ABSTRACT

SCHRECK, STEVEN DANIEL. Characterization of Halophilic Thioesterase from *Chromohalobacter salexigens* for Use in Biofuel Production. (Under the direction of Dr. Amy M. Grunden.)

Lipases and esterases are enzymes which hydrolyze ester bonds between a fatty acid moiety and an esterified conjugate, such as a glycerol or phosphate. These enzymes have a wide spectrum of use in industrial applications where their high activity, broad substrate specificity, and stability under harsh conditions have made them integral in biofuel production, textile processing, waste treatment, and as detergent additives. To date, these industrial applications have mainly leveraged enzymes from mesophilic and thermophilic organisms. However, increasingly, attention has turned to halophilic enzymes as catalysts in environments where high salt stability is desired.

Acyl-CoA thioesterases are enzymes which catalyze the hydrolysis of acyl-CoAs at thioester linkages to form free fatty acids and coenzyme A. These enzymes are potential catalysts for the enrichment of fatty acids from oil-producing biofuel feedstocks. This technology coupled with an increasing interest in sustainable biofuels has led researchers to investigate algae based systems of fuel production. Since a number of marine algae that are being evaluated for biofuel production prefer moderately halophilic environments, it follows that thioesterases/lipases originating from a halophilic organism may be best suited for use with a marine algae-based biofuel system. Recent studies have shown increased production of free fatty acids in *Escherichia coli* and cyanobacteria following overexpression of recombinant, leaderless thioesterase I (TesA) from *E. coli*. A homolog of TesA from the moderate halophilic bacterium *Chromohalobacter salexigens* has been identified, cloned, and

recombinantly expressed in *E. coli* strains BL21 and M15. Because previous studies indicated that histidine tag position alters TesA substrate specificity, three different recombinant versions of the *C. salexigens* TesA were produced for this study; an N-terminal histidine-tagged enzyme, a C-terminal histidine-tagged enzyme, and one in which the N-terminal histidine-tag was removed via DAPase digestion. Introduction of a C-terminal histidine tag shifted substrate preference to shorter (<C10) carbon chain lengths, while introduction of an N-terminal tag resulted in overall reduced activity. Finally, removal of the N-terminal tag restored overall activity levels while slightly increasing enzyme preference for longer (C10/C12) carbon chain substrates. Given the requirement of lipolytic activity to generate free fatty acids for biofuel synthesis, this study has important implications for the use of thioesterases/lipases in marine algae based biofuel systems.

Characterization of Halophilic Thioesterase from *Chromohalobacter salexigens* for Use in Biofuel Production

by
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requirements for the degree of
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DEDICATION

Dedicated to my parents, Charles and Lucille Schreck. You never gave up on me and always pushed me to be the best I could be. You gave me the tools to succeed and all the love and support imaginable. I couldn't have done it without you.

Thanks Mom and Dad.

BIOGRAPHY

Steven Daniel Schreck was born in Jefferson City, Missouri and spent the first year of his life in Tipton, a small farming town in the middle of the state. When he was one year old, his father moved the family to Key West, Florida where Steve grew up. He attended the University of Florida in Gainesville, Florida where he was first introduced to research while doing an undergraduate project on *Salmonella typhimurium* pathogenesis in Dr. Paul Gulig's lab. That experience instilled in him a love for research and science. He graduated in 1992 with a B.S. in microbiology. His first job was as a researcher for a small start-up company in Mobile, Alabama developing a piezoelectric-based biosensor for tuberculosis detection. From there, he moved on to Raleigh, North Carolina where he worked as a contractor for Bayer Pharmaceuticals and he assisted in developing a novel isolation and purification process for recovery of IgG from fractionated human blood plasma. At the beginning of the dot com boom in the late 1990s, Steve transitioned his career into computers. He taught himself how to program in C++ and earned a programming certificate from North Carolina State University. He worked for the next fifteen years as a quality assurance automation developer and software developer for a subsidiary of the Charles Schwab Corporation. He re-entered the world of biological sciences in 2009 when he embarked on his M.S. degree in microbiology while working both at Charles Schwab and in Dr. Amy Grunden's lab at North Carolina State University. He will complete his Master of Science degree under the direction of Dr. Amy Grunden in June, 2013.

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CHAPTER 1

Halophilic Lipases – A Review

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Abstract

Lipases and esterases are enzymes which hydrolyze ester bonds between a fatty acid moiety and an esterified conjugate, such as a glycerol or phosphate. These enzymes have a wide spectrum of use in industrial applications where their high activity, broad substrate specificity, and stability under harsh conditions have made them integral in biofuel production, textile processing, waste treatment, and as detergent additives. To date, these industrial applications have mainly leveraged enzymes from mesophilic and thermophilic organisms. However, increasingly, attention has turned to halophilic enzymes as catalysts in environments where high salt stability is desired. This review provides a brief overview of lipases and esterases and examines specific structural motifs and evolutionary adaptations of halophilic lipases. Finally, we examine the state of research involving these enzymes and provide an in depth look at an exciting algal-based biofuel production system. This system uses a halophilic lipase to increase oil production efficiency by cleaving algal lipids into free fatty acids, which can be used directly in existing fuel conversion processes.

Introduction

Lipases and esterases are enzymes which hydrolyze ester bonds between a fatty acid moiety and an esterified conjugate, such as a glycerol or phosphate. The Enzyme Commission number for these proteins falls under EC 3.1: Enzymes Acting on Ester Bonds. Not surprisingly, there is a wide variety of esterase types depending on the bond and substrates acted upon. For the purposes of this review, we will be restricting our attention to members of the carboxylic ester hydrolases (EC. 3.1.1) and thioester hydrolases (EC. 3.1.2) and focusing on triacylglycerol lipases (EC 3.1.1.3) and acetyl-CoA hydrolases (3.1.2.1) from those respective groups.

Acetyl-CoA hydrolases are enzymes which catalyze the hydrolysis of acetate from acetyl-CoA. They accomplish this by breaking the thio-ester bond between the coenzyme-A molecule and the acetate residue at the end of acetyl-CoA. These enzymes are important in controlling intracellular concentrations of acetyl-CoA and are strongly inhibited by the presence of CoA. Similar hydrolases exist to remove other carbon moieties from the end of coenzyme-A, including succinyl-CoA hydrolase, and palmitoyl-CoA hydrolase which generate succinate and CoA and palmitate and CoA, respectively.

Another class of carboxylic ester hydrolases is the phospholipase. This class is usually categorized into four families, phospholipases A, B, C, and D, with phospholipase A further subdivided into phospholipase A1 and A2. These enzymes are responsible for breaking down phospholipids into free fatty acids and various other intermediates depending on which fatty acid chain was hydrolyzed. Phospholipase A1 and A2 are responsible for

hydrolysis of the SN-1 and SN-2 acyl chains, respectively, while phospholipase B cleaves both SN-1 and SN-2 chains. Phospholipase C cleaves the ester bond before the phosphate group at the C3 position of the glycerol in the phospholipid, while phospholipase D cleaves after the phosphate.

Triacylglycerol lipases are enzymes which catalyze the stepwise hydrolysis of free fatty acids from long chain triacylglycerides. They accomplish this by breaking the ester bond between the fatty acid side chain and the glycerol backbone of the triacylglyceride. Since the substrates acted upon are only slightly water soluble, the lipase must work at the interface between the hydrophobic fatty acid and the surrounding aqueous medium This unique operating environment, coupled with specificity for longer fatty acid side chains distinguishes the lipase from other types of esterases, which generally hydrolyze water soluble shorter chain acyl esters [1].

Both classes of enzymes are well established in the biotechnology arena where their high activity, broad substrate specificity, and stability under harsh industrial conditions have made them integral in biofuel production, textile processing, waste treatment, as detergent additives and many other applications [2]. These qualities may be further optimized by using specific lipases or thioesterases from extremophiles that are best suited for activity under specific industrial or environmental conditions. For example, halophilic microorganisms have been shown to degrade petroleum hydrocarbons in marine environments [3, 4] and lipases can function to break down fatty acids and oils; therefore, it is possible that a lipase

or thioesterase from a halophilic organism could be more adept at functioning in a marine environment and may provide for more efficient bio-remediation of oil spills.

Halophilic or halotolerant organisms are bacteria or archaea that have evolved to live under high salt conditions such as a marine environment or salterns where seawater or brine is collected and allowed to evaporate for salt harvesting. Halophilic organisms can be divided into five groups [5] according to salt tolerance: non-halophiles (< 0.2 M salt), slight halophiles (0.2-0.5 M salt), moderate halophiles (0.5-2.5 M salt), borderline extreme halophiles (1.5-3.0M salt), and extreme halophiles (2.5-5.2M salt). The proteins produced by these organisms have a number of adaptations which allow them to remain active in such high salt concentrations [6-9]. These adaptations, coupled with the existing robust nature of lipases in general make halophilic lipases especially attractive for industrial and biotechnological use. To date, research on lipases and enzymes from extremophiles has largely been directed towards thermophiles, but with the advent of marine algae based biofuel systems, more attention is being given to these salt loving micro-organisms and the enzymes they produce.

General Structure of Lipases

The general structure of most lipases and thioesterases is conserved and consists of an α/β 'hotdog' fold structure where seven or eight anti-parallel beta sheets form the 'bun' and wrap around a five or six turn alpha helical hotdog [10, 11]. A representative schematic diagram is shown in Figure 1-1. The actual solved three dimensional structure of *Escherichia coli* TesA is shown in Figure 1-2.

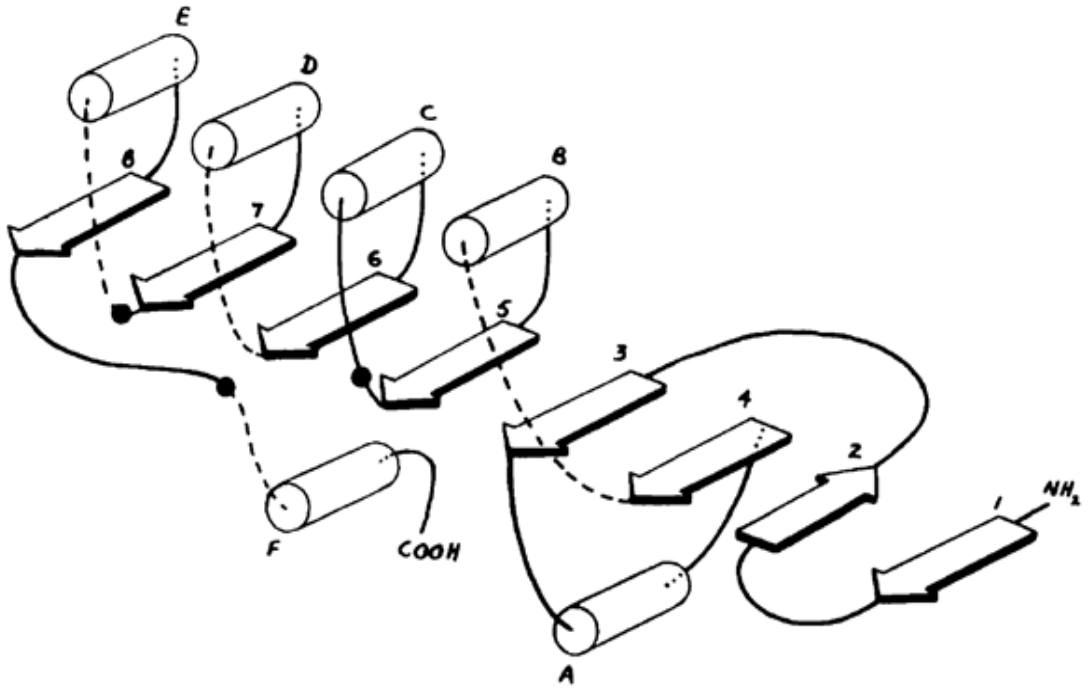

Figure 1-1. A schematic diagram of the α/β hydrolase fold. (Reproduced with permission, from David L. Ollis, Eong Cheah, Miroslaw Cygler, Bauke Dijkstra, Felix Frolow, Sybille M. Franken, Michal Harel, S. Jamse Remington, Israel Silman, Joseph Schrag, Joel L. Sussman, Koen H.G. Verschueren, Adrian Goldman, (1992), Protein Engineering, Design and Selection, 5(3):197-211. © Oxford University Press.)

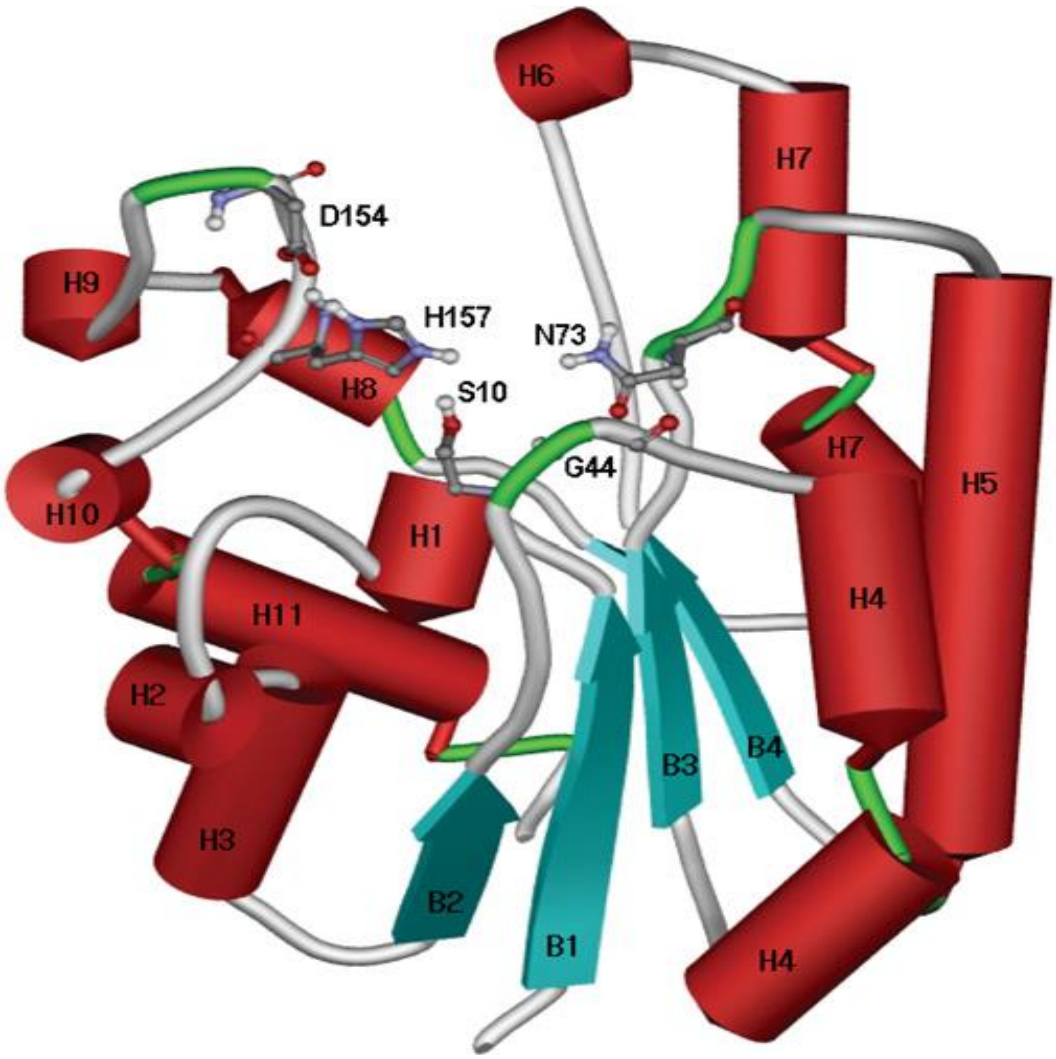

Figure 1-2. The three-dimensional structure of TAP was solved by crystallography (PDB code 1IVN), and prepared using the ViewerLite (version 5.0) program, showing 11 helices and a five-stranded parallel β-sheet. The indicated residues are the catalytic triad, Ser10-Asp154-His157, and the stabilizing oxyanion residues, Gly44 and Asn73. H represents helices, including α-helices and 310-helices. B represents β-strands, and B5 located behind B4 is not labelled. Ser10 serves as a nucleophile, and its amide proton can be devoted to hydrogen-bonding with oxyanion, a tetrahedral-carbon-covalent acyl-enzyme intermediate. The Gly44 residue, whose amide proton is devoted to hydrogen-bonding with oxyanion, is located in a loop region. Asp73 is located in α3 by NMR or in a loop region of the crystal, and its side-chain Hδ serves as a proton-donor in the oxyanion hole. Both Asp154 and His157 are located in a loop region, and they combine with catalytic Ser10 to form the catalytic triad. (Reproduced with permission, from Lee, LC, Lee, YL, Leu, RJ and Shaw, JF (2006), Biochemical Journal, 397(1) 69-76. © the Biochemical Society.)

