ALGINATE AND LAMINARIN DEGRADING ENZYMES FROM *VIBRIO SPLENDIDUS* AND *VIBRIO BREOGANII*

BY

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DISSERTATION

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Abstract

The marine bacteria *V. splendidus* 12B01, *V. splendidus* 13B01, and *V. breoganii* 1C10 metabolize brown seaweeds. Brown seaweeds have many advantages over terrestrial feedstocks, including fast growth and non-recalcitrant carbon feedstocks, so brown seaweeds are an attractive target for industrial fermentation. Alginate and laminarin are two major components of brown seaweeds, comprising up to 60% of the dry weight. Alginate is a polysaccharide consisting of the 1,4-linked epimers α-L-guluronate and β-D-mannuronate. Laminarin is a storage glucan consisting of β-1,3- and β-1,6-linked glucose monomers. In order to utilize these carbon sources, many organisms express enzymes that cleave the bonds linking the constituent monomers within alginate and laminarin. These enzymes are called alginate lyases and laminarinases.

V. splendidus 12B01, *V. splendidus* 13B01, and *V. breoganii* 1C10 each contain between four and twelve putative alginate lyases. We have over-expressed and purified 21 alginate lyases from these organisms and determined under what conditions these enzymes are most active. We found these enzymes are optimally active between pH 6.5 and 10 and between 20 to 30 °C. Additionally, these enzymes were broadly salt tolerant between 50 mM and 1 M NaCl. We also determined the enzyme kinetics for these enzymes and found K_m parameters towards alginate between 22 and 300 μM alginate. The computed turnover numbers range from 0.6 to 18 s^{-1}. Alginate lyases have preferential specificity toward specific dyads within alginate. We found alginate lyases with all potential dyad specificities: G-G, G-M, M-G, and M-M specific alginate lyases.

Having characterized the alginate lyases in 12B01, 13B01, and 1C10, we can begin to understand the metabolism of alginate by these organisms. 12B01 was found to poorly degraded and metabolize alginate, and we found 12B01 to express and secrete its enzymes at low levels. In addition we found the 12B01 alginate lyases have low enzymatic activity and narrow dyad specificity. 13B01 was found to degrade and metabolize alginate at high levels. We identified the presence of a unique enzyme to 13B01, which upon knockout, resulted in eight-fold less secreted alginate lyase activity. We found that this high activity enzyme allows 13B01 to degrade alginate efficiently and

then metabolize the liberated monomers of alginate. 1C10 contains eleven alginate lyases within its genome. While this organism has 70% of the 13B01 secreted alginate lyase activity, we found that the 1C10 lyases do not have large enzymatic activity. Rather, the concerted action of enzymes with broad dyad specificity allow 1C10 to efficiently degrade alginate. Overall, we identified several attractive alginate lyases for future metabolic engineering to produce biofuels from alginate. While this would require expression of additional metabolic pathways, we present the first step to the industrial utilization of alginate.

V. breoganii 1C10 contains four laminarinases which we over-expressed and purified. These enzymes had optimal enzymatic activity between pH 6.5 and 8.0 and between 25 and 40 °C. These enzymes were shown to have especially broad tolerance to salt between 50 mM and 1 M NaCl. The 1C10 laminarinases had K_m parameters towards laminarin between 3.4 and 6 mM laminarin. These enzymes also had computed turnover numbers ranging from 0.69 to 6.1 s^{-1}. As the degraded monomer of laminarin is glucose, these enzymes can be expressed in fermentative hosts with no additional metabolic pathways, so laminarin utilization is an attractive target for biofuel production.

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Chapter 1 Introduction

1.1 An overview of brown algae

Brown algae are eukaryotic organisms that fix carbon using photosynthesis in marine environments. Brown algae are macroalgae: meaning that they are comprised of multicellular organism, as opposed to the unicellular microalgae. All macroalgae are typically comprised a leaf-like structures termed a thallus. The thallus contains a lamina (leaf) and stipe (stem) attached to a holdfast. Each thallus is then connected to a supporting structure like a rock or other algae.

