Primers used to Identify Gene Copy Number in the Knockout Strains.

Gene Product (Short Reference)	Protein / Nucleotide Accession Number	Primer (5' to 3')
WS/DGAT #1	YP_957462	ACCGGTTCCCGTCGTTTCGC AGCTGTCGTCCCGGCGTAGT
WS/DGAT #2	YP_960328	GGCCCGGTTTCCGTGCTCAA ATACCGGCCGTCAGTGGGGT
FACoA Reductase	YP_959769	GTTCCGTTCCGCATCTACCG CCAGTGCATCGACCACGAAA
FAld Reductase	YP_959486	CCGTCTTCGCGAGGCCGATT TGATGGCCAGCGCCTTGTCG
16S rDNA	NR_027551 (3 copies)	CCGGCTAACTCCGTGCCAGC ACGCATTTCACCGCTACACAGG
Recombinase A	YP_959349	TCCTGCCGTATCCACCGGCT AAACCGGGTCCAGAGCGTGC
Kanamycin		CGCCGCCAAGCTCTTCAGCAAT AACTGTTCGCCAGGCTCAAGGC
Mobilization Element		TCGGACGTTCTTTGCGGACGGT TGCCCCAACAGCGATTCGTCCT

Once isolated, each of the individual deletion strains Δ*facoar*, Δ*faldr*, and the wild-type *M. aquaeolei* VT8 were grown under wax ester accumulating conditions (18). Figure 14A shows the results of the wax ester analysis from three independent growths of these strains. Due to differences in peak lipid production there is some degree of variance seen in Figure 14A because of the increase and decrease in levels of wax esters in the cell during growth, yet the general trend of the levels of wax esters in the cell are reproducible (n>5 independent experiments). The wild-type and Δ*facoar* strain yielded similar quantities of wax esters (Figure 14A) while the Δ*faldr* strain resulted in a significant decrease in the amounts of wax esters found, yet neither deletion strain resulted in a complete wax ester deletion phenotype, indicating that both enzymes are capable of fulfilling this role in the wax ester biosynthesis pathway in the absence of the other gene.

Single gene deletions were also obtained for strain *Δws1*, which containing the deletion of the gene coding for WS1, and another strain, *Δws2*, which contained a single

deletion of the gene coding for WS2. When grown under conditions which supported wax ester accumulation (18), these two strains resulted in no significant change in wax esters produced when compared to wild-type *M. aquaeolei* VT8 (Figure 14A) demonstrating the redundancy encoded for in this step of the pathway.