The active site in these molecules is also largely conserved and consists of a catalytic triad of amino acids Ser-Asp-His. In *E. coli* TesA (thioesterase I EC 3.1.2.2) these three amino acids are in position Ser^{10}, Asp^{154}, and His^{157} as shown in Figure 1-3.

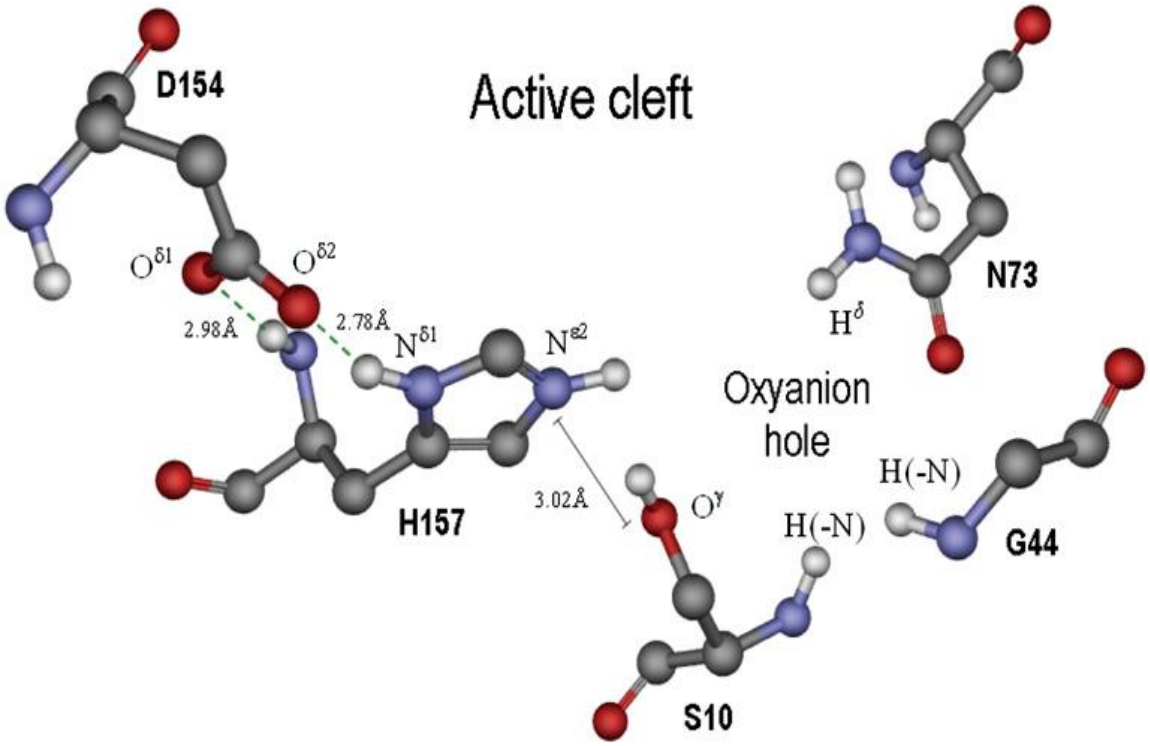

Figure 1-3. The active cleft of TAP solved by crystallography. The left-hand side is lined with three catalytic triad resides, Asp154, His157 and Ser10. When active Ser10 attacks the ester substrate, an oxyanion intermediate will form, which will be stabilized with the residues on the right-hand side, Gly44 and Asn73. Each atom of the amino acid residues that is involved in the interaction with substrate or with the other residues in the diagram is denoted using amino acid side-chain nomenclature. Nε2 of His157 in the imidazole ring is protonated in Figure, but the catalytic activity can only be performed when the proton is dissociated, by which the lone electron pair of Nε2-His157 is provided for the attraction of Hγ -Ser10. Reproduced with permission, from Lee, LC, Lee, YL, Leu, RJ and Shaw, JF (2006), Biochemical Journal, 397(1) 69-76. © the Biochemical Society.)

These residues coordinate with two additional amino acids, Gly^{44} and Asn^{73} to form an oxyanion hole. Ser^{10} not only participates in the catalytic site, it forms part of the oxyanion hole. It is thought that this hole allows for stabilization of the oxyanion intermediate formed in the hydrolysis reaction when the Ser^{10} hydroxyl group attacks the ester's carbonyl carbon, and also provides a hole through which the long chain fatty acids may extend and provide more stability and proper orientation for the catalytic residues to do their work [10]. Of the three catalytic residues, His^{157} and Ser^{10} are the most vital. The serine residue initiates a nucleophilic attack on the ester's carbonyl carbon while the histidine and aspartic acid residues stabilize the structure via hydrogen bonding between active site residues and substrate. Finally, site directed mutagenesis experiments where each of the catalytic and oxyanion hole residues were mutated to alanine showed the strongest reduction in activity when Ser^{10}, and His^{157} were changed [10].

Most, but not all, esterases and lipases exhibit the α/β-hydrolase structure mentioned above. A novel acyl-CoA thioesterase from *E. coli* TesB (thioesterase II, EC. 3.1.2.1) behaves in a different manner [12]. This enzyme has a catalytic triad, but it is formed by Asp^{204}, Gln^{278}, and Thr^{228}. Instead of an active site serine conducting a nucleophilic attack, this triad stabilizes and orients a water molecule to attack the substrate's ester linkage. The overall structure of this enzyme is different as well, behaving as a 'double hotdog' dimer (Figure 1-4A) with the active site and oxyanion hole existing at the interface between the two dimers and their structural repeats (Figure 1-4B) [12].

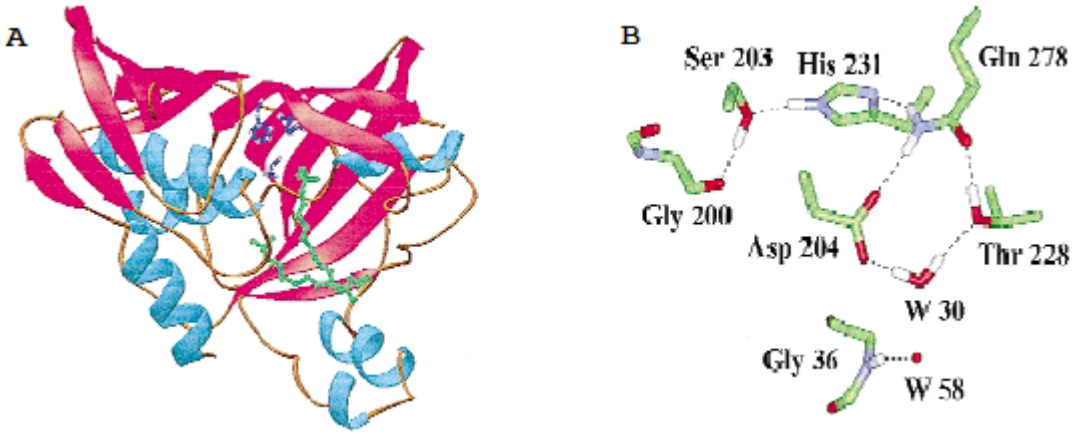

Figure 1-4. A, Ribbon stereo diagram showing the location of the active site residues (colored blue) in relation to the putative substrate binding pocket. **B**, Stereo view of the hydrogen bond network in the active site. W30 is the putative nucleophilic water and has two protons (shown in white) located to optimize the hydrogen bond geometry. Protein protons that can be placed according to stereochemical constraints are all shown. W58 is the water molecule that occupies the putative oxyanion hole. (Reproduced with permission, from Jia Li,Urszula Derewenda, Zbigniew Dauter, Stuart Smith and Zygmunt S. Derewenda, (2000), (Nature Structural and Molecular Biology), 7(7):555-9. © Nature Publishing Group.)

Properties of Halophilic and Halotolerant Enzymes

In order to understand why certain halophilic organisms have evolved the adaptations they have, one must understand the constraints forced on them by their environment and how they have developed mechanisms to deal with those constraints. There are two main strategies used by halophiles to cope with their high salt environment [6, 7]. Examples are listed in Table 1-1. One strategy is called ‘the compatible solute’ strategy while the other is called ‘the salt in’ strategy. Organisms using the former strategy cope with high salt conditions by having active ion transporters on their surface which actively pump salt out of the cell while importing solutes that are more compatible with its intracellular contents such

as ectoine and glycine betaine [9, 13, 14]. These organisms generally do not exhibit large adaptive changes in intracellular enzymes, but their cell walls, transporters, and periplasmic proteins typically are modified for function in high salt environments.

Organisms following the 'salt in' strategy do not appear to rely on export of salt ions from the cell, choosing instead to adapt to high salt concentrations inside the cell. The presence of high levels of salt inside a cell presents a number of challenges. It dramatically reduces the amount of water available to cellular components and promotes aggregation of proteins that have hydrophobic regions on their surface. It also interferes with ionic and electrostatic interactions between proteins. To deal with these challenges, 'salt in' organisms and periplasmic enzymes from 'compatible solute' organisms have evolved several adaptations described in turn below [6, 7, 15-17]. To prevent protein aggregation and electrostatic interaction hindrance, halophilic enzymes have many acidic amino acid residues and a reduced number of hydrophobic residues on exteriors of proteins [18, 19]. The high negative charge associated with the acidic amino acids is offset by the high cation concentration inside the cell. Normally this would be expected to be sodium ions, but these organisms prefer to use potassium counter ions instead because potassium has a much lower water binding rate than sodium [20]. This preference for potassium also helps maximize the amount of water available for the enzymes. Lastly, since an organism cannot divest itself of all hydrophobic amino acids, halophiles will preferentially use hydrophobic amino acids with short side chains (Val, Gly, and Ala) [8, 15, 21-23]. It should be noted that one of the reasons halophilic lipases and enzymes are interesting for biotechnological applications stems from

their ability to retain activity in the presence of low water conditions. These adaptations would prove useful in harsh industrial environments where enzymatic reactions may need to be carried out in an organic solvent, for example DMSO [24].

Work by Siglioccolo et al. investigated many of these haloadaptations from the perspective of three dimensional structure [9]. The researchers looked at fifteen different enzymes from halophiles, some of which used the 'salt in' approach and some of which used the 'compatible solutes' approach of adaptation. They compared the three dimensional structures of these proteins with their non-halophilic homologs and found an overall reduction in surface hydrophobicity in halophiles coupled with an increase in negative surface charge due to increased oxygen and nitrogen containing amino acid side chains. They also determined that the most common amino acid substitution in the halophilic enzymes was a substitution of an isoleucine with a valine.

Table 1-1. Halophilic Adaptations and Examples

Halophilic Adaptation	Examples
Salt In Adaption Strategy	
Minimize basic and positively charged amino acids in favor of acidic and negatively charged amino acids	DNA-protecting protein from *Halobacterium salinarum* [9] Dodecin from *Halobacterium salinarum* [9] Nucleoside diphosphate kinase from *Halobacterium salinarum* [9] LipC from *Haloarcula marismortui* [25] Periplasmic binding components of ABC transport systems in *Chromohalobacter salexigens* [17] Cytoplasmic membrane-bound transferrin and carbonic anhydrase in *Dunaliella salina* [26]
When hydrophobic amino acids must be used, use amino acids with short side chains	Catalase-peroxidase from *Haloarcula marismortui* [9] Nucleoside diphosphate kinase from *Halobacterium salinarum* [9] LipC from *Haloarcula marismortui* [25]
Use potassium instead of sodium as counter-ions inside cell to reduce water binding.	*Halobacterium* NRC-1 [17] *Halobacterium salinarum* [6] *Haloarcula marismortui* [6]
Compatible Solute Adaptation Strategy	
Import high levels of organic solutes	*Dunaliella* sp producing glycerol [6] *Natronococcus occultus* producing 2-sulfo-trehalose [27] *Halomonas halodenitrificans, Marinococcus* strain M52,*Nocardiopsis lucentensis* A5-1 producing ectoine [27]

Table 1-1 Continued

Active pumping of ions out of cytoplasm	*Vibrio alginolyticus* [27] *Halomonas variabilis* [27] *Salinivibrio costicola* [27] *Pseudomonas halosaccharolytica* [27]

Halophilic Lipase Research and Industrial Applications

To date, limited research has been undertaken regarding halophilic lipases or esterases and their applications in industrial processes. Instead, thermophilic lipases have garnered the lion's share of attention. However, as researchers search for additional enzymes to improve existing biotechnological applications, or design entirely new ones, halophilic organisms and the enzymes they produce are gaining traction. Increasingly, researchers are turning to archaea as sources of halophilic enzymes, and several studies have been done to assess their applicability for lipase production in particular [28-33]. Table 1-2 summarizes these enzymes, the organism from which they originated, and their characteristics.