The term macroalgae is a loose term that does not refer to a genetic or biologic group, but rather marine organisms that follow the general physical thallus structure. There are three groups of macroalgae: brown algae, green algae, and red algae. These three macroalgae do not share a common multicellular ancestor, and as such, seaweeds are a polyphyletic group.

Macroalgae are targeted as a carbon source for biofuel production. Macroalgae possess many attributes that are favorable over terrestrial crops and microalgae. Some microalgae have been predicted to produce up to 80% oil content of its dry weight (1). Additionally, since microalgae are unicellular, harvesting of microalgae crops is dependent only on cell cycle. Some microalgae have doubling times of 3.5 hours (2). However, since microalgae are unicellular, a culture and harvest system must be enclosed and contained. This system would also require short light paths for maximal growth and a large supply of CO_2 to the closed system. Taking these together, a microalgae-based biofuel process would require large initial costs to build a facility to contain and grow the unicellular organisms.

Macroalgae would not necessitate a closed harvesting area, but rather require interaction with ecosystems for the proper nutrients. As such, the cultivation of macroalgae would be similar to that of terrestrial crops. Macroalgae have a number of advantages in comparison to current terrestrial crops. Macroalgae do not require arable land for cultivation and hence avoid the conflict between food and fuel (3, 4).

Macroalgae also are among the fastest growing organisms on the planet (5). Finally, macroalgae lack crystalline cellulose and lignin (6, 7), thus alleviating a key obstacle to biofuel production.

While the outlined advantages of macroalgae hold for green, red, and brown macroalgae, there are key differences between macroalgae. An ideal macroalgae for biofuel production would be have fast growth in industrial manufacturing and easy downstream processing for biofuel fermentation, so evaluation of macroalgae must take these factors into account. Red macroalgae are currently cultivated for their cell wall polysaccharides, mainly agars and carrageenans. While red macroalgae might seem as an attractive biofuel feedstock, since they are currently cultivated, red macroalgae have slower growth rates than other macroalgae (8). Additionally, the polysaccharides found within red macroalgae are a valuable foodstuff, thus abolishing the "food-vs.-fuel" benefit of macroalgae. Overall, red algae do not meet the requirements for a macroalgae based carbon source. Green macroalgae do have fast growth rates (8), however, their primary cell wall polysaccharide, ulvan, is highly sulfated (9). The sulfated ulvan would complicate any downstream bioprocessing, as fermentation would resulted in hydrogen sulfide production. Additionally, the breakdown of ulvan is poorly understood (10), again complicating downstream processing. Brown macroalgae possess fast growth rates (11) and polysaccharides very amenable to enzymatic degradation (12). As such, brown macroalgae based feedstocks would be easily treated for downstream fermentation. Overall, brown macroalgae are the best candidate feedstock for the production of biofuel.

1.2 Economics of macroalgae

An important question for the future of macroalgae based biofuels is the carrying capacity of marine ecosystems. Previous work has sought to characterize the flux of carbon in both terrestrial and oceanic ecosystems (13). The carbon flux through algal beds and reefs and estuaries comprise approximately 10^9 tons of carbon per year. These populations comprise approximately two thirds of the total ocean carbon biomass. Additionally, these populations, algae and estuary plants, are not directly consumed by animals, but rather are decomposed into CO_2, which is then cycled into the dissolved carbon pools. The decomposed and dissolved CO_2 serves as an important flux of carbon

into the deep ocean that normally doesn't interact with atmospheric carbon. Algae and estuary plants have a turnover time an order magnitude faster than terrestrial plants (1 year vs 10 years) indicating that these populations serve an important role in global carbon flux.

An important factor in the economics of macroalgae for the production of biofuels is the current production of macroalgae. In 2003, the global production of macroalgae was estimated at $5.5 – 6 billion with the majority of macroalgae for human consumption (14). By 2006, the global production of macroalgae had grown to $7.2 billion (15). This corresponds to more than 15 million tons per year of cultivated macroalgae.