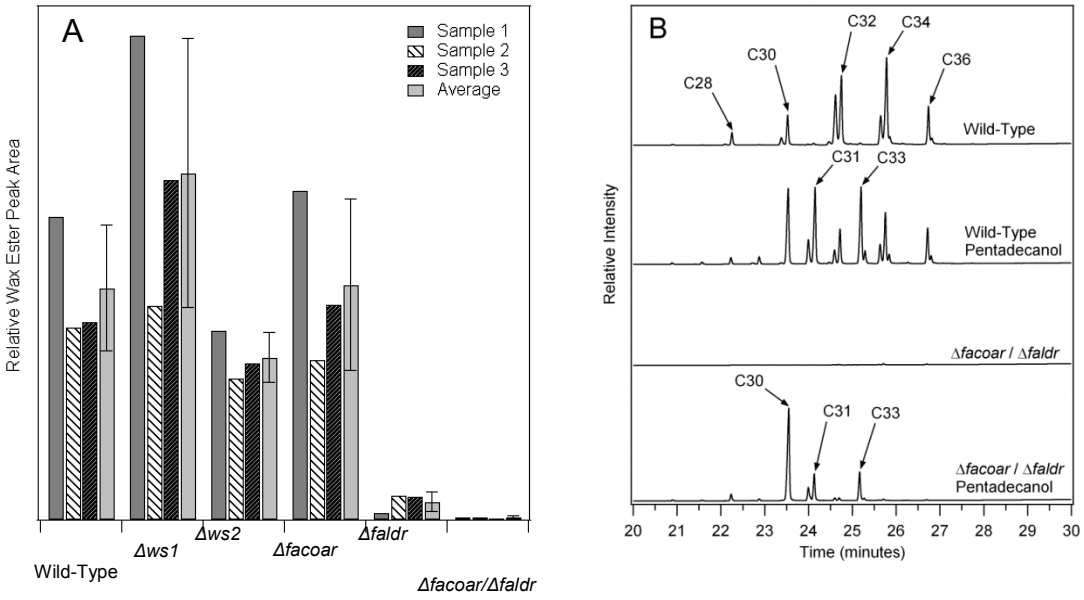

Figure 14. Gene Deletion and Wax Ester Production Rescue Studies. Shown on the left panel (A) are the results of the quantities of wax esters obtained from replicate growths under wax ester accumulating conditions of wild-type *M. aquaeolei* VT8 cells versus the single gene deletion strains and the double deletion strain. Shown on the right (B) are gas chromatography chromatograms illustrating the results of studies with wild-type cells and the double deletion strain grown under wax accumulation conditions and under the same conditions with added extraneous pentadecanol resulting in the accumulation of odd numbered wax esters (for wild-type) and the rescue of wax ester production based on only these odd alcohols (for the double deletion).

Double gene deletions of both facoar and faldr. To probe and confirm the specific role of both enzymes, a further effort was initiated to construct a double deletion strain. Here, the first deletion was accomplished by using a single homologous recombination event with a selectable marker for counter selection. We chose to utilize the *sacB* gene that results in a sucrose sensitive phenotype in certain bacteria (49, 52, 53). Following isolation and confirmation of the single homologous recombination based on antibiotic selection, *M. aquaeolei* VT8 colonies were grown in liquid culture containing sucrose in a minimal media, and enriched by several subsequent transfers to fresh media before plating and screening for loss of the antibiotic marker. Strains containing the markerless deletion were confirmed by colony PCR. The second gene deletion was then isolated using the double homologous selection method described above for single deletions. The toxicity of *sacB* in *M. aquaeolei* VT8 was not as potent as was found for controls tested in *Escherichia coli*, and is best characterized as a screening protocol under the current conditions. We suspect this may be related to poor sugar uptake by *M. aquaeolei* VT8, which does not grow well on simple sugars. However, utilizing this procedure, a double deletion strain Δ*facoar*/Δ*faldr* was obtained. Characterization of wax ester production in the Δ*facoar*/Δ*faldr* strain (Figure 14A) revealed only a minimal background of wax esters. This confirms that either enzyme is capable of supporting the wax biosynthetic pathway independent of one another, though the Δ*faldr* gene product seems to play a greater role in *M. aquaeolei* VT8 under wax ester accumulating conditions based on these gene deletion studies. It also supports the proposal that these are the two primary genes capable of producing fatty alcohols, as only trace amounts of wax esters were found in the absence of both genes.

Wax ester production phenotype rescue through addition of extraneous alcohols. To confirm the loss of the wax ester production in these deletion strains is based on the lack of this enzymatic step in the pathway, and not a secondary affect related to poor culture health for example, we utilized a strategy taken previously to add foreign fatty alcohols, specifically the odd carbon number pentadecanol that results in unique wax ester products, in an attempt to rescue the wax ester production phenotype in this double

deletion strain (18). This results in the production of odd numbered wax esters when provided extraneously to wild type cells (18) as shown in Figure 14B. This strategy relies on the fact that waxes found in *M. aquaeolei* VT8 under the growth conditions utilized here are derived primarily from C16 and C18 fatty acids (18). Thus, addition of pentadecanol (C15) to a strain lacking only the enzyme(s) involved in the reduction of fatty acids to fatty alcohols should result in wax esters containing the even fatty acids C16 and C18, and only the fatty alcohol pentadecanol. This result is confirmed in Figure 14B, with an additional large peak corresponding to a C30 wax ester resulting from pentadecanol entry and oxidation through alternative directions in the pathway (Figure 4 Chapter 2, pg. 20) resulting in some C15 fatty acid derived wax ester accumulating in the cell as well. Importantly, predominant wax esters such as C32 derived from C16 fatty acids and fatty alcohols are not present in the double deletion strain. This confirms that the wax ester biosynthesis pathway can be reconstituted *in vivo* in the double deletion strain by adding extraneous alcohols (pentadecanol).

Agreement between RT-qPCR and gene deletion experiments. The results from transcriptional levels of mRNA for the fatty aldehyde reductase and fatty acyl-CoA reductase presented in Chapter 2 correlate well with what was found in the gene deletion studies. The fatty aldehyde reductase transcription appears to be up-regulated during lipid accumulation. The fatty acyl-CoA reductase transcriptional levels are elevated primarily during exponential growth under the conditions utilized here, but dropped substantially once the cell entered into the wax ester accumulation stage (Figure 10 Chapter 2, pg. 32). Thus, the deletion of the gene coding the fatty acyl-CoA reductase (Δ*facoar*) had very little effect on levels of wax esters. However, deletion of the gene coding for the fatty aldehyde reductase (Δ*faldr*) dramatically decreased the levels of wax esters accumulated, but did not completely delete the wax ester accumulation phenotype.