Boutaiba et al. [28] isolated fifty four *Halobacteria* from a salt lake in the Algerian Sahara and screened thirty five of these strains for lipolytic activity. Of those 35 strains, strain TC6 was selected for further study. Identified as belonging to the *Natronococcus* genus, TC6 was assayed for optimal lipolytic activity over a range of parameters, including substrate, temperature, pH and salt concentration. They found that the culture supernatant from cultures in stationary phase contained a lipase that was optimally active at 4M NaCl, pH 7 and 50^{o}C and was most active against the substrate p-nitro-phenyl palmitate (C16). Activity was inhibited by PMSF, indicating the lipase(s) had a serine catalytic site. Long term stability

at 4°C was investigated and results indicated a stable protein with no loss of function after six months of storage at 4°C. Heat stability of the enzyme along with 4 M NaCl stabilization was investigated with 80% relative activity being retained in both buffers with and without 4 M NaCl following incubation at 50°C for one hour. NaCl stabilization was evident at 80°C incubation where activity decay curves showed non-NaCl containing buffers were 50% degraded after 35 min at 80°C while the 4 M NaCl containing buffers did not reach 50% degradation until 76 min had passed at 80°C. Additionally, this lipase was shown to be a true lipase as it was capable of hydrolyzing olive oil over a variety of salt concentrations. This study provided the first report of a true lipase identified in archaea.

Muller-Santos et al. [30] took a different tack with an esterase in the archaeon *Haloarcula marismortui*. They cloned an esterase coding gene *lipC* from *H. marismortui* into *E. coli*, over-expressed, purified and biochemically characterized it. Optimal activity for the enzyme was detected against short chain fatty acids and monoesters, inhibition by PMSF was demonstrated, and a high acidic amino acid composition for the enzyme was shown. Most notably, this work was the first to show salt dependent folding of an esterase from an archaeon. Circular dichroism assays indicated that the esterase was completely unfolded in the absence of salt, and the protein began to re-fold in the presence of 0.25-0.5 M KCl. Ultimately, refolded protein recovered approximately 60% of its activity at 2 M KCl. Finally, the investigators generated a series of structural models for their enzyme and analyzed it in terms of amino acid composition and charge distribution. The later was estimated from the electrostatic potentials over the model. This analysis demonstrated that the surface of the

enzyme was populated with negatively charged acidic amino acid residues. This is in keeping with earlier observations of haloadaptations using the 'salt-in' approach, where the large numbers of charged amino acid residues likely assist in solvation and stability of the enzyme under high salt conditions.

Ozcan et al. [31, 32] screened 118 archaeal strains for lipolytic activity and found eighteen strains that possessed it. Five strains were characterized, and it was shown that the esterase component provided more activity than the lipase component of the crude cell preparations. The highest esterase activity was obtained at pH 8 – 8.5, temperatures between 60 – 65°C and NaCl concentrations ranging from 3 – 4.5 M. The same parameters for the highest lipase activities were found to be pH 8, temperatures between 45 – 65°C and NaCl concentration from 3.5 – 4 M. The esterase component preferred para-nitro phenol butyrate (C4) as the substrate while the lipase component preferred para-nitro phenol palmitate (C16).

Additionally, several groups have isolated and characterized lipases and esterases from halophilic bacteria. [24, 25, 34-36] Amoozegar et al. [34] isolated and characterized fifty halophilic bacteria from various environments in Iran and assayed them for extracellular lipase activity. One strain, SA-2 was identified for further study. It was shown to be from the genus *Salinivibrio* and to produce an extracellular lipase that exhibited optimum activity at a salt concentration, pH, and temperature of 0.5 M NaCl, pH 7.5, and 50^{o}C, respectively. The enzyme showed temperature tolerance as well as salt tolerance, retaining approximately 40% of its activity after one hour of incubation at 80^{o}C.

Jiang et al. [35] cloned, over-expressed and purified an esterase PE10 from *Pelagibacterium halotolerans* and found it to be active in 3M NaCl and that it preferred para-nitro phenyl acetate (C2) as a substrate. The enzyme was shown to be unstable at temperatures above 40^{o}C for longer than 10 minutes, losing 60% of its activity. The enzyme was further characterized with respect to its three dimensional structure, and again a large number of negatively charged (acidic) amino acids were shown to be on its surface. This is in keeping with known haloadaptations mentioned earlier. PE10 was also stable in the presence of some organic solvents and in detergent, maintaining more than 50 % of its activity in individual reaction mixes containing 15 % DMSO or 15 % methanol or 1 % Triton X-100. These characteristics could be useful in industrial applications where activity under non-aqueous conditions may be desired, but the long term thermostability issues noted earlier will likely limit its practicality in most industrial settings.

Sana et al [24] isolated 138 obligate marine bacteria and screened them for extracellular esterase production. Five promising microorganisms producing high levels of esterase were further assayed for activity under high salt and acidic pH conditions. From these assays, one organism was selected for further purification and study. The organism was grown to stationary phase and the culture media containing the esterase was harvested. The enzyme was subjected to ammonium sulfate precipitation followed by anion and cation exchange column purification. Ultimately, a pure sample of esterase containing buffer was isolated and characterized. That characterization revealed a DMSO tolerant esterase from a *Bacillus* species of bacteria and showed that it could tolerate 1 M NaCl with maximal activity

at pH 8 and 40°C. PMSF inhibited the enzyme's activity indicating that it is a serine esterase. The presence of detergents (5 mM SDS) and reducing agents (5 mM B-mercapto-ethanol, and 5 mM dithiothreitol) also led to activity inhibition. The last two results supported the data discovered in SDS-PAGE analysis of the enzyme showing it to be a dimer. They found that incubation and reaction in the presence of 30% DMSO increased activity by 2-3 fold when compared to aqueous incubation alone. Additionally, the enzyme was stable in 30-80% DMSO for 10 days at 30°C. This stability, coupled with organic solvent tolerance suggests this enzyme could find a use as an immobilized biocatalyst in a non-aqueous process.

Finally, interesting work regarding a more concrete industrial application for halophilic lipases came from Perez et al. [36] These investigators cloned a lipolytic enzyme LipBL from the moderate halophile *Marinobacter lipolyticus* SM19 into *E. coli*, over-expressed and purified it. Notably, this enzyme's maximal activity was found to be in the absence of NaCl. Addition of 0.5 M NaCl inhibited activity by 80%. Yet, the enzyme showed 20% activity at NaCl concentrations up to 4M. While not a truly halophilic enzyme, the protein showed remarkable stability in a wide variety of organic solvents including DMSO, DMF, methanol, ethanol, toluene, diethylether, propanol and acetone, all at 30% concentration. LipBL was immobilized on various solid supports and retained 92% of its activity against para-nitro phenol substrates when immobilized on a CNBr support. Under these conditions, LipBL was assayed for its ability to produce both EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid) from fish oil. The fish oil used in the experiment

contained 18% of EPA, increasing to 27% in the released fatty acids by the activity of the CNBr derivative. This represents an enrichment of 45%. DHA, however, was only enriched 4%. These polyunsaturated fatty acids are important components in health food preparations and dietary supplements so any improvement in their production would be of interest to the food and health industry [37-41]. These results indicate that immobilized LipBL might be a good candidate for preparation of polyunsaturated fatty acids on an industrial scale.

Table 1-2. Halophilic Esterases and Lipases and Their Characteristics

Organism	Halophilic Esterase, Thioesterase, or Lipase	Characteristics
Natronococcus sp. TC6	Culture supernatant	**Substrate**: p-nitrophenyl palmitate **Opt. Temp:** 50^{o}C **Opt. pH:** 7.0 **Opt. salt conc:** 4M NaCl [28]
Haloarcula marismortui	LipC	**Substrate**: vinyl butyrate **Opt. Temp:** ND **Opt. pH:** 8.5 **Opt. salt conc:** 3M KCl [30]
Salinivibrio sp. SA2	Whole cells	**Substrate**: p-nitrophenyl butyrate **Opt. Temp:** 50^{o}C **Opt. pH:** 7.5 **Opt. salt conc:** 0.5M NaCl [34]
Pelagibacterium halotolerans	PE10	**Substrate**: p-nitrophenyl acetate **Opt. Temp:** 50^{o}C **Opt. pH:** 7.5 **Opt. salt conc:** 3M NaCl [35]

Table 1-2 Continued

Bacillus sp.	Unknown. Culture supernatant purified via ammonium sulfate precipitation followed by anion/cation exchange	**Substrate**: p-nitrophenyl acetate **Opt. Temp:** 45^{o}C **Opt. pH:** 7.5 **Opt. salt conc:** 1M NaCl [24]
Marinobacter lipolyticus SM19	LipBL	**Substrate**: p-nitrophenyl hexanoate **Opt. Temp:** 80^{o}C **Opt. pH:** 7 **Opt. salt conc:** 0 - 0.25M NaCl [36]
Chromohalobacter salexigens	TesA	**Substrate**: p-nitrophenyl octanoate **Opt. Temp:** 45^{o}C **Opt. pH:** 8.0 **Opt. salt conc:** 1M NaCl [This study]

Future Opportunities for Halophilic Lipases in Biotechnology

One particular interest in halophilic lipases is their application in microalgae based biofuel systems. One approach, which has already been shown to work in bacteria [42] and cyanobacteria [43], is to genetically modify marine algae such as *Dunaliella* sp. to overproduce triacylglycerides of specific desired fatty acid chain lengths and saturation. These triacylglycerides would subsequently be hydrolyzed into free fatty acids and glycerol by recombinant lipases and used as feedstock for biofuel production. This approach, when combined with an effective lipid sink [44] in the culture medium, could yield significant improvements over existing algae based systems, as it would allow the free fatty acids to be secreted from the algae and harvested on an ongoing basis. This would avoid the expensive

and time consuming steps involved in physical destruction of the algae and mechanical harvesting of lipid content [45]. In addition, this approach would avoid the need for extensive degumming of the extracted free fatty acids as sulfur and phosphorus contaminants could be contained in the microalgae when the fatty acids are secreted.

The requirement of lipolytic activity to form free fatty acids from triacylglycerides is not the only reason for the inclusion of a recombinant lipase in the system. There is also a metabolic need for the lipase, which may be understood by looking at the fatty acid synthesis cycle in bacteria, archaea and microalgae. The first committed and regulated step in fatty acid synthesis is the conversion of acetyl-CoA to malonyl-CoA by acetyl-CoA carboxylase (ACCase) [46, 47]. This enzyme, if over-expressed, has been shown to flux carbon through the fatty acid synthesis pathway resulting in increased production of long chain acyl-ACPs [48]. These long chain hydrocarbons, in turn, feedback inhibit the recombinant ACCase [49]. The presence of a recombinant lipase would allow for hydrolysis of the long chain acyl-ACPs and relieve the feedback inhibition, effectively pulling carbon through the fatty acid synthesis pathway [48].

Because the environment in which most marine algae thrive is moderately halophilic, and since increased salt concentrations in the microalgae culture media may influence length and saturation of fatty acids produced, the system could utilize a recombinant lipase from the moderate halophile *Chromohalobacter salexigens.* This bacterium was isolated from a saltern in the Netherlands [50]. Since both *Dunaliella* and *C. salexigens* normally grow under

aerobic, halophilic conditions, it is reasonable to expect that transgenic ACCase and lipase enzymes from *C. salexigens* would be active if expressed in the microalgae.

One particular lipase that could be expressed in *Dunaliella* is a *C. salexigens* homolog of the *E. coli* TesA protein. The TesA protein has been shown to be active over a wide range of conditions and is specific for fatty acid chains with twelve to eighteen carbons in length [51]. This substrate specificity is important because algal oils with lengths in this range are most amenable to direct conversion to transportation fuels. *E. coli* TesA lipase is a small monomeric enzyme 20.5 kDa in size. Structurally, the enzyme falls into the α/β-hydrolase family of esterases and possesses two short consensus sequences common to the enzymes in that family [52]. These sequences are the N-terminal Gly-X-Ser-X-Gly motif followed by the C-terminal Gly-X-His motif, separated from one another by 133 – 170 amino acids (Figure 1-4). It should be noted that these motifs are functionally identical to the Ser^{10}, Asp^{154}, and His^{157} and Ser^{10}, Gly^{44}, Asn^{73} motifs mentioned earlier.

One notable difference between the halophilic enzyme and its mesophilic counterpart is that the *C. salexigens* homolog (CsTesA) does not exhibit a membrane specific N-terminal leader sequence. This indicates that, while very similar, the two enzymes are not identical and probably serve different roles in the two organisms and may have different activity and substrate specificity. Catalytically, however, both enzymes possess the conserved serine esterase family sequences mentioned above including the N-terminal active site serine and C-terminal active site histidine.

Another promising reason for the selection of a TesA homolog from *C. salexigens* is the success seen in production of free fatty acid from cyanobacteria using a recombinant, leaderless version of the *E. coli* TesA enzyme [43]. In these experiments, a series of recombinant *Synechocystis* sp. cyanobacteria strains were modified to over-express a leaderless TesA protein. Additionally, successive modifications were made to components of the fatty acid synthesis system in order to optimize free fatty acid secretion. The results were encouraging, showing dramatic increase in secretion of saturated C12, C16, and C18 fatty acids [43].

Other groups have shown similar results when they combined modifications to fatty acid synthesis with over-expression of leaderless TesA in *E. coli* [42, 53]. These experiments also showed increased secretion of C12, C14, and C16 free fatty acids at high levels. Additionally, it was shown that specific modifications to the fatty acid synthesis system, coupled with optimized growth conditions and high levels of TesA activity, resulted in specific chain length and saturation of free fatty acids [42, 53].

Given that saturated algal oils with carbon chain lengths of C12 to C14 are most amenable to jet fuel production, it is important that the lipase introduced into the algae be able to hydrolyze fatty acids of that length or greater. The research mentioned above indicates that in both cyanobacteria and *E. coli*, saturated fatty acid products of appropriate length are produced from over-expression of TesA. However, additional research indicates that modification of TesA via attachment of a C-terminal histidine tag results in a shift in

substrate preference towards shorter carbon chain lengths [54]. The effects of such tagging would need to be evaluated prior to implementation of the transgenic construct in *Dunaliella.*

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CHAPTER 2

Characterization of a Halophilic Acyl-CoA Thioesterase from *Chromohalobacter salexigens* for Use in Biofuel Production

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Abstract

Acyl-CoA thioesterases are enzymes which catalyze the hydrolysis of acyl-CoAs at thioester linkages to form free fatty acids and coenzyme A. These enzymes, along with lipases, have a wide spectrum of use in industrial applications including the enrichment of fatty acids from oil-producing biofuel feedstocks. This technology coupled with an increasing interest in sustainable biofuels has led researchers to investigate algae based systems of fuel production. Since a number of marine algae that are being evaluated for biofuel production prefer moderately halophilic environments, it follows that thioesterases/lipases originating from a halophilic organism may be best suited for use with a marine algae-based biofuel system. Recent studies have shown increased production of free fatty acids in *Escherichia coli* and cyanobacteria following overexpression of recombinant, leaderless thioesterase I (TesA) from *E. coli*. A homolog of TesA from the moderate halophilic bacterium *Chromohalobacter salexigens* has been identified, cloned, and recombinantly expressed in *E. coli* strains BL21 and M15. Because previous studies indicated that histidine tag position alters TesA substrate specificity, three different recombinant versions of the *C. salexigens* TesA were produced for this study; an N-terminal histidine-tagged enzyme, a C-terminal histidine-tagged enzyme, and one in which the N-terminal histidine-tag was removed via DAPase digestion. Introduction of a C-terminal histidine tag shifted substrate preference to shorter (<C10) carbon chain lengths, while introduction of an N-terminal tag resulted in overall reduced activity. Finally, removal of the N-terminal tag restored overall activity levels while slightly increasing enzyme preference

for longer (C10/C12) carbon chain substrates. Given the requirement of lipolytic activity to generate free fatty acids for biofuel synthesis, this study has important implications for the use of thioesterases/lipases in marine algae based biofuel systems.