1.3 Composition of macroalgae

Macroalgae are comprised of various polysaccharides and glucans that are used for structural support. These polysaccharides comprise up to 60% of the dry weight of macroalgae (16). The composition of macroalgae varies dramatically between species, however, most macroalgae do not contain cellulose, lignin, nor hemicellulose, and thus macroalgae are a different paradigm from current terrestrial feedstocks for fermentation. Cellulose, lignin, and hemicellulose are found in several macroalgae species, albeit at low amounts (less than 10% of the dry weight) (17).

The majority components of macroalgae typically consist of a mixture of several polymers classified as either polysaccharides or glucans. The macroalgae *Laminaria digitata* is targeted as a potential source of biomass for macroalgae-based fermentation. Within this macroalgae, up to 45% of its dry weight is comprised of the polysaccharide alginate, up to 18% of its dry weight is the glucan laminarin, a small amount (2-4% dry weight) is comprised of the polysaccharide fucoidan, and up to 22% of its dry weight is mannitol (16, 18). These compositional percentages vary dramatically on the life-cycle of each macroalgae and the cultivation conditions. The polysaccharide and glucan compositions also vary between different macroalgae species. The compositions of other macroalgae can be found in **Table 1.1**.

1.4 Alginate

Alginate is a polymer consisting of α-L-guluronate (G), and β-D-mannuronate (M) linked together, **Figure 1.1**. Guluronate and mannuronate are epimers of one another in that they differ only in chiral center. The interlinkage of the M and G block within alginate can take on one of three forms: polyguluronate (polyG), polymannuronate (polyM), and heteropolymeric M/G (polyMG) (19). The heteropolymeric form can take the form of either alternating G or M or alternating short stretches of polyG and polyM. Alginate is a polysaccharide found within the cell wall and intercellular region of brown seaweeds. Commercial production of alginate is extracted primarily from brown seaweed genera *Macrocystis*, *Laminaria*, and *Ascophyllum*.

Alginate is also produced from the bacterial families *Azotobacteriaceae* and *Pseudomonadaceae*. Alginate derived from bacterial sources differs from algae alginates, in that bacterial alginates contain *O*-acetyl groups on the 2 or 3 carbon positions of D-mannuronate (20). An initial investigation of an alginate lyase derived from an *Azotobacter vinelandii* phage suggested that acetylation of the D-mannuronate protects its conversion to L-guluronate via a C-5-mannuronate epimerase (21, 22). This hypothesis was confirmed (20) via epimerization of acetylated and deacteylated alginate from *A. vinelandii*. The acetylated alginate had a minor increase in guluronate fraction, while the deacteylated alginate has a more than 20% increase in L-guluronate fraction, indicating that acetylation of the D-mannuronate protects epimerization. Only 20% of the D-mannuronate residues were acetylated, indicating that acetylation of a D-mannuronate residue has a protective effect on neighboring D-mannuronate residues.

Interestingly, the synthesis of alginate within *A. vinelandii* has been proposed (23) to involve conversion of fructose into mannose-6-phosphate via fuctokinase and phosphoglucose isomerase. Mannose-6-P is then converted into GDP-mannose via the enzymes phosphomannomutase and GDP-mannose pyrophosphorylase. Finally, GDP-mannose is converted into GDP-mannuronic acid via GDP-mannose dehydrogenase. The GDP-mannuronic acid is linked together to form polymannuronic acid, which is then converted to alginate via the selective epimerization of mannuronic acid residues by C-5-mannuronate epimerase. The acetylation of mannuronate allows for the partial conversion of polymannuronic acid. The formation of alginate in brown seaweeds has

been proposed as having a similar pathway (24), however, GDP-guluronic acid has been detected in algal seaweeds, so conversion of the assembled polymer is not needed, and thus acetylation is not present in the brown seaweed derived alginate.