Summary of findings and future efforts. These results demonstrate that while *M. aquaeolei* VT8 has incorporated redundancy within the wax ester production pathway, evolution has selected one specific branch of the reaction which yields the fatty alcohols

required for wax ester production, as the clear preferred route when producing wax esters from simple organic acids such as succinate or citrate.

The findings from these studies which looked at features of these enzymes *in vivo* in the indigenous organism contrasts with what has been found previously for these fatty alcohol producing enzymes through *in vitro* experiments (54). Studies with isolated enzymes indicate that the fatty acyl-CoA reductase is the more active of the two enzymes following purification (34, 36). Homologs to the fatty acyl-CoA reductase are also more prevalent in other model wax ester accumulating species (*Acinetobacter*, *Psychrobacter* and *Rhodococcus*), while the fatty aldehyde reductase is found less frequently (36). This was a key motivator to pursue these studies, as *M. aquaeolei* VT8 provides an ideal test case to analyze which fatty alcohol yielding enzyme evolution has selected to optimize wax ester production when both options exist within the repertoire of enzymes in the cell. As mentioned previously, additional activities have been reported for the fatty aldehyde reductase from *M. aquaeolei* VT8 since the initial characterization of this enzyme (38-39).

Future work is needed to explore means of altering the types of waxes produced in order to tailor this organism to produce specific types of wax esters which may have higher value within the industrial field. We found the plasmid pBBR1MCS-2 (Table 3) to be a suitable replication vector for *M. aquaeolei* VT8 with a copy number of approximately 33 (± 2.03) and could be used as a means of expressing heterologous genes. Copy number was determined utilizing the qPCR method described above for determining copy number of the knockout strains. Primers were designed (Table 6) for the mobilization element of the plasmid as well as the kanamycin cassette, and normalized against recombinase A (37).

Future work is also needed to reintroduce the deleted genes into the deletion strains to further verify their roles in producing the fatty alcohol substrate. Currently, the kanamycin cassette currently being used in *M. aquaeolei* VT8 has been found to be the only reliable antibiotic selection marker in this strain, leaving us without an additional antibiotic selection marker for selection of a strain with the reintroduced genes. Because

of this, future work is needed to identify alternative antibiotic selection markers or a more optimal counter selection method needs to be derived, whether it be optimizing the *sacB* method or utilizing a different method.

Once a more reliable counter-selection method has been established further studies of the four homologs to the wax synthase enzyme, which catalyzes the final step in wax production, will need to be completed. Though the data presented here offers some insight into the intricacies of this pathway by demonstrating that loosing either of the WS1 or WS2 enzymes, wax esters are still produced. Deletions of two or more of these enzymes will allow a better understanding of the redundancy in this step of the pathway and may uncover how this organism uses these redundancies to its advantage.

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References

1. Cho, J. Y. (2012) Algicidal activity of marine *Alteromonas* sp KNS-16 and isolation of active compounds, *Bioscience Biotechnology and Biochemistry* 76, 1452-1458.
2. Jung, S. W., B. H.Kim, T. Katano, D. S. Kong, and M. S. Han. (2008) *Pseudomonas fluorescens* HYK0210-SK09 offers species-specific biological control of winter algal blooms caused by freshwater diatom *Stephanodiscus hantzschii*, *Journal of Applied Microbiology* 105, 186-195.
3. Kang, Y. K., S. Y. Cho, Y. H. Kang, T. Katano, E. S. Jin, D. S. Kong, and M. S. Han. (2008) Isolation, identification and characterization of algicidal bacteria against *Stephanodiscus hantzschii* and *Peridinium bipes* for the control of freshwater winter algal blooms, *Journal of Applied Phycology* 20, 375-386.
4. Kim, D., J. F. Kim, J. H. Yim, S. K. Kwon, C. H. Lee, and H. K. Lee. (2008) Red to red - the marine bacterium *Hahella chejuensis* and its product prodigiosin for mitigation of harmful algal blooms, *Journal of Microbiology and Biotechnology* 18, 1621-1629.
5. Kim, J. D., B. Kim, and C. G. Lee. (2007) Alga-lytic activity of *Pseudomonas fluorescens* against the red tide causing marine alga *Heterosigma akashiwo* (Raphidophyceae), *Biological Control* 41, 296-303.
6. Kim, J. D., J. Y. Kim, J. K. Park, and C. G. Lee. (2009) Selective control of the *Prorocentrum minimum* harmful algal blooms by a novel algal-lytic bacterium *Pseudoalteromonas haloplanktis* AFMB-008041, *Marine Biotechnology* 11, 463-472.
7. Lee, S. J., Y. Kim, H. G. Kim, G. M. Seo, J. H. Jeong, and Y. K. Hong. (2000) Algalytic activity of alpha-mannosidase on harmful marine microalgae, *Journal of Applied Phycology* 12, 191-193.
8. Mu, R. M., Z. Q. Fan, H. Y. Pei, X. L. Yuan, S. X. Liu, and X. R. Wang. (2007) Isolation and algae-lysing characteristics of the algicidal bacterium B5, *Journal of Environmental Science (China)* 19, 1336-1340.