Introduction

Acyl-CoA thioesterases are enzymes which hydrolyze Acyl-CoAs at thioester linkages to form free fatty acids and coenzyme A. This activity differentiates them from lipases which catalyze the hydrolysis of free fatty acids from long chain triacylglycerides. This specificity for longer, water insoluble fatty acids differentiates lipases from esterases which generally only hydrolyze water soluble shorter chain acyl esters [1]. Both classes of enzymes are well established in the biotechnology arena where they are integral in biofuel production, textile processing, waste treatment, as detergent additives and many other applications [2].

Of particular interest is the application of thioesterases/lipases in microalgae based biofuel systems. One approach, which has already been shown to work in bacteria [3] and cyanobacteria [4], is to genetically modify marine algae such as *Dunaliella* sp. to overproduce triacylglycerides of specific desired fatty acid chain lengths and saturation. These triacylglycerides would subsequently be hydrolyzed into free fatty acids and glycerol by recombinant thioesterases/lipases and used as feedstock for biofuel production. This approach, when combined with an effective lipid sink [5] in the culture medium, could yield significant improvements over existing algae based systems, as it would allow the free fatty acids to be secreted from the algae and harvested on an ongoing basis. This would avoid the expensive and time consuming steps involved in physical destruction of the algae and mechanical harvesting of lipid content [6]. In addition, this approach would avoid the need

for extensive degumming of the extracted free fatty acids as sulfur and phosphorus contaminants could be contained in the microalgae when the fatty acids are secreted.

The requirement of lipolytic activity to form free fatty acids from triacylglycerides is not the only reason for the inclusion of a recombinant lipase in the system. There is also a metabolic need for the lipases and thioesterases, which may be understood by looking at the fatty acid synthesis cycle in bacteria, archaea and microalgae. The first committed and regulated step in fatty acid synthesis is the conversion of acetyl-CoA to malonyl-CoA by acetyl-CoA carboxylase (ACCase) [7, 8]. This enzyme, if over-expressed, has been shown to flux carbon through the fatty acid synthesis pathway resulting in increased production of long chain acyl-ACPs [9]. These long chain hydrocarbons, in turn, feedback inhibit the recombinant ACCase [10]. The presence of recombinant thioesterases would allow for hydrolysis of the long chain acyl-ACPs and relieve the feedback inhibition, effectively pulling carbon through the fatty acid synthesis pathway [9].

Because the environment in which most marine algae thrive is moderately halophilic, and since increased salt concentrations in the microalgae culture media may influence length and saturation of fatty acids produced, the system could use recombinant thioesterases and lipases from the moderate halophile *Chromohalobacter salexigens.* This bacterium was isolated from a saltern in the Netherlands [11]. Its optimal growth conditions are similar to the *Dunaliella* species of microalgae, and the two microorganisms share sufficient homology to suggest that transgenic ACCase and thioesterases and lipases from *C. salexigens* would be active in the microalgae.

The acyl-coA thioesterases we have chosen for expression in *Dunaliella* is a homolog of *E. coli*'s TesA protein which has been shown to be active over a wide range of conditions and is specific for fatty acid chains with twelve to eighteen carbons in length [12]. This substrate specificity is important because algal oils with lengths in this range are most amenable to direct conversion to transportation fuels. TesA is a small monomeric enzyme 20.5 kDa in size and possesses a 26 amino acid leader peptide which targets it for export to the periplasm [13]. Structurally, the enzyme falls into the family of esterases known as serine esterases and possesses two short consensus sequences common to the enzymes in that family [13]. These sequences are the N-terminal Gly-X-Ser-X-Gly motif followed by the C-terminal Gly-X-His motif, separated from one another by 133 – 170 amino acids. The specific sequences present in both the *E. coli* and *C. salexigens* enzymes are shown in Figure 2-1. It should be noted that in both enzymes the Gly-X-Ser-X-Gly motif is not followed explicitly, but the active site serine (S^{10}) is conserved as is the very important downstream histidine in the Gly-X-His sequence.

The *C. salexigens* homolog (CsTesA) does not possess a membrane specific N-terminal leader sequence. This indicates that, while very similar, the two enzymes are not identical and likely serve different roles in the two organisms and may have different activity and substrate specificity. Catalytically, however, both enzymes have the conserved serine esterase family sequences mentioned above including the N-terminal active site serine (*C. salexigens* TesA amino acid residue number 18) and C-terminal active site histidine (*C. salexigens* TesA amino acid residue number 165).

Another promising reason for the selection of a TesA homolog from *C. salexigens* is the success seen in production of free fatty acids from cyanobacteria using a recombinant, leaderless version of the *E.coli* TesA enzyme [4]. In these experiments, a series of recombinant *Synechocystis* sp. cyanobacteria strains were modified to over-express a leaderless TesA protein, as well as successive modifications to components of the fatty acid synthesis system to optimize free fatty acid secretion. The results were encouraging, showing dramatic increase in secretion of saturated C12, C16, and C18 fatty acids [4].

Other studies have shown similar results when modifications to fatty acid synthesis were combined with over-expression of leaderless TesA in *E.coli* [3, 14]. These experiments also showed increased secretion of C12, C14, and C16 free fatty acids at high levels. Additionally, it was shown that specific modifications to the fatty acid synthesis system, coupled with optimized growth conditions and high levels of TesA activity, resulted in specific chain length and saturation of free fatty acids [3, 14].

Given that saturated algal oils with carbon chain lengths of C12 to C14 are most amenable to jet fuel production, it is important that the thioesterases and lipases introduced into the algae be able to hydrolyze fatty acids of that length or greater. The research mentioned above indicates that in both cyanobacteria and *E. coli*, saturated products of appropriate length are produced from overexpression of TesA. However, additional research indicates that modification of TesA via attachment of a C-terminal histidine tag results in a shift in substrate preference towards shorter carbon chain lengths [15]. To further evaluate the effects of histidine tag position on CsTesA activity, C-terminal and N-terminal tagged

variants of the enzyme were expressed and biochemically characterized. In addition, a third variant in which the N-terminal histidine tag has been removed after protein purification was also characterized. This information will guide our selection of an appropriate lipase and will suggest possible tagging options that may be of use when optimizing the expression of recombinant lipase in transgenic *Dunaliella.*

Materials and Methods

Bacterial Strains, Plasmids, Enzymes, and Reagents: The halophile, *Chromohalobacter salexigens* ATCC BAA-138, was purchased from ATCC. The *E. coli* XL1-Blue strain (Novagen, EMD Biosciences) was used for cloning and maintaining the recombinant plasmid. BL21(DE3)LysS (Novagen, EMD Biosciences) cells or M15[pRep4] cells (Qiagen) were used for the over-expression of *C. salexigens* TesA. The expression plasmids pET21b and pQE-1 were obtained from Novagen, EMD Biosciences and Qiagen, respectively. Restriction endonucleases, T4 DNA ligase, and DNA polymerase were purchased from New England Biolabs. Synthetic oligonucleotides were synthesized by Eurofins MWG Operon. The PCR products were purified using QIAquick PCR purification kit (Qiagen). The plasmids were purified using QIAquick gel extraction kit (Qiagen). All 4-nitrophenyl-based substrates were purchased from Sigma-Aldrich. TAGZyme™ DAPase enzyme and Cysteamine-HCl for histidine tag removal were obtained from Qiagen.

Cloning of Halophilic tesA from Chromohalobacter salexigens: Basic Local Alignment Search Tool (BLAST) analysis was used to identify sequences in the *C. salexigens* genome that are similar to the *E. coli* thioesterases. The *C. salexigens* open reading frame CSal2620

(YP_574666) was identified which has 64% DNA sequence homology to *E. coli TesA*. The protein encoded in this open reading frame possesses 50% identity and 78% similarity to TesA. *C. salexigens* was cultured in nutrient broth with 10% NaCl, according to the supplier's recommendation. The *C. salexigens* genomic DNA was isolated using the UltraClean® Microbial DNA Isolation kit by Mo Bio Laboratories, Inc. according to the manufacturer's instructions. The gene for *C. salexigens* TesA homologue CSal2620 was amplified by PCR for subsequent cloning into the T7-polymerase-driven expression vector pET-21b (Novagen) and the T5-polymerase-driven expression vector pQE-1 (Qiagen). The former expression system enables production of C-terminal histidine tag-fused proteins, while the later system allows for production of removable N-terminal histidine tag-fused protein. For PCR amplification of the *C. salexigens tesA* homolog gene, primers were designed for each expression vector. The primers used to amplify the CSal2620 gene for expression in pET-21b were 5'-**CATATG**GGGCCCGTCGCGAATGTCCATGCCGAT-3'; forward, containing an *Nde1* restriction site shown in bold and 5'-**AAGCTT**TGATTCGTTCCGGTCGGTGTCCCGCGACAG-3'; reverse, containing a *Hind*III restriction site shown in bold. The annealing temp used for amplification with these primers was 55°C**.** The primers used to amplify the CSal2620 gene for expression in pQE-1 were 5'- **GCATGC**AATGGGGCCCGTCGCGAATGTCCATGCCGA -3'; forward, containing an *Sph1* restriction site shown in bold and 5'-**AAGCTT**TCATTCGTTCCGGTCGGTGTCCCGCGACAG -3'; reverse, containing a *Hind*III restriction site shown in bold. The annealing temp used for amplification with these

primers was 67°C. All primers were designed using MacVector (Accelrys, San Diego, CA) computer software. PCR amplification was performed in a 50 µl reaction containing 5 µl 10× Taq buffer, 0.4 µl dNTP (25 mM), 0.5 µl forward primer (40 µM), 0.5 µl reverse primer (40 µM), 0.5 µl Taq polymerase, and 1 µl *C. salexigens* genomic DNA (~300 ng/µl). The PCR was performed using a Bio-Rad iCycler thermal cycler programmed with the following parameters: One initial cycle for 5 min at 94 °C for denaturation, followed by 30 cycles of 94 °C for 30 sec, annealing temp listed above depending on primers for 30 sec, 72 °C for elongation for 1 min; and one final cycle at 72 °C for 7 min. The *tesA* PCR product size was 585 bp for CSal2620. PCR products were electrophoresed through a 1% agarose gel for visual inspection.

The amplified *tesA* genes for CSal2620::pET-21b, and CSal2620::pQE-1 were subsequently cloned into the *EcoR*V site of plasmid pCR-Script (Stratagene). Plasmids were transformed into *E. coli* strain XL1-Blue, and the transformed cells were plated on LB plates supplemented with ampicillin (100 µg/ml) and X-gal (40 µg/ml) and incubated at 37°C overnight. Blue-white screening was used to select colonies for plasmid isolation. pCR-Script plasmids with insert were isolated and digested with N*de*I and *Hind*III to excise the gene for CSal2620 from the CSal2620::pCR-Script plasmid, and *Sph*I and *Hind*III to excise the gene for CSal2620 from the CSal2620::pCR-Script. The pCR-Script excised *tesA* gene was subsequently cloned into the N*de*I and *Hind*III (NEB) sites in expression vector pET-21b (Novagen) or the *Sph*I and *Hind*III sites in expression vector pQE-1 (Qiagen). All plasmids

were sent to MWG Biotech for sequencing to ensure that no sequence changes occurred in the cloning process.

Over-expression of Recombinant Halophilic tesA: The CSal2620::pET-21b expression plasmid was transformed into *E. coli* BL21(λDE3) cells, which have IPTG- inducible expression of T7-RNA polymerase encoded on the chromosome. Transformants were selected on LB–ampicillin (100 μg/ml) plates after incubation at 37°C overnight. Expression plasmids based on pQE-1 (CSal2620::pQE-1) were transformed into *E. coli* M15[pRep4] cells (Qiagen). The pQE-1 plasmid contains a T5 phage promoter under the control of two *lac* operator sequences which allows for IPTG induction of cloned gene expression. The M15 strain contains the pRep4 plasmid which provides *lac* repressor in *trans* with pQE-1. Large-scale protein expression was done for all constructs by inoculating 2 L cultures of LB media supplemented with 100 μg/ml of ampicillin for pET-21b based constructs, and 100 μg/ml ampicillin with 50 μg/ml kanamycin was used for pQE-1 systems ensuring plasmid maintenance. Cells were grown with shaking (200 rpm) at 37°C until an OD_{600} of 0.6 to 0.8 was reached. Expression was induced by adding IPTG (0.2 mm final concentration). The cells were allowed to continue growing for two hours before being harvested by centrifugation and were stored at −80°C prior to preparing cell lysates. Recombinant protein expression was evaluated throughout this process using SDS-PAGE analysis.

Recombinant Halophilic TesA Purification: Cell pellets containing recombinant CSal TesA were suspended in 50 mM sodium phosphate buffer (pH 8.0) containing 1 M NaCl,1 mM benzamidine–HCl and 10 mM imidizole. The cell suspension was passed through a French

pressure cell (20,000 lb/in^2) three times. The lysed suspension was centrifuged at 15,000 x g for 60 min at 4^{o}C to remove cell debris. The supernatant was filtered through 0.45 µm syringe filters to further remove debris. The filtered extract was applied to a 5 ml HisTrap HP Nickel Sepharose™ affinity column (GE Healthcare Life Sciences) and washed with five column volumes of wash buffer (50 mM sodium phosphate buffer, pH 8.0, 1.0 M NaCl, 20 mM imidizole). The binding buffer used was 50 mM sodium phosphate buffer, pH 8.0, 1.0 M NaCl, 10 mM imidizole, and the elution buffer was 50 mM sodium phosphate buffer, pH 8.0, 1.0 M NaCl, 250 mM imidizole. Elution was done using 20 column volumes of a linear gradient of 0% to 100% elution buffer. All fractions were visualized on 12.5% SDS-polyacrylamide gels. Following affinity chromatography, the samples containing active protein were pooled and dialyzed using a 10,000 Da molecular weight cutoff (MWCO) dialysis cassette against 50 mM Tris-HCl, pH 8.0, 1.0 M NaCl to remove imidazole from the fractions. Final protein concentrations were estimated using Bradford assays (BioRad).

DAPase Removal of Histidine Tags from Recombinant Halophilic TesA: Purified *C. salexigens* TesA was desalted by dialysis (10,000 MWCO) against TAGZyme™ buffer (20 mM NaH_2PO_4, pH 7.0, 150 mM NaCl, 0.001% Tween-20). N-terminal fused histidine tags on proteins expressed by the pQE-1 system were removed using the TAGZyme ™ DAPase Enzyme kit (Qiagen). Removal of DAPase enzymes from untagged TesA was accomplished by subtractive immobilized-metal affinity chromatography (IMAC) using Ni-NTA Spin Columns (Qiagen). Final protein concentrations were estimated using the Bradford assay (Bio-Rad).