1.5 Alginate lyases and alginate metabolism

Alginate lyases are a class of enzymes that degrade alginate through β-elimination of the glycosidic bond between polyG (EC 4.2.2.11), polyM (EC 4.2.2.3), or polyGM. Alginate lyases have been discovered that are characterized as polyG, polyM, or polyMG-specific alginate lyases based on substrate specificity. Additionally, alginate lyases can have either endo-cleaving or exo-cleaving specificity with the majority of alginate lyases having endo-cleaving preference (19). The mechanism of action for alginate lyase has been proposed as the following three sequential events: 1) removal of the negative charge on the carboxyl anion, possibly through action of a arginine or glutamine residue, 2) abstraction of the C-5 proton on the sugar ring by an aspartic acid, glutamic acid, histidine, lysine, or cysteine residue, and 3) transfer of electrons from the carbonyl group to form a double bond between C-4 and C-5. The culmination of these steps results in the β-elimination of the glycosidic bond (19, 25, 26), as depicted in **Figure 1.1**. The action of an alginate lyase, along with other polysaccharide lyases (PLs), differs from the action of glycoside hydrolases (GHs). GHs cleave the glycosidic bonds through hydrolysis, wherein the addition of a water molecule across the cleaved bond maintaining the 4-OH group on the new non-reducing end. PHs cleave the glycosidic bond without water, as outlined above for alginate lyases, generating a double-bond on the new non-reducing end termed a hexeneuronic acid moiety.

Some microorganisms can use alginate as a carbon source, and as such, must possess a means of converting alginate to a form metabolized by common metabolic pathways. The action of an alginate lyase will cleave alginate using a β-elimination reaction that generates an L-guluronate or a D-mannuronate and a non-reducing end. The non-reducing end contains 4-deoxy-L-erythro-hex-4-enepyranosyluronate residue that is signified as Δ to emphasize that it is structurally different from both L-guluronate and D-mannuronate (27). The L-guluronate and D-mannuronate monomers are non-

enzymatically converted into 4-deoxy-L-erythro-hexoseulose uronic acid (DEH) and then the α-keto acid is enzymatically converted into 2-keto-3-deoxy-D-gluconic acid (KDG) via the NADPH-dependent enzyme DEH reductase (28). KDG is then converted into 2-keto-3-deoxy-6-phosphogluconic acid (KDPG) via the enzyme KDG kinase **(Figure 1.1)** (12). KDPG is then converted to pyruvate and glyceraldehyde-3-phosphate via the enzyme KDG-6-phosphate aldolase (12).

In order to transport the extracellularly degraded alginate derived L-guluronate and D-mannuronate oligomers into the cell, a means of transport is required on both the outer-membrane and inner-membrane. The marine bacterium *Vibrio splendidus* contains both transport systems that allow transport of oligomers into the cytoplasm (12). The outer-membrane porin KdgMN is used to transport longer chain oligoalginate polymers into the periplasm. The longer chain oligoalginate polymers are degraded into chains of length 2-to-4 within the periplasm, which are then transported into the cytoplasm via the symporter ToaABC. The short chain oligomers are then degraded and metabolized. An alternative means to transport alginate can be found in the bacterium *Sphingomonas sp.* strain A1 which contains a periplasmic alginate binding protein-dependent ATP binding cassette (ABC) transporter (29). The transporter contains three components: a pit on the cell surface which allows the alginate polymer into the periplasm, alginate-binding proteins within the periplasm, and an ABC transporter in the inner-membrane. Alginate lyases are then contained within the cytoplasm that degrades alginate. The two transport mechanisms outlined above demonstrate that alginate must be carefully transported into the cell either following initial degradation or via chaperone-like transport to continuously metabolize alginate.