9. Paul, C., and G. Pohnert. (2011) Interactions of the algicidal bacterium *Kordia algicida* with diatoms: Regulated protease excretion for specific algal lysis, *Plos One* 6.
10. Shilo, M. (1970) Lysis of blue-green algae by myxobacter, *Jounal of Bacteriology* 104, 453-461.
11. Chisti, Y. (2007) Biodiesel from microalgae, *Biotechnology Advances 25*, 294-306.
12. Hu, Q., M. Sommerfeld, E. Jarvis, M. Ghirardi, M. Posewitz, M. Seibert, and A. Darzins. (2008) Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances, *Plant Journal* 54, 621-639.
13. Halim, R., M. K. Danquah, and P. A. Webley. (2012) Extraction of oil from microalgae for biodiesel production: A review, *Biotechnology Advances* 30, 709-732.
14. Mercer, P., and R. E. Armenta. (2011) Developments in oil extraction from microalgae, *European Journal of Lipid Science and Technology* 113, 539-547.
15. Chisti, Y. (2008) Biodiesel from microalgae beats bioethanol, *Trends in Biotechnology 26*, 126-131.
16. Borowitzka, M. A. (1999) Commercial production of microalgae: ponds, tanks, tubes and fermenters, *Journal of Biotechnology* 70, 313-321.
17. Raja, R., S. Hemaiswarya, and R. Rengasamy. (2007) Exploitation of *Dunaliella* for beta-carotene production, *Applied Microbiology and Biotechnology* 74, 517-523.
18. Barney, B. M., B. D. Wahlen, E. Garner, J. S. Wei, and L. C. Seefeldt. (2012) Differences in substrate specificities of five bacterial wax ester synthases, *Applied and Environmental Microbiology* 78, 5734-5745.
19. Gouveia, L., A. E. Marques, T. L. da Silva, and A. Reis. (2009) *Neochloris oleabundans* UTEX #1185: A suitable renewable lipid source for biofuel production, *Journal of Industrial Microbiology and Biotechnology* 36, 821-826.

20. Hulatt, C. J., A. M. Lakaniemi, J. A. Puhakka, and D. N. Thomas. (2012) Energy demands of nitrogen supply in mass cultivation of two commercially important microalgal species, *Chlorella vulgaris* and *Dunaliella tertiolecta*, *Bioenergy Research* 5, 669-684.

21. Tang, H. Y., N. Abunasser, M. E. D. Garcia, M. Chen, K. Y. S. Ng, and S. O. Salley. (2011) Potential of microalgae oil from *Dunaliella tertiolecta* as a feedstock for biodiesel, *Applied Energy* 88, 3324-3330.

22. Araujo, G. S., L. J. B. L. Matos, L. R. B. Goncalves, F. A. N. Fernandes, and W. Farias. R. L. (2011) Bioprospecting for oil producing microalgal strains: Evaluation of oil and biomass production for ten microalgal strains, *Bioresource Technology* 102, 5248-5250.

23. Shi, S. Y., D. S. Tang, and Y. D. Liu. (2009) Effects of an algicidal bacterium *Pseudomonas mendocina* on the growth and antioxidant system of *Aphanizomenon flos-aquae*, *Current Microbiology* 59, 107-112.

24. Cowan, A. K., P. D. Rose, and L. G. Horne. (1992) *Dunaliella salina*: a model system for studying the response of plant cells to stress, *Journal of Experimental Botany* 43, 1535-1547.