O-nitrophenyl Fatty Acid Based Activity Assays: A typical 1 ml reaction mixture contains 988 µl 50 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 0.001% Tween-20, 10 µl 100 mM o-nitrophenyl linked substrate (ex. 4-nitrophenyl butyrate (Sigma-Aldrich)), and 2 µl of enzyme at a dilution sufficient to give an OD_{410} between 0.1 – 0.8. The buffer was heated to 37ºC for 5 min, after which the substrate and enzyme were added. The reaction was allowed to continue for 5 min, at which time it was stopped by addition of 250 µl of 0.25 M Na_2CO_3. The absorbance was immediately read at 410 nm with an extinction coefficient of 13,635 M^{-1} cm^{-1}. One unit of thioesterase activity is defined as the amount of enzyme that liberates one µmole of o-nitrophenyl per min per mg of enzyme. For assays conducted at different pH values, the following buffers were used: pH 2.0 - 5.5, 50 mM sodium acetate, 50 mM Tris-HCl, 0.001% Tween-20; pH 6.0–9.5, 50 mM Tris-HCl, 0.001% Tween-20. When evaluating substrate specificity the following substrates were used: 4-nitrophenyl-acetate, 4-nitrophenyl-butyrate, 4-nitrophenyl-octanoate, 4-nitrophenyl-decanoate, 4-nitrophenyl-dodecanoate, and 4-nitrophenyl-palmitate, each at a final concentration of 1 mM.

Results

Identification of C. salexigens tesA Homolog Gene: C. salexigens TesA (CsTesA) and *E. coli* TesA amino acid sequence show 50% identity excluding the N-terminal leader sequence (Figure 2-1). Neither enzyme contains the specific Gly-X-Ser-X-Gly motif found in mammalian and avian serine esterases [13]; however, they both contain a similar active site sequence consisting of Gly^{8}-Asp-Ser-Leu-Ser-Ala-Gly^{14} in the case of TesA [13] and Gly^{16}-Asp-Ser-Leu-Ser-Ala-Ala^{22} in the case of CsTesA. Additionally, both enzymes contain the

C-terminal Gly-X-His motif found in mammalian and avian serine esterases [13], with both enzymes containing the Gly-Ile-His sequence starting at Gly^{155} in TesA and Gly^{163} in CsTesA. Further analysis of the two enzymes reveals additional homology in terms of specific residues and their catalytic function. In particular, TesA contains conserved residues forming a catalytic triad (Ser^{10}-Asp^{154}-His^{157}) and an oxyanion hole (Ser^{10}-Gly^{44}-Asn^{73}) [16]. These six conserved residues are also found in CsTesA, (Ser^{18}-Asp^{162}-His^{165} and Ser^{18}-Gly^{52}-Asn^{81}).

Figure 2-1. Clustal alignment of E. coli TesA and CsTesA. Black and gray shading indicates identical and similar residues respectively. Important sequences of homology are underlined and key amino acids are marked with an asterisk above them. The end of the leader sequence is indicated by an arrow.

Expression and Purification of Recombinant TesA from C. salexigens: C. salexigens TesA (CSal2620) was expressed in either *E. coli* BL21 or M15[pRep4] cells and isolated using

Ni^{2+}affinity chromatography. An untagged variant of CSal2620 was generated by DAPase digestion of the N-terminal histidine tag. This work gave rise to three separate variants of CSal2620, a C-terminal histidine tagged enzyme provided by CSal2620 being cloned into pET21b and expressed in *E. coli* strain BL21; an N-terminal histidine tagged enzyme provided by CSal2620 being cloned into pQE-1 and expressed in *E. coli* strain M15[pRep4]; and an untagged enzyme generated by DAPase digestion of the N-terminal tagged variant.

Figure 2-2A shows a representative SDS-PAGE gel illustrating the stepwise purification of N-terminal histidine tagged CSal2620 (TesA).

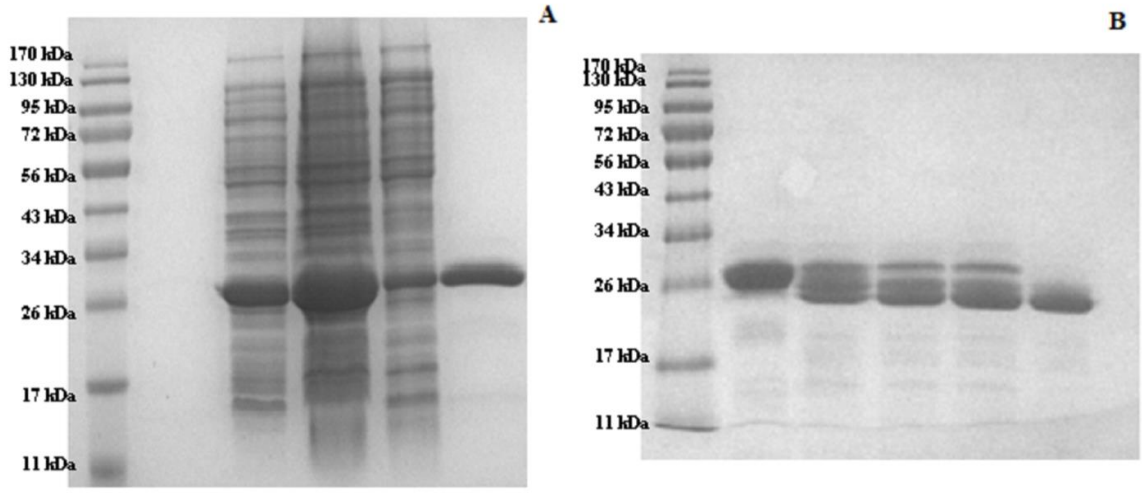

Figure 2-2. A. SDS-PAGE gel showing purification of N-terminal tagged CSal2620 (TesA) expressed in M15[pRep4]. Lane 1 – Protein Ladder. Lane 2: Empty. Lane 3: Whole cell free extract. Lane 4: Soluble protein pre-filtration. Lane 5: Soluble protein post filtration. Lane 6: 5 ug of purified, N-Terminal Tagged CSal2620 post dialysis. **B.** SDS-PAGE gel showing removal of the N-Terminal Histidine tag by DAPase digestion over time. Lane 1: Protein ladder. Lane 2: DAPase + N-terminal tagged CSal2620 - 0 min digestion. Lane 3-5: DAPase + N-terminal tagged CSal2620 – 10, 20, and 30 min. digestion. Lane 6: Untagged CSal2620 following subtractive IMAC via Ni2+ spin column. The expected molecular weight of tagged CsTesA is ~27kDa, untagged is ~25kDa.

Following purification of the N-terminal histidine-tagged variant of CsTesA, an aliquot of purified tagged protein was subjected to DAPase digestion to remove the N-terminal histidine tag. Figure 2-2B shows an SDS-PAGE gel of a time-course series demonstrating the removal of the histidine tag over time. Visible are the 0, 10, 20, and 30 minute time points in the DAPase digestion (Lanes, 2-5) followed by the subtractive IMAC purified untagged TesA (Lane 6). Lane 2, the zero time point, shows a protein band corresponding to undigested N-terminal tagged CsTesA. The DAPase enzyme co-migrates with the CsTesA and is not visible as a separate band. Lane 3 shows a smeared band corresponding to enzymes with partially digested tags after ten minutes. Lanes 4 and 5 show two separate bands. The upper band is the DAPase enzyme (27 kDa), while the lower band is CsTesA (25 kDa) which has had its tag removed. Following digestion for 30 min, the entire reaction mixture was loaded onto a Ni^{2+} spin column and the flow through collected. This flow through contained only untagged CsTesA and is shown in Lane 6. The DAPase enzyme itself is histidine tagged on its C-terminal end. Thus it, along with any undigested CsTesA were retained on the Ni^{2+} spin column.

Biochemical Properties of C. salexigens TesA*:*

Determination of optimum NaCl concentration: Purified CsTesA was incubated at 37ºC in the presence of 4-nitro phenyl linked substrates and varying concentrations of NaCl at pH 8.0 for five min. Activity was measured spectrophotometrically at 412 nm. Figure 2-3 shows the activity of tagged and untagged CsTesA against eight and ten carbon length substrates and

indicates that enzymatic activity is present at all salt concentrations tested with optimum activity occurring between 0.25 M - 2.0 M NaCl. Note that similar activity profiles exist for all three enzymes. Activity levels for C-terminal tagged enzyme and untagged enzyme are comparable at all but the lowest NaCl concentrations, while in most cases, the N-terminal histidine-tagged enzyme shows reduced activity. This suggests that the N-terminal histidine-tag is interfering with the enzyme's function and as a result may not be a good choice for use in the algae biofuel system.

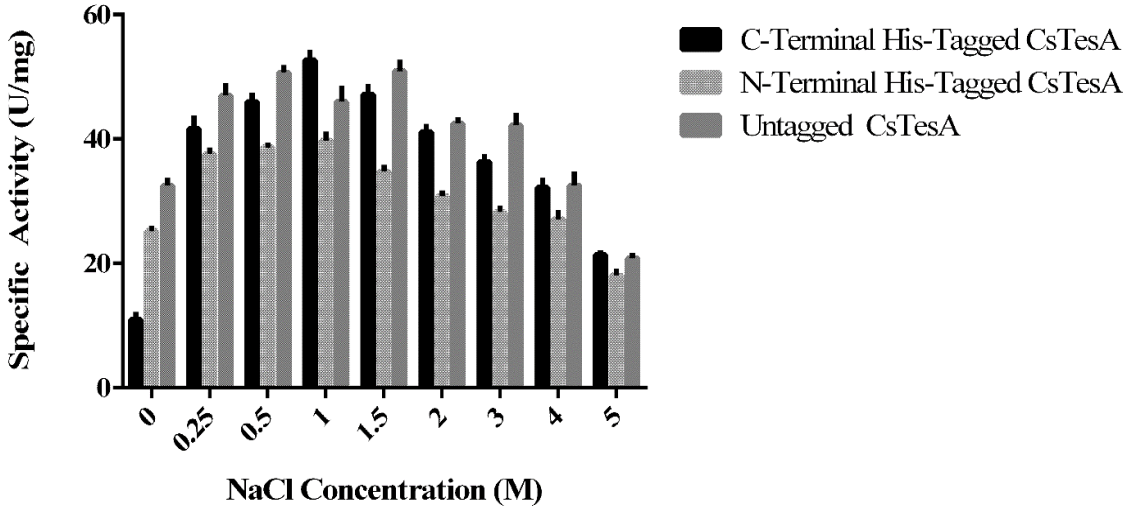

Figure 2-3. Activity of recombinant CsTesA over a zero to 5 M range of NaCl concentrations. Assays were performed at 37ºC at a pH of 8.0 against 1mM 4-nitrophenyl-octanoate, 50 mM Tris buffer, and 0.001%(v/v) Tween-20 for five min. 1 unit of enzyme activity is defined as the amount of enzyme that liberates 1 µmol of 4-nitro-phenyl per minute per mg protein.

Determination of optimum pH for C. salexigens TesA activity: Purified CsTesA was incubated at 37^{o}C in the presence of 4-nitro phenyl linked substrates, 0.5 M NaCl, and varying pH for five min. Activity was measured spectrophotometrically at 412 nm. Figure 2-4 shows the activity of histidine-tagged and untagged CsTesA against eight and ten carbon length substrates and indicates that enzymatic activity is present at pH values above 5.0, with optimum activity occurring between pH values of 7.5 - 9.0.

Note that similar activity profiles exist for all three enzymes. Activity levels between C-terminal histidine-tagged enzyme and untagged enzyme are comparable at most pH values, but the untagged enzyme shows higher activity across the optimum range, in particular at pH 7.5 and 8.0. Again, in most cases the N-terminal histidine-tagged enzyme shows reduced activity. This continues to support the earlier data suggesting that N-terminal histidine-tagged CsTesA is less active than the C-terminal tagged and untagged variants.

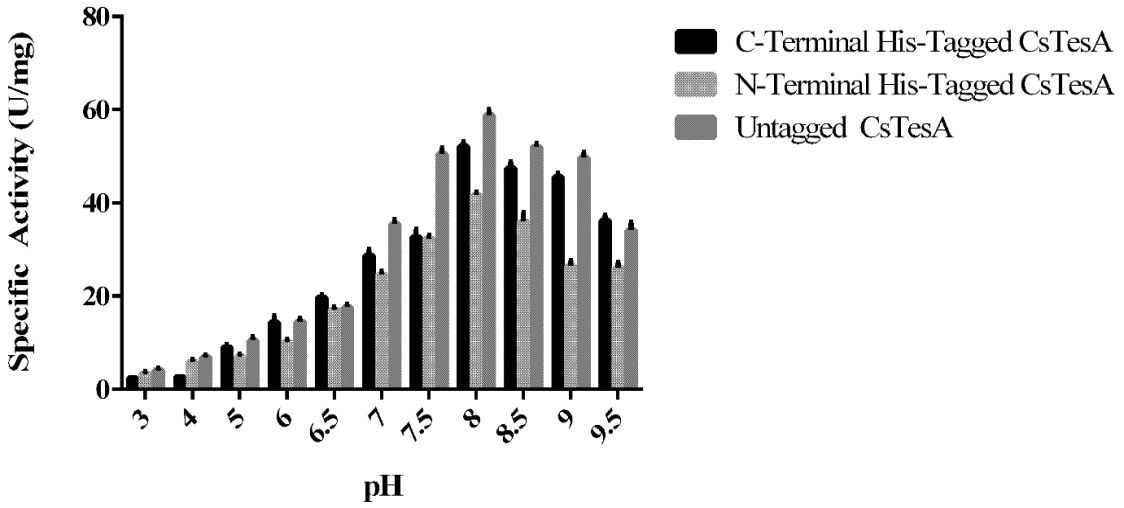

Figure 2-4. Activity of recombinant CsTesA over a 3 to 9.5 pH range. Assays were performed at 37ºC with 1.0 M NaCl against 1mM 4-nitrophenyl-octanoate and 0.001%(v/v) Tween-20 for five minutes. For pH 3-5.5 the reaction buffer contained 1M NaCl, and 50mM Tris/50mM Na Acetate. For pH 6-9.5 the reaction buffer contained 1M NaCl, and 50mM Tris. 1 unit of enzyme activity is defined as the amount of enzyme that liberates 1 µmol of 4-nitro-phenyl per minute per mg protein.

Determination of optimum temperature for C. salexigens TesA activity: Purified CsTesA was incubated at varying temperatures in the presence of 4-nitro phenyl linked substrates, 0.5 M NaCl, and a pH of 8.0 for five min. Activity was measured spectrophotometrically at 412 nm. Figure 2-5 shows the activity of histidine-tagged and untagged CsTesA against eight and ten carbon length substrates and indicates that enzymatic activity is present at all temperatures, with optimum activity occurring between 35ºC - 50ºC. Note, again, that similar activity profiles exist for all three recombinant TesA enzymes. Activity is evident at all temperatures with an optimum range of temperature between 35ºC and 50ºC. Activity levels between C-terminal histidine-tagged enzyme and untagged enzyme are comparable at higher

temperatures, but the untagged enzyme shows higher activity across the entire optimum range. Again, in most cases the N-terminal histidine-tagged enzyme shows reduced activity.