In analyzing the known alginate lyases, a number of different lytic motif structures have appeared, termed PL families. PL families show wide variety in fold types, including β-helicies and α/α barrels. Since, PLs are characterized by the catalytic function rather than their structures, PLs can have the same catalytic function, but vastly different structures, indicating that PLs have been invented many times. Alginate lyases belong to seven PL families (30, 31): PL5, PL6, PL7, PL14, PL15, PL17, and PL18. PL5 and PL15 contain an $(\alpha/\alpha)_6$ barrel structure, which consists of α-helices interlinked. PL7, PL14, and PL18 contain a β-jelly roll that consists of β-sheets in an antiparallel, adjacent

barrel forming a cleft. PL6 contains a parallel β-helix forming a long series of β-strands. The crystal structure of PL17 has many α-helices in a large 3-D structure (32). PLs belonging to the same family are expected to have a similar evolutionary origin. Thus, analysis of PLs must take into account the PL families, in order to make an equal comparison.

Alginate lyases are a prevalent class of enzyme within prokaryotes and eukaryotes. Alginate itself is produced in a number of organisms, both terrestrial and aquatic environments. Alginate lyases have been discovered that are produced by micro-organisms that co-inhabit these environments. A survey of bacterial isolates associated with brown algae *Fucus distichus* found dramatically variable alginate lyase activity and M and G specificity (33). Interestingly, while alginate is produced by many organisms, an alginate lyase from the soil bacterium *Azotobacter vinelandii* has been found to degrade alginate produced from aquatic algae and the bacterium *Pseudomonas aeruginosa*, despite having different chemical properties (34).

An alginate lyase Aly from *Pseudomonas alginovora* has been overexpressed and classified based on its substrate specificity (35). Aly was found to have a polyM specificity based on its high enzymatic activity with a polyM substrate, intermediate to low activity with an alginate substrate, and an almost 14-fold decrease under a polyG substrate in comparison to the polyM substrate. Additionally, Aly from *P. alginovora* was found to have 50% lower activity in a 75 mM sodium phosphate (pH 7.5)/450 mM NaCl buffer in comparison to a 225 mM Tris/HCl (pH 7.5). Additionally, a preliminary classification of Aly from *P. alginovora* assigned the gene with the corresponding Aly gene from *K. pneumoniae*. Further studies of Aly using NMR analysis confirmed the substrate specificity of polyM substrates (36). The NMR analysis found Aly to act only on the M-M dyads. No cleavage occurred between M-MG, G-MM, or G-MG. Following expectations, the only oligosaccharides found were ΔM, ΔMM, ΔMMM, and ΔMMMM. The NMR analysis confirms that Alg from *P. alginovora* has indeed polyM specificity.

The alginate lyase, AlgL, from the bacterium *Azotobacter chroococcum* was cloned and overexpressed (37). This lyase has high sequence similarity to another *Azotobacter* species lyase (90% identity), while has low homology (15 to 20% identity) to other alginate lyase, so AlgL can serve as a representative from a distinct PL5 lyase. It

was found that this lyase had optimal activity at 30 °C and pH 7.5. Additionally, by combinatorially adding potassium, sodium, and magnesium ions, the authors found that maximal activity was found at 100 mM $K^+/Na^+/Mg^+$ in a 10 mM Tris-HCl (pH 7.5) buffer. The addition of Ca^{2+}, Co^{2+}, Mn^{2+}, and Zn^{2+} had no effect on the activity of AlgL.

An alginate lyase from the soil bacterium *Azotobacter chroococcum* was isolated from the supernatant during growth on 0.3% sodium alginate (38). The lyase was found to have optimal activity at pH 5.5-6.0 and 60 °C. Additionally, the lyase was found to be stable for 24 h exposure at pH 6.0-10.0 and 30-60 °C. The metal ion effect was also investigated; 1 mM Ca^{2+} was found to increase enzymatic activity 50%, while 1 mM Hg^{2+} was found to abolish activity. Other metal ions, 1 mM Ba^{2+}, Co^{2+}, Cu^{2+}, Fe^{3+}, and Mn^{2+}, were found to have moderate decrease to no effect on enzymatic activity. Using polyG and polyM as a substrate, the alginate lyase was found have activity only with polyM substrate.