25. Pund, R. R., and D. Theegarten. (2008) The importance of aeromonads as a human pathogen, *Bundesgesundheitsblatt-Gesundheitsforschung-Gesundheitsschutz* 51, 569-576.

26. Minnaganti, V. R., P. J. Patel, D. Iancu, P. E. Schoch, and B. A. Cunha. (2000) Necrotizing fasciitis caused by *Aeromonas hydrophila*, *Heart Lung* 29, 306-308.

27. Chi, C. Y., C. H. Lai, C. P. Fung, and J. H. Wang. (2005) Pseudomonas mendocina spondylodiscitis: A case report and literature review, *Scandinavian Journal of Infectious Diseases* 37, 950-953.

28. Yen, K. M., M. R. Karl, L. M. Blatt, M. J. Simon, R. B. Winter, P. R. Fausset, H. S. Lu, A. A. Harcourt, and K. K. Chen. (1991) Cloning and characterization of a *Pseudomonas mendocina* Kr1 gene-cluster encoding toluene-4-monooxygenase, *Journal of Bacteriology* 173, 5315-5327.

29. Huu, N.B., E.B.M. Denner, D.T.C. Ha, G. Wanner, and H. Stan-Lotter. (1999) *Marinobacter aquaeolei* sp. nov., a halophilic bacterium isolated from a Vietnamese oil-producing well, *International Journal of Systematic and Evolutionary Microbiology* 49, 367-375.

30. Singer, E., E.A. Webb, W.C. Nelson, J.F. Heidelberg, N. Ivanova, A. Pati, and K.J. Edwards. (2011) Genomic Potential of *Marinobacter aquaeolei*, a Biogeochemical "Opportunitroph", *Applied and Environmental Microbiology* 77, 2763-2771.

31. Wältermann, M., A. Hinz, H. Robenek, D. Troyer, R. Reichelt, U. Malkus, H.J. Galla, R. Kalscheuer, T. Stöveken, P. von Landenberg, and A. Steinbüchel. (2005) Mechanism of lipid-body formation in prokaryotes: how bacteria fatten up, *Molecular Microbiology* 55, 750-763.

32. Manilla-Pérez, E., A.B. Lange, S. Hetzler, and A. Steinbüchel. (2010) Occurrence, production, and export of lipophilic compounds by hydrocarbonoclastic marine bacteria and their potential use to produce bulk chemicals from hydrocarbons, *Applied Microbiology and Biotechnology* 86, 1693-1706.

33. Wahlen, B.D., W.S. Oswald, L.C. Seefeldt, and B.M. Barney. (2009) Purification, characterization, and potential bacterial wax production role of an NADPH-dependent fatty aldehyde reductase from *Marinobacter aquaeolei* VT8, *Applied and Environmental Microbiology* 75, 2758-2764.

34. Wältermann, M., T. Stöveken, and A. Steinbüchel. (2007) Key enzymes for biosynthesis of neutral lipid storage compounds in prokaryotes: properties, function and occurrence of wax ester synthases/acyl-CoA: diacylglycerol acyltransferases, *Biochimie* 89, 230-242.

35. Willis, R.M., B.D. Wahlen, L.C. Seefeldt, and B.M. Barney. (2011) Characterization of a Fatty Acyl-CoA Reductase from *Marinobacter aquaeolei* VT8: A Bacterial Enzyme Catalyzing the Reduction of Fatty Acyl-CoA to Fatty Alcohol, *Biochemistry-US* 50 10550-10558.

36. Stöveken, T., and A. Steinbüchel. (2008) Bacterial acyltransferases as an alternative for lipase-catalyzed acylation for the production of oleochemicals and fuels, *Angewandte Chemie International Edition* 47, 3688-3694.

37. Takle, G.W., I.K. Toth, and M.B. Brurberg. (2007) Evaluation of reference genes for real-time RT-PCR expression studies in the plant pathogen *Pectobacterium atrosepticum*, *BMC Plant Biology* 7.

38. Hofvander, P., T.T.P. Doan, and M. Hamberg. (2011) A prokaryotic acyl-CoA reductase performing reduction of fatty acyl-CoA to fatty alcohol, *FEBS Letters* 585, 3538-3543.

39. McDaniel, R., B. Behrouzian, L. Clark, D. Hattendorf, and F. Valle. Feb 14, 2013 2013. Production of fatty alcohols with fatty alcohol forming acyl-CoA reductases (FAR) patent US20130040352.