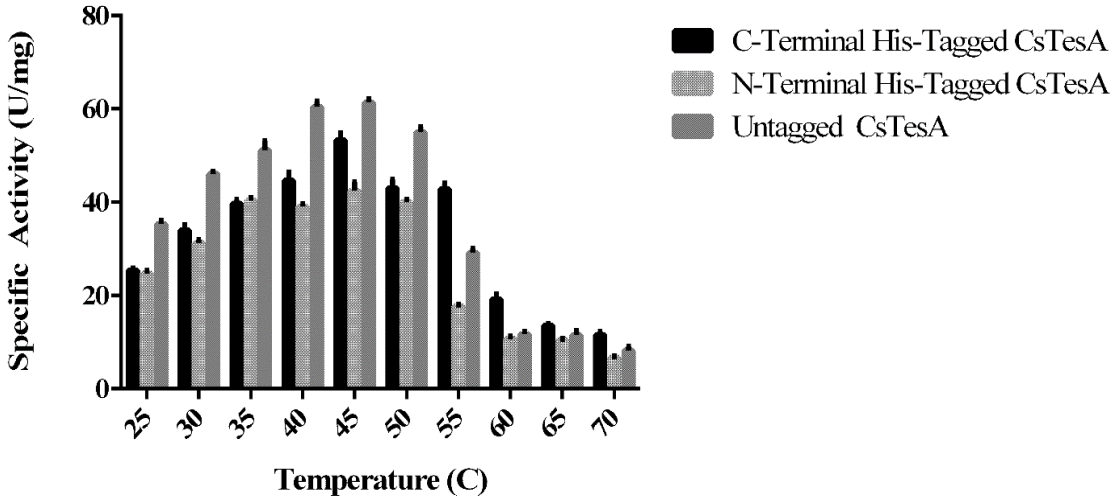

Figure 2-5. Activity of recombinant CsTesA over a 25^0C to 70^0C range of temperature. Assays were performed at a pH of 8.0 with 1.0 M NaCl/50 mM Tris against 1mM 4-nitrophenyl-octanoate and 0.001%(v/v) Tween-20 for five minutes. 1 unit of enzyme activity is defined as the amount of enzyme that liberates 1 µmol of 4-nitro-phenyl per minute per mg protein.

Determination of preferred substrate: Purified CsTesA was incubated at 45ºC in the presence of 4-nitro phenyl linked substrates, 0.5 M NaCl, and pH of 8.0 for five min. Activity was measured spectrophotometrically at 412 nm. Figure 2-6 shows the activity of CsTesA against two, four, eight, ten, twelve, and sixteen carbon chain length substrates and indicates that enzymatic activity is present against all but the nitrophenyl linked substrates except the C16 version, with optimum activity occurring with C8 substrates. Note that the C-terminal histidine-tagged variant shows enhanced activity against shorter C2/C4 substrates. These

results are consistent with earlier published findings for *E.coli* TesA which indicate C-terminal tag introduction skews substrate specificity towards shorter chain lengths [15]. It is noteworthy that N-terminal histidine-tagged and untagged enzymes show higher activity against C8-C10 substrates. Again, in most cases the N-terminal histidine-tagged enzyme shows reduced activity across the substrates as compared to the untagged enzyme.

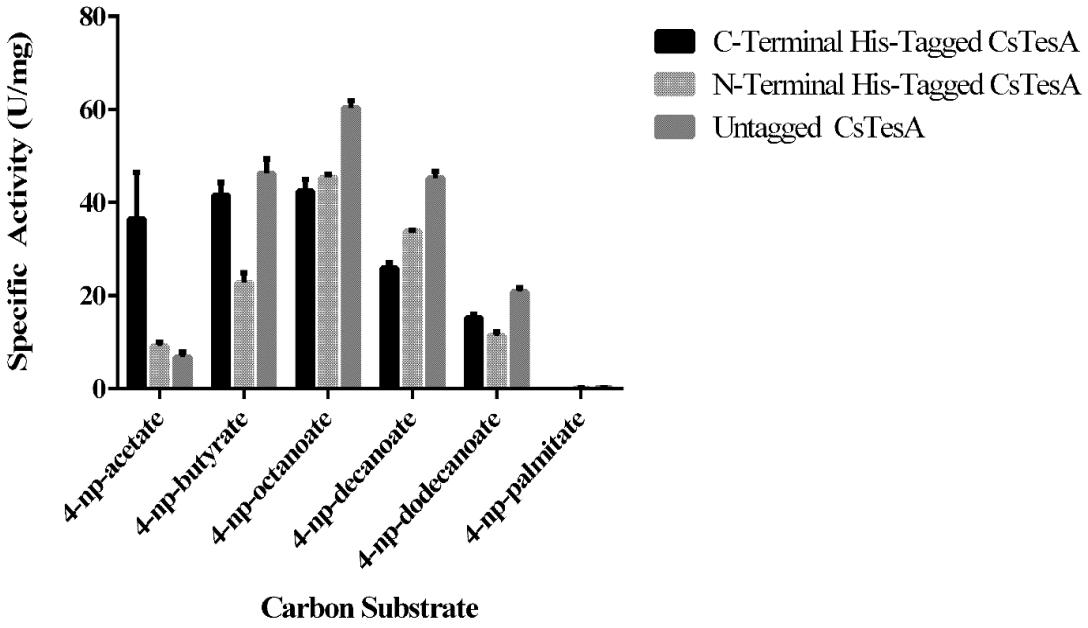

Figure 2-6. Activity of recombinant CsTesA against 2, 4, 8, 10, 12 and 16 carbon chain length esters. Assays were performed at a pH of 8.0 with 1.0 M NaCl/50 mM Tris and 0.001%(v/v) Tween-20 for five minutes. 1 unit of enzyme activity is defined as the amount of enzyme that liberates 1 µmol of 4-nitro-phenyl per minute per mg protein.

Viewed collectively, the data suggest that the untagged version of CsTesA is more active across a wider variety of substrates, temperatures, and pH values than either histidine-tagged

enzyme. This increased stability and activity against longer carbon substrates, in particular C10 and C12 substrates, make it a better choice for use as part of the algae based biofuel system.

Discussion

There are many factors that play a role in determining optimum enzyme activity against a given series of substrates. External factors such as salt concentration, pH, and temperature play well known roles. Equally important in determining the optima for a given enzyme is alteration of enzyme structure by amino acid substitution and histidine tagging. Since the purpose of this work is to ultimately express recombinant thioesterases and lipases in marine algae that are capable of hydrolyzing triacylglycerides of twelve or higher carbon chain lengths, it is necessary to understand which combination of factors yields the best activity for those needs.

Another factor to be considered anytime one is investigating fatty acid chain hydrolysis by esterases is solubility of the fatty acid in the aqueous media. If the esterase is not able to access the ester bond between the 4-nitrophenyl group and the hydrophobic side chain due to micelle or emulsion formation, estimation of its activity will be under-reported. To ensure that our substrates were indeed soluble and available to the recombinant TesA enzymes, we investigated the effects of various solvents and detergents on the activity of the enzyme against 4-nitrophenyl-decanoate. This substrate was chosen because preliminary assays (data not shown) in 50 mM Tris buffer without detergent or solvent showed likely solubility problems for that substrate, yet activity was still high enough that an increase in

activity was expected under assay conditions in which substrate solubility could be increased. Towards that end, the effects of varying concentrations of ethanol, methanol, butanol, acetone, and dimethyl sulfoxide on TesA activity was investigated. In addition, the influence of varying concentrations of detergents such as Triton-X and Tween-20 on recombinant TesA activity was also evaluated. It was found that solubility and enzymatic activity for all of the recombinant TesA enzymes was dramatically increased in the presence of 0.001% (v/v) Tween-20. It should be noted that the derivatized nitrophenyl substrates are not what the enzyme will be acting against *in situ*; however, within the algae cells it is expected that the glycerol backbone and cytoplasmic milieu will provide access to soluble substrates for any recombinant thioesterase/lipase.

The NaCl concentration optimum for the three CsTesA enzymes presented in Figure 2-3 shows a broad tolerance towards a range of salt concentrations. This is consistent with the expected enzyme properties from a thioesterase originating from a moderate halophile. The increased activity towards the substrates demonstrated by the untagged CsTesA variant is also to be expected as it is understood the fusion tags can interfere with enzyme structure and function. The fact that the untagged TesA is directly derived from the N-terminal histidine-tagged version and shows such a significant increase in activity over nearly all salt concentrations, suggests that the untagged recombinant TesA is indeed superior in comparison. Given the similar results presented in Figures 2-4, 2-5, and 2-6, it is clear that the presence of the N-terminal histidine-tag inhibits activity and is a suboptimal choice for our purposes.

However, when comparing the untagged variant against the C-terminal histidine-tagged variant a comparable activity against NaCl concentration, temperature and pH is observed. It is only when substrate specificity is investigated is a marked difference in performance seen. Figure 2-6 shows that the C-terminal histidine-tagged enzyme is more active against shorter carbon chain lengths than it is against longer ones (100% relative activity against 4-nitro-phenyl butyrate versus 63% against 4-nitro-phenyl decanoate). Both of these observations are consistent with existing literature which shows that residues in both the N-terminal and C-terminal domains of TesA contain amino acids specific for catalytic activity and govern substrate specificity [16]. It should be noted that the large variation in the 4-nitrophenyl-acetate assay is due to relative instability of the substrate at a pH of 8 or higher resulting in higher background values.

It is not clear why the untagged CsTesA enzyme differs so markedly in terms of substrate preference when compared to published data regarding *E. coli* TesA [12]. Both enzymes contain all of the conserved residues necessary for serine esterase activity, the main difference being the absence of a leader sequence and an offset of eight amino acids from the N-terminal end of the CsTesA protein. It is possible that the eight amino acid shift is enough to alter the alignment of the catalytic and oxyanion domains. It may also be possible that the artificial o-nitrophenyl group on the substrates is interfering with access to the ester bond. Alternately, it may be that the presence of the histidine tag on either end of the enzyme altered the initial folding of the protein slightly, resulting in a modified substrate pocket.

Overall, the NaCl concentration optimum for untagged *C. salexigens* TesA shows a broad range from 0.25 M - 2.0 M NaCl. The optimum NaCl concentration for *Dunaliella* falls within that range [11, 17-19]. Similarly, optimum pH, and temperature ranges for CsTesA are within the preferred ranges for *Dunaliella* as well [11, 17-19]. Finally, the higher activity of the untagged enzyme against C-12 substrates shown in Figure 6 suggests that not only will the transgenic enzyme be active in the microalgae but that it may be able to relieve the feedback inhibition of both native and recombinant ACCases by acyl-ACPs.

Conclusion

A recombinant *C. salexigens* TesA homolog was successfully cloned, expressed, purified and biochemically characterized. The enzyme, regardless of histidine tag presence or position, showed activity against 4-nitro-phenyl linked substrates with carbon chain lengths of 2, 4, 8, 10 and 12 carbons. The presence of a histidine tag has deleterious effects on activity and substrate specificity of CsTesA. The N-terminal histidine-tagged enzyme showed inhibited activity compared to the untagged enzyme, and the C-terminal tagged variant showed a skewed preference towards shorter substrates. CsTesA shows a broad tolerance to salt concentrations, pH, and temperature, which correspond well to optimal growth conditions of the microalga *Dunaliella.* Overall, the activity optima for untagged CsTesA are acceptable for use in marine algal biofuel systems.

Acknowledgements

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APPENDICES

APPENDIX A

Abstract

Acyl-CoA thioesterases are enzymes which catalyze the hydrolysis of acyl-CoAs at thioester linkages to form free fatty acids and coenzyme A. These enzymes, along with lipases, have a wide spectrum of use in industrial applications including the enrichment of fatty acids from oil-producing biofuel feedstocks. Recent studies have shown increased production of free fatty acids in *Escherichia coli* and cyanobacteria following overexpression of recombinant, leaderless thioesterase I (TesA) from *E. coli*. Recent work in our lab investigated the biochemical characteristics of a homolog of TesA from the moderate halophilic bacterium *Chromohalobacter salexigens.* That work indicated the TesA from *C. salexigens* possessed the highest activity against eight and ten carbon chain substrates. While promising, an enzyme with specificity for twelve or longer carbon chain substrates would be optimal for creating jet fuel based bio-feedstocks. Since TesB in *E.coli* functions via a different mechanism than TesA, and literature indicates a specificity of twelve to eighteen carbons for the fatty acid substrate, we isolated the *C. salexigens* homolog of this enzyme and attempted to characterize it as well. The *tesB* gene from *C. salexigens* has been identified, cloned, and recombinantly expressed in *E. coli* strains BL21 and M15. Because previous studies indicated that histidine tag position alters TesA substrate specificity, two different recombinant versions of the *C. salexigens* TesB were produced for this study; an N-terminal histidine-tagged enzyme, and a C-terminal histidine-tagged enzyme. Minimal

activity was detected with N-terminal histidine tagged CsTesB against 4-nitrophenyl linked substrates or acyl-CoA linked substrates.

Introduction

Acyl-CoA thioesterases are enzymes which hydrolyze Acyl-CoAs at thioester linkages to form free fatty acids and coenzyme A. This activity differentiates them from lipases which catalyze the hydrolysis of free fatty acids from long chain triacylglycerides. This specificity for longer, water insoluble fatty acids differentiates lipases from esterases which generally only hydrolyze water soluble shorter chain acyl esters [1]. Both classes of enzymes are well established in the biotechnology arena where they are integral in biofuel production, textile processing, waste treatment, as detergent additives and many other applications [2].

Of particular interest is the application of thioesterases/lipases in microalgae based biofuel systems. One approach, which has already been shown to work in bacteria [3] and cyanobacteria [4], is to genetically modify marine algae such as *Dunaliella* sp. to overproduce triacylglycerides of specific desired fatty acid chain lengths and saturation. These triacylglycerides would subsequently be hydrolyzed into free fatty acids and glycerol by recombinant thioesterases/lipases and used as feedstock for biofuel production. This approach, when combined with an effective lipid sink [5] in the culture medium, could yield significant improvements over existing algae based systems, as it would allow the free fatty acids to be secreted from the algae and harvested on an ongoing basis. This would avoid the

expensive and time consuming steps involved in physical destruction of the algae and mechanical harvesting of lipid content [6]. In addition, this approach would avoid the need for extensive degumming of the extracted free fatty acids as sulfur and phosphorus contaminants could be contained in the microalgae when the fatty acids are secreted.

The requirement of lipolytic activity to form free fatty acids from triacylglycerides is not the only reason for the inclusion of a recombinant lipase in the system. There is also a metabolic need for the lipases and thioesterases, which may be understood by looking at the fatty acid synthesis cycle in bacteria, archaea and microalgae. The first committed and regulated step in fatty acid synthesis is the conversion of acetyl-CoA to malonyl-CoA by acetyl-CoA carboxylase (ACCase) [7, 8]. This enzyme, if over-expressed, has been shown to flux carbon through the fatty acid synthesis pathway resulting in increased production of long chain acyl-ACPs [9]. These long chain hydrocarbons, in turn, feedback inhibit the recombinant ACCase [10]. The presence of recombinant thioesterases would allow for hydrolysis of the long chain acyl-ACPs and relieve the feedback inhibition, effectively pulling carbon through the fatty acid synthesis pathway [9].

Because the environment in which most marine algae thrive is moderately halophilic, and since increased salt concentrations in the microalgae culture media may influence length and saturation of fatty acids produced, the system could use recombinant thioesterases and lipases from the moderate halophile *Chromohalobacter salexigens.* This bacterium was isolated from a saltern in the Netherlands [11]. Its optimal growth conditions are similar to the *Dunaliella* species of microalgae, and the two microorganisms share sufficient homology

to suggest that transgenic ACCase and thioesterases and lipases from *C. salexigens* would be active in the microalgae.

An alternate acyl-coA thioesterase that was considered for expression in *Dunaliella* is a homolog of *E. coli*'s TesB protein which has been shown to be active over a wide range of conditions and is specific for medium fatty acid chains with fourteen to eighteen carbons in length[12, 13]. This substrate specificity is important because algal oils with lengths in this range are most amenable to direct conversion to transportation fuels. TesB is a tetrameric enzyme with a size of 120 kDa [12]. Structurally, the enzyme falls into a novel family of esterases with four specific residues. The specific sequences present in both the *E. coli* and *C. salexigens* enzymes are shown in Figure A1-1. It should be noted that the one substitution, Thr^{228} for a Ser is a known substitution [14]. Another difference between the two enzymes is the long stretch of eighteen amino acids that is present between position 140 and 157 in the *E. coli* enzyme but is absent in the *C. salexigens* homolog.

Neither enzyme possesses a membrane specific N-terminal leader sequence. Catalytically, both enzymes have the conserved residues mentioned above including the N-terminal active site histidine (*C. salexigens* TesB amino acid residue number 58) and C-terminal active sites Asp^{204}, Thr/Ser^{228}, and Gln^{278}.

Another reason for the investigation of a TesB homolog from *C. salexigens* is the success seen in production of free fatty acids from cyanobacteria using a recombinant, leaderless version of the *E.coli* TesA enzyme [4]. In these experiments, a series of recombinant *Synechocystis* sp. cyanobacteria strains were modified to over-express a

leaderless TesA protein, as well as successive modifications to components of the fatty acid synthesis system to optimize free fatty acid secretion. The results were encouraging, showing dramatic increase in secretion of saturated C12, C16, and C18 fatty acids [4].

Other studies have shown similar results when modifications to fatty acid synthesis were combined with over-expression of leaderless TesA in *E.coli* [3, 15]. These experiments also showed increased secretion of C12, C14, and C16 free fatty acids at high levels. Additionally, it was shown that specific modifications to the fatty acid synthesis system, coupled with optimized growth conditions and high levels of TesA activity, resulted in specific chain length and saturation of free fatty acids [3, 15].

Given that saturated algal oils with carbon chain lengths of C12 to C14 are most amenable to jet fuel production, it is important that the thioesterases and lipases introduced into the algae be able to hydrolyze fatty acids of that length or greater. The research mentioned above indicates that in both cyanobacteria and *E. coli*, saturated products of appropriate length are produced from over-expression of TesA.

While no such studies exist using TesB, it is possible that TesB's unique mechanism of hydrolysis of acyl-CoAs may result in different specificity and activity levels resulting in a better choice for inclusion in our biofuel process.

Our research confirms that presence of a C-terminal histidine tag on CsTesA shifts substrate preference towards shorter carbon chain lengths. This is also reported in literature with *E. coli* TesA [16]. This observation led us to evaluate the effects of histidine tag position on CsTesB activity. C-terminal and N-terminal tagged variants of the enzyme were expressed

and biochemically characterized. This approach will also allow us to remove the tag from the N-terminal tagged enzyme should the need arise. This information will guide our selection of an appropriate lipase and will suggest possible tagging options that may be of use when optimizing the expression of recombinant lipase in transgenic *Dunaliella.*

Materials and Methods

Bacterial Strains, Plasmids, Enzymes, and Reagents: The halophile, *Chromohalobacter salexigens* ATCC BAA-138, was purchased from ATCC. The *E. coli* XL1-Blue strain (Novagen, EMD Biosciences) was used for cloning and maintaining the recombinant plasmid. BL21(DE3)LysS (Novagen, EMD Biosciences) cells or M15[pRep4] cells (Qiagen) were used for the over-expression of the proteins. The expression plasmids pET21b and pQE-1 were obtained from Novagen, EMD Biosciences and Qiagen, respectively. Restriction endonucleases, T4 DNA ligase, and DNA polymerase were purchased from New England Biolabs. Synthetic oligonucleotides were synthesized by Eurofins MWG Operon. The PCR products were purified using the QIAquick PCR purification kit (Qiagen). Plasmids were purified using the QIAquick gel extraction kit (Qiagen). All 4-nitrophenyl-based substrates were purchased from Sigma-Aldrich.

Cloning of Halophilic tesB from Chromohalobacter salexigens: Basic Local Alignment Search Tool (BLAST) analysis was used to identify sequences in the *C. salexigens* genome that are similar to the *E. coli* thioesterases. The *C. salexigens* open reading frame CSal0838 (YP_572894) was identified which has 58% DNA sequence homology to *E. coli tesB*. The protein encoded in this open reading frame possesses 46% identity and 60% similarity to

TesB. *C. salexigens* was cultured in nutrient broth with 10% NaCl, according to the supplier's recommendation. The *C. salexigens* genomic DNA was isolated using the UltraClean® Microbial DNA Isolation kit by Mo Bio Laboratories, Inc. according to the manufacturer's instructions. The gene encoding the *C. salexigens* TesB homolog CSal0838 was amplified by PCR for subsequent cloning into the T7-polymerase-driven expression vector pET-21b (Novagen) and the T5-polymerase-driven expression vector pQE-1 (Qiagen). The former expression system enables production of C-terminal histidine tag-fused proteins, while the later system allows for production of removable N-terminal histidine tag-fused protein. For PCR amplification of the *C. salexigens tesB* homolog gene, primers were designed for each expression vector. The primers used to amplify the CSal0838 gene for expression in pET-21b were 5'-**CATATG**ACGCAACCACTCGACACCCTCGTC GAT-3'; forward, containing an *Nde1* restriction site shown in bold and 5'-**GGATCC**CACTCGACCAGGCGCGT CAGCCCCTCCTGCGCCACCGAGGCCACCAG -3'; reverse, containing a *BamHI* restriction site shown in bold. The annealing temp used for amplification with these primers was 65°C**.** The primers used to amplify the CSal0838 gene for expression in pQE-1 were 5'-AGAG**GCATGC**AATGACGCAACCACTCGACACCCTCGTCGATCTTCTGGGCCTCG A-3'; forward, containing an *Sph1* restriction site shown in bold and 5'-AGAG**AAGCTT**TCACTCGACCAGGCGCGTCAGCCCCTCCTGCGC CACCGAGGCCAC-3'; reverse, containing a *Hind*III restriction site shown in bold. The annealing temp used for amplification with these primers was 67°C. All primers were

designed using MacVector (Accelrys, San Diego, CA) computer software. PCR amplification was performed in a 50 µl reaction containing 5 µl 10× Taq buffer, 0.4 µl dNTP (25 mM), 0.5 µl forward primer (40 µM), 0.5 µl reverse primer (40 µM), 0.5 µl Taq polymerase, and 1 µl *C. salexigens* genomic DNA (~300 ng/µl). The PCR was performed using a Bio-Rad iCycler thermal cycler programmed with the following parameters: One initial cycle for 5 min at 94 °C for denaturation, followed by 30 cycles of 94 °C for 30 sec, annealing temp listed above depending on primers for 30 sec, 72 °C for elongation for 1 min; and one final cycle at 72 °C for 7 min. The *tesB* PCR product size was 807 bp for CSal0838. PCR products were electrophoresed through a 1% agarose gel for visual inspection. The amplified *tesB* gene for CSal0838::pET-21b was subsequently cloned into the *Eco*RV site of plasmid pCR-Script (Stratagene). Plasmids were transformed into *E. coli* strain XL1-Blue, and the transformed cells were plated on LB plates supplemented with ampicillin (100 µg/ml) and X-gal (40 µg/ml) and incubated at 37°C overnight. Blue-white screening was used to select colonies for plasmid isolation. The amplified lipase gene for CSal0838::pQE-1 was subsequently digested with *Sph*I and *Hind*III to generate sticky ends on the amplified products and then cloned into the *Sph*I and *Hind*III sites of plasmid pQE-1 (Qiagen). Plasmids were transformed into *E. coli* strain XL1-Blue, and the transformed cells were plated on LB plates supplemented with ampicillin (100 µg/ml) and kanamycin (50 µg/ml) and incubated at 37°C overnight. pCR-Script plasmids with CSal0838 insert were isolated and digested with *Nde1* and *BamH1* to excise the gene for CSal0838 from the CSal0838::pCR-Script. The pCR-Script excised *tesB* gene was subsequently cloned into the

N*de*I and *BamH1* (NEB) sites in expression vector pET-21b (Novagen). All plasmids were sent to MWG Biotech for sequencing to ensure that no sequence changes occurred in the cloning process.

Over-expression of Recombinant Halophilic tesB: The CSal0838::pET-21b expression plasmid was transformed into *E. coli* BL21(λDE3) cells, which have IPTG- inducible expression of T7-RNA polymerase encoded on the chromosome. Transformants were selected on LB–ampicillin (100 µg/ml) plates after incubation at 37°C overnight. Expression plasmids based on pQE-1 (CSal0838::pQE-1) were transformed into *E. coli* M15[pRep4] cells (Qiagen). The pQE-1 plasmid contains a T5 phage promoter under the control of two *lac* operator sequences which allows for IPTG induction of cloned gene expression. The M15 strain contains the pRep4 plasmid which provides *lac* repressor in *trans* with pQE-1. Large-scale protein expression was done for all constructs by inoculating 2 L cultures of LB media supplemented with 100 µg/ml of ampicillin for pET-21b based constructs, and 100 µg/ml ampicillin with 50 µg/ml kanamycin was used for pQE-1 systems ensuring plasmid maintenance. Cells were grown with shaking (200 rpm) at 37°C until an OD_{600} of 0.6 to 0.8 was reached. Expression was induced by adding IPTG (0.2 mm final concentration). The cells were allowed to continue growing for two hours before being harvested by centrifugation and were stored at −80°C prior to preparing cell lysates. Recombinant protein expression was evaluated throughout this process using SDS-PAGE analysis.

Recombinant Halophilic TesB Purification: Cell pellets containing recombinant CSal TesB were suspended in 50 mM sodium phosphate buffer (pH 8.0) containing 1 M NaCl,1 mM

benzamidine–HCl and 10 mM imidizole. The cell suspension was passed through a French pressure cell (20,000 lb/in^2) three times. The lysed suspension was centrifuged at 15,000 x g for 60 min at 4ºC to remove cell debris. The supernatant was filtered through 0.45 µm syringe filters to further remove debris. The filtered extract was applied to a 5 ml HisTrap HP Nickel Sepharose™ affinity column (GE Healthcare Life Sciences) and washed with five column volumes of wash buffer (50 mM sodium phosphate buffer, pH 8.0, 1.0 M NaCl, 20 mM imidizole). The binding buffer used was 50 mM sodium phosphate buffer, pH 8.0, 1.0 M NaCl, 10 mM imidizole, and the elution buffer was 50 mM sodium phosphate buffer, pH 8.0, 1.0 M NaCl, 250 mM imidizole,. Elution was done using 20 column volumes of a linear gradient of 0% to 100% elution buffer. All fractions were visualized on 12.5% SDS-polyacrylamide gels. Following affinity chromatography, the samples containing active protein were pooled and dialyzed using a 10,000 Da molecular weight cutoff (MWCO) dialysis cassette against 50 mM Tris-HCl, pH 8.0, 1.0 M NaCl to remove imidazole from the fractions. Final protein concentrations were estimated using Bradford assays (BioRad).

O-nitrophenyl Fatty Acid Based Activity Assays: A typical 1 ml reaction mixture contains 988 µl 50 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 0.001% Tween-20, 10 µl 100 mM o-nitrophenyl linked substrate (ex. 4-nitrophenyl butyrate (Sigma-Aldrich)), and 2 µl of enzyme at a dilution sufficient to give an OD_{410} between 0.1 – 0.8. The buffer was heated to 37ºC for 5 min, after which the substrate and enzyme were added. The reaction was allowed to continue for 5 min, at which time it was stopped by addition of 250 µl of 0.25 M Na_2CO_3. The absorbance was immediately read at 410 nm with an extinction coefficient of

13,635 M^{-1} cm^{-1}. One unit of thioesterase activity is defined as the amount of enzyme that liberates one µmole of o-nitrophenyl per min per mg of enzyme. For assays conducted at different pH values, the following buffers were used: pH 2.0 - 5.5, 50 mM sodium acetate, 50 mM Tris-HCl, 0.001% Tween-20; pH 6.0–9.5, 50 mM Tris-HCl, 0.001% Tween-20. When evaluating substrate specificity the following substrates were used: 4-nitrophenyl-acetate, 4-nitrophenyl-butyrate, 4-nitrophenyl-octanoate, 4-nitrophenyl-decanoate, 4-nitrophenyl-dodecanoate, and 4-nitrophenyl-palmitate, each at a final concentration of 1 mM.

DTNB/Acyl-CoA Based Activity Assays: A typical 1 ml reaction mixture contains 988 µl 50 mM Tris-HCl/125 uM DTNB, pH 8.0, 0.5 M NaCl, 0.001% Tween-20, 10 µl 400 uM acyl-CoA linked substrate (ex. Octanoyl-CoA (Sigma-Aldrich)), and 2 µl of enzyme at a dilution sufficient to give an OD_{410} between 0.1 – 0.8. The buffer was heated to 37ºC for 5 min, after which the substrate and enzyme were added. The reaction was allowed to continue for 10 min, at which time it was immediately read at 410 nm with an extinction coefficient of 13,635 M^{-1} cm^{-1}. One unit of thioesterase activity is defined as the amount of enzyme that liberates one µmole of NTB per min per mg of enzyme. When evaluating substrate specificity the following substrates were used: octanoyl-CoA and dodecanoyl-CoA, each at a final concentration of 4 uM.

Results and Discussion

Identification of C. salexigens tesB Homolog Gene: C. salexigens TesB (CsTesB) and *E. coli* TesB amino acid sequence show 46% identity and 60% similarity (Figure A1-1). Neither enzyme contains the specific Gly-X-Ser-X-Gly motif found in mammalian and avian serine

esterases [17] as the TesB enzyme acts through a completely different mechanism than TesA [14]. However, they both contain a similar series of active site residues consisting of His^{58}, Asp^{204}, Gln^{278}, and Thr^{228} where CsTesB substitutes a Ser in place of Thr^{228}. This is a common substitution.[14]. Additionally, neither enzyme contains a membrane sequence, indicating that they are cytoplasmic in nature. One notable difference between the two TesB sequences is that CsTesB lacks a long stretch of eighteen amino acids between position 140 and position 157 that are found in the *E. coli* TesB.