40. Ishige, T., A. Tani, Y. Sakai, and N. Kato. (2000) Long-chain aldehyde dehydrogenase that participates in n-alkane utilization and wax ester synthesis in *Acinetobacter* sp strain M-1, *Applied and Environmental Microbiology* 66, 3481-3486.

41. McLeod, M.P., R.L. Warren, W.W.L. Hsiao, N. Araki, M. Myhre, C. Fernandes, D. Miyazawa, W. Wong, A.L. Lillquist, D. Wang, M. Dosanjh, H. Hara, A. Petrescu, R.D. Morin, G. Yang, J.M. Stott, J.E. Schein, H. Shin, D. Smailus, A.S. Siddiqui, M.A. Marra, S.J.M. Jones, R. Holt, F.S.L. Brinkman, K. Miyauchi, M. Fukuda, J.E. Davies, W.W. Mohn, and L.D. Eltis. (2006) The complete genome of *Rhodococcus* sp RHA1 provides insights into a catabolic powerhouse, *Proceedings of the National Academy of Sciences USA* 103, 15582-15587.

42. Holtzapple, E., and C. Schmidt-Dannert. (2007) Biosynthesis of isoprenoid wax ester in *Marinobacter hydrocarbonoclasticus* DSM 8798: identification and characterization of isoprenoid coenzyme A synthetase and wax ester synthases, *Journal of Bacteriology* 189, 3804-3812.

43. Wei, J., J.G. Timler, and B.M. Barney. (2013) Branched-chain 2-keto acid decarboxylases derived from cold-tolerant bacteria, *FEMS Letters* Under revision.

44. Saltikov, C. W., and D. K. Newman. (2003) Genetic identification of a respiratory arsenate reductase, *Proceedings of the National Academy of Sciences USA* 100, 10983-10988.

45. Bakermans, C., R. E. Sloup, D. G. Zarka, J. M. Tiedje, and M. F. Thomashow. (2009) Development and use of genetic system to identify genes required for efficient low-temperature growth of *Psychrobacter arcticus* 273-4, *Extremophiles* 13, 21-30.

46. Sonnenschein, E. C., A. Gärdes, S. Seebah, I. Torres-Monroy, H. P. Grossart, and M. S. Ullrich. (2011) Development of a genetic system for *Marinobacter adhaerens* HP15 involved in marine aggregate formation by interacting with diatom cells, *Journal Microbiological Methods* 87, 176-183.

47. Kovach, M. E., P. H. Elzer, D. S. Hill, G. T. Robertson, M. A. Farris, R. M. Roop 2nd, and K. M. Peterson. (1995) Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes, *Gene* 166, 175-176.

48. Taylor, L. A., and R. E. Rose. (1988) A correction in the nucleotide sequence of the Tn9O3 kanamycin resistance determinant in pUC4K, *Nucleic Acids Research* 16, 358.

49. Blomfield, I. C., V. Vaughn, R. F. Rest, and B. I. Eisenstein. (1991) Allelic Exchange in *Escherichia coli* using the *Bacillus subtilis sacB* gene and a temperature-sensitive Psc101 replicon, *Molecular Microbiology* 5, 1447-1457.

50. Reiser, S., and C. Somerville. (1997) Isolation of mutants of *Acinetobacter calcoaceticus* deficient in wax ester synthesis and complementation of one mutation with a gene encoding a fatty acyl coenzyme a reductase, *Journal of Bacteriology* 179, 2969-2975.

51. Röttig, A., and A. Steinbüchel. (2013) Acyltransferases in Bacteria. *Microbiology and Molecular Biology Reviews* 77, 277-321.

52. Pelicic, V., J. M. Reyrat, and B. Gicquel. (1996) Generation of unmarked directed mutations in *Mycobacteria*, using sucrose counter-selectable suicide vectors. *Molecular Microbiology* 20, 919-925.

53. van der Geize, R., G. I. Hessels, R. van Gerwen, P. van der Meijden, and L. Dijkhuizen. (2001) Unmarked gene deletion mutagenesis of *kstD*, encoding 3-ketosteroid Delta(1)-dehydrogenase, in *Rhodococcus erythropolis* SQ1 using *sacB* as counter-selectable marker, *FEMS Letters* 205, 197-202.

54. Liu, A., X. Tan, L. Yao, and X. Lu. (2013) Fatty alcohol production in engineered *E. coli* expressing *Marinobacter* fatty acyl-CoA reductases, *Applied Microbiology Biotechnology* 97, 7061-7071.