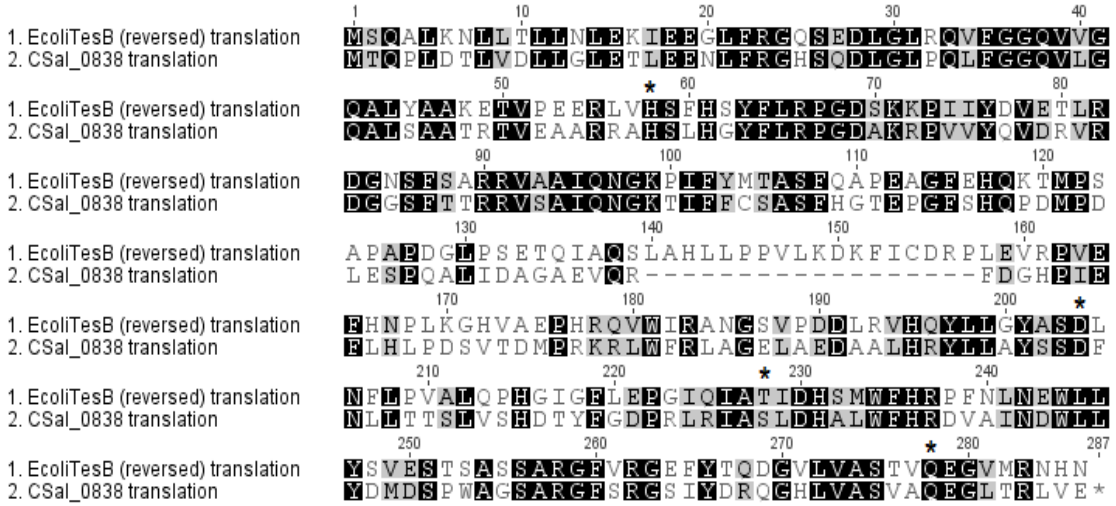

Figure A1-1: Clustal alignment of E. coli TesB and CsTesB. Black and gray shading indicates identical and similar residues respectively. Key amino acids are marked with an asterisk above them.

Expression and Purification of Recombinant TesB from C. salexigens: C. salexigens TesB (CSal0838) was expressed in either *E. coli* BL21 or M15[pRep4] cells and isolated using Ni^{2+}affinity chromatography. An untagged variant of CSal0838 could have been generated

by DAPase digestion of the N-terminal histidine tag had preliminary assays indicated activity in either of the tagged variants. However, since no activity was observed against any substrate with either tagged variant, only two separate variants of CSal0838 were generated. The first was a C-terminal histidine tagged enzyme provided by CSal0838 being cloned into pET21b and expressed in *E. coli* strain BL21; and the second was an N-terminal histidine tagged enzyme provided by CSal0838 being cloned into pQE-1 and expressed in *E. coli* strain M15[pRep4].

Figure A1-2 shows a representative SDS-PAGE gel illustrating the stepwise purification of N-terminal histidine-tagged CSal0838 (TesB).

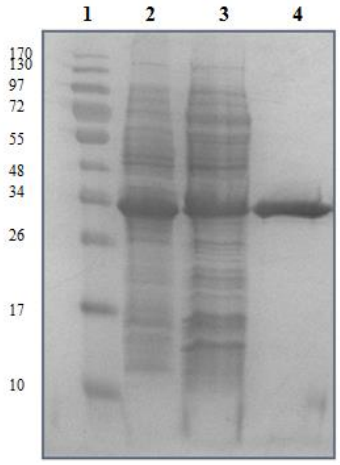

Figure A1-2: SDS-PAGE showing purification of CSal-0838 halophilic lipase. Lane 1: Protein ladder; Lane 2: Whole cell; Lane 3: Cell-free extract; Lane 4: Purified pooled elution fractions of CSal-0838 (5 µg).

We were unable to obtain satisfactory enzymatic activity for CsTesB regardless of assay conditions or substrate. We tried troubleshooting the assay conditions by varying the

standard 4-nitrophenyl linked assay described in the materials and methods section. We varied the salt concentration from 0.5M to 1.0M. We varied the pH from 8.0 to 7.0, and we allowed the incubation time to double to ten minutes from the regular five. We also obtained acyl-CoA linked substrates and tested them according to the conditions listed in the materials and methods section. Table A1-1 shows the relative activity between N-terminal histidine-tagged CsTesA and CsTesB for reactions in which a variety of substrates were used. In all cases, CsTesB activity was minimal. The conditions under which these assays were run were identical to the examples given in the materials and methods section and do not include variations for troubleshooting. Against all of the 4-nitrophenyl-linked substrates, CsTesA was significantly more active. CsTesB activity was less than 1% of the CsTesA activity against all substrates except 4-nitrophenyl palmitate which was the least favorable of all substrates for CsTesA. We do not have a good explanation for the lack of activity seen with CsTesB against 4-nitrophenyl linked substrates. It is possible that the eighteen amino acid deletion in the CsTesB enzyme has resulted in a non-functional protein. Alternately, the CsTesB gene may code for a protein with a completely different function than suggested by sequence analysis.

Table A1- 1. Percent Relative Specific Activity Between N-terminal Histidine Tagged CsTesA and N-terminal Tagged CsTesB.

Substrate	Activity of N-terminal tagged CsTesA	Activity of N-terminal tagged CsTesB	% Relative Activity
4-nitrophenyl butyrate	22.7 ± 2.12	0.05 ± 0.01	0.22

Table A1-1 Continued

4-nitrophenyl octanoate	45.4 ± 0.61	0.04± 0.02	0.09
4-nitrophenyl decanoate	33.8 ± 0.22	0.09 ± 0.04	0.27
4-nitrophenyl dodecanoate	11.4 ± 0.82	0.08± 0.04	0.70
4-nitrophenyl palmitate	0.11 ± 0.06	0.008± 0.005	7.2
Octanoyl-CoA	0.057 ± 0.01	0.046 ± 0.01	80.1
Dodecanoyl-CoA	0.057 ± 0.01	0.04 ± 0.01	70.1

Finally, it should be noted that neither enzyme performed well against the acyl-coA linked substrates. These results could reflect the possibility that neither enzyme as purified is active against acyl-CoA esters, or the results could be artifacts caused by a poorly tuned assay. Investigations into these results are ongoing.

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APPENDIX B

Abstract

Dunaliella are photosynthetic microalgae which lack a rigid cell wall. These microorganisms have the capacity to produce large amounts of triacylglycerides and other algal oils. These oils, coupled with the algae's photosynthetic ability and tolerance to moderate salt conditions has made them attractive to biofuel researchers. One approach being investigated to maximize algal oil production in *Dunaliella* involves introduction of recombinant genes from the moderate halophile *Chromohalobacter salexigens* into the algae. These genes would modify carbon flux through endogenous fatty acid synthesis pathways resulting in an over-production of oils for use as biofuel feedstocks. Introduction of inducible ACCase genes from *C. salexigens* into *Dunaliella* would flux carbon into the fatty acid synthesis pathway, while simultaneous expression of a transgenic thioesterase would relieve feedback inhibition of ACCase and help pull carbon through the fatty acid synthesis pathway. These genes, along with a selectable antibiotic resistance marker would be introduced into the algae via electroporation. The transgenes are integrated into an rbcS cassette containing a transit peptide for chloroplast targeting. The algae will then be monitored for increased triacylglyceride production during both active growth and senescence. This oil will be collected and processed for biofuels.

Introduction

Dunaliella are photosynthetic microalgae which thrive in moderately halophilic environments. They lack a rigid cell wall which enables harvesting of algal oil via osmotic lysis, possess fast growth kinetics, and are able to synthesize a variety of products, including glycerol, carotenoids and antibiotics that have biotechnological relevance [1]. They are also able to produce large amounts of triacylglycerides that can be used as feedstock for biofuel production [1-4].

Plant oils, animal fat or waste cooking oils currently serve as biofuel feedstocks. However, none of these sources are scalable to meet transportation fuel demands. The use of food crops, such as corn and soybeans for biofuel production are established, but any attempt to leverage them results in less land that can be dedicated to food crop production. As an example, if oil palm production were scaled to meet 50% of the nation's transportation fuel demand, approximately one quarter of all available cropland in the United States would need to be dedicated to its growth [2]. Microalgae, on the other hand, offer up to 40 times as much oil per unit area of land than soybeans and canola [5].

To realize such gains in biofuel production from microalgae, and for these gains to be economically viable, large scale algae production facilities are required. The most efficient of these for biofuel production is the open raceway pond [2, 6]. These ponds are suitable for *Dunaliella* culturing because *Dunaliella* are able to grow in halophilic environments that other organisms cannot tolerate. Also, these microalgae are fast growers and can out-compete most of the common contaminants in the raceway pond [2, 4, 6].

Finally, not only are the algal oils themselves attractive for biofuel production, the biomass created by the algae themselves can be used for production of many different biofuels including bioethanol, biodiesel, biohydrogen [4, 7] and may be concomitantly used for waste water treatment while still producing feedstocks for transportation fuel [5, 6]. If this rich availability of biofuel feedstocks can be supplemented with molecular approaches to maximize yield, it is possible that microalgae based biofuel production can realize the goal of reducing our dependence on fossil fuels while securing our cropland for food production.

Materials and Methods

The project to transform *D. viridis* with recombinant *C. salexigens tesA* (*CsTesA*) has just begun. Thus, formal materials and methods for the entire process are not available. Details for well-defined tasks have been provided. Less defined areas are covered by a general overview concerning high level strategy.

Selection of D. viridis for transformation: *D. viridis* was chosen to be transformed because the NCSU researchers involved in the algae cultivation for the NSF EFRI project determined that it provided a higher level of fatty acid synthesis than other strains, and because it lacks a cell wall, making transformation and manipulation easier. Finally, *D. viridis* grows better at higher temperatures and is sensitive to zeocin for antibiotic selection. Codon usage analysis of C. salexigens tesA (CsTesA) and Metallosphaera sedula tesA (MsTesA): Codon usage analysis was carried out to determine which thioesterase gene, CsTesA or MsTesA, would be the best candidate for initial attempts at *D. viridis*

transformation. CsTesA required fewer codon changes and was chosen for transformation.

Site directed mutagenesis of C. salexigens tesA: Three codons were identified as needing to be changed in *C. salexigens tesA.* Site directed mutagenesis of *C. salexigens tesA* was carried out using Stratagene's QuickChange II kit according to manufacturer's instructions. The primers used to modify the *C. salexigens tesA* gene were 5'-CCTGCGCGGACTGTCCCCCCAGCAAATGAAGC-3'; forward, and 5'-GCTTCATTTGCTGGGGGGACAGTCCGCGCAGGC-3'; reverse. The annealing temp used for amplification with these primers was: 55°C**.** All primers were designed using MacVector (Accelrys, San Diego, CA) computer software. PCR amplification was performed in a 50 µl reaction containing 5 µl 10× PFU Ultra HF Taq buffer, 1.0 µl dNTP (25 mM), 1.3 µl forward primer (10 µM), 1.3 µl reverse primer (10 µM), 1.0 µl PFU Ultra HF polymerase, and 1 µl *C. salexigens tesA* DNA in pET-21b (~30 ng/µl). The PCR was performed using a Bio-Rad iCycler thermal cycler programmed with the following parameters: One initial cycle for 30 sec at 95 °C for denaturation, followed by 16 cycles of 95 °C for 30 sec, 55°C for annealing for 1 min, 68 °C for elongation for 5 min. Amplified, mutagenized plasmids were treated with 1 ul DpnI enzyme for 1 hour at 37°C and then transformed into SuperCompetent *E. coli* strain XL1-Blue cells provided as part of the mutagenesis kit. The transformed cells were plated on LB plates supplemented with ampicillin (100 µg/ml) and incubated at 37°C overnight. All plasmids were sent to MWG Biotech for sequencing to ensure that proper mutagenesis occurred and that no unwanted sequence changes were introduced in the cloning process.

Transformation of D. viridis with C. salexigens tesA: Transformation of *D. viridis* will be accomplished vie electroporation. The gene sequence coding for N-terminal histidine tagged CsTesA will be cloned into an algal expression construct containing a RUBISCO promoter/transit peptide and a RUBISCO terminator (Figure A2-1A). Selection of transformed algae cells will be accomplished via co-transformation of a bleomycin resistance gene driven by a RUBISCO promoter/terminator construct (Figure A2-1B).

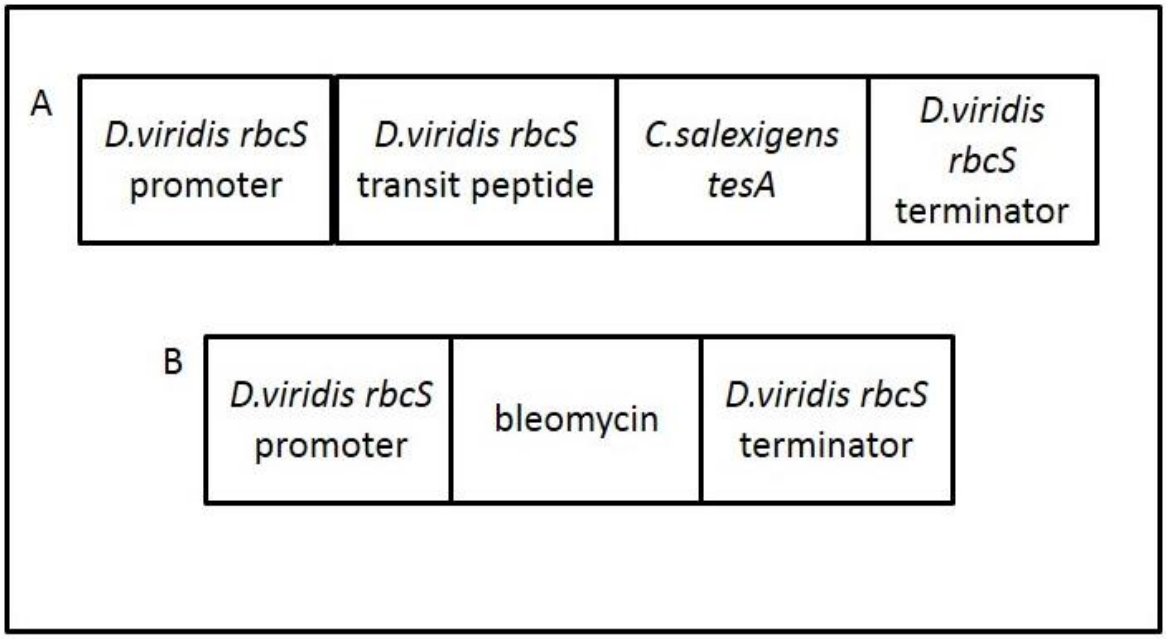

Figure A2-1: **A**: Construct map for expression of *C. salexigens tesA* in *D. viridis.* **B.** Construct map for the selection cassette containing a bleomycin gene for zeocin resistance.

Results and Discussion

The *D. viridis* transformations are currently underway and as such there are no results to report at this point.

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