The alginate lyase $AlxM_B$ from the marine bacterium ATCC 433367 (39) has been overexpressed and characterized (40). $AlxM_B$ was found to have two-fold increase in activity on alginate derived from *P. aeruginosa* in comparison to the brown seaweed *L. hyperborean*. Increasing ionic strength was found to increase enzymatic activity; above 1 M ionic strength enzymatic activity plateaus, while enzymatic activity is linear below 1 M ionic strength. The maximal enzymatic activity of $AlxM_B$ under high ionic strengths is expected, and should follow for other alginate lyases derived from marine bacteria.

The vertebrate sea hare *Aplysia kurodai* contains two alginate lyases AkAly28 and AkAly33 (41). The lyases were isolated from the gastric lumen and then dialyzed and centrifuged in order to isolate the lyases. The crude enzymes were then purified to yield ~28 kDa and ~33 kDa products. Both lyases had an identical optimal pH of 6.7 and temperature of 40 °C. The NaCl concentration effect on enzymatic activity differed between AkAly28 and AkAly33: AkAly28 showed no activity in the absence of NaCl, while AkAly33 showed 20% of maximal activity without NaCl. Both AkAly28 and AkAly33 showed optimal activity at approximately 0.2 M NaCl. In analyzing the substrate specificity, the authors found that both AkAly28 and AkAly33 did not activity with a polyG-rich substrate, while activity was found with polyM- and polyMG-rich substrates, indicating that AkAly28 and AkAly33 have preference to polyM substrates.

1.6 Laminarin

Laminarin is a storage glucan present in macroalgae. Laminarin is composed of β-1,3- and β-1,6- linked glucose monomers (42), **Figure 1.2**. The precise composition of laminarin varies dependent on its origin; the composition can vary from less than 3% to almost 30% of the dry weight of macroalgae (43). The unicellular algae *Phaeocystis globosa* was found to contain only β-1,3-glucose within its laminarin (44). Another unicellular algae, *Pleurochrysis haptonemofera*, was found to contain 50% more β-1,3-linked glucose than β-1,6-linked glucose in its laminarin (45). The macroalgae genus *Laminarina* (brown seaweed) contains a laminarin with a terminal mannitol residues. These terminal mannitol residues outnumber non-terminated residues 3:1 (46). Further work demonstrated that mannitol terminated laminarin is more insoluble than non-terminated polymers (47).

Since laminarin is primarily a β-1,3-glucan, much analogy can be made to other β-1,3-glucans. β-1,3-glucans are a prevalent interlinkage in storage glucans found in nature with the majority of those storage glucans containing other linkages in their structure. Yeast cell walls contain storage glucans with β-1,3-linked glucose and either β-1,2-, β-1,4-, or β-1,6-linked glucose (47). Additionally, the glucans with β-1,3- and β-1,4-linked glucose can be found in oat β-glucans (47). Since storage glucans with similar structures to laminarin can be found, these glucans can serve as important points of comparison to the study of laminarin and its degradation

1.7 Laminarinases

The enzymes that degrade the β-1,3 and β-1,6 linkages within laminarin are called laminarinases. These enzymes belong to a larger class of enzymes called glycoside hydrolases (GHs) (48). Glycoside hydrolases catalyze the hydrolysis of glycosidic bonds within glucans. Using a molecule of water, GHs cleave a glycosidic bond within a glucan, as depicted in **Figure 1.2**. The cleavage of laminarin occurs in two steps. First, in the glycosylation step, one of the catalytic residues acts as a nucleophile by attacking the anomeric center. Simultaneously, the other residue acts as an acid by protonating the

glycosidic oxygen, thus cleaving the glycosidic bond (49). These actions result in the formation of a complex between one of the catalytic residues and the cleaved laminin molecule. In the second step, called deglycosylation, water now hydrolyzes the complex, thus freeing the amino acid from the cleaved laminarin. The action of a GH results in the formation of a hemiacetal and an aglycon (48). The residues that catalyze the hydrolysis of a glycosidic bond are commonly glutamic and aspartic acids (50, 51).

Glycoside hydrolases are classified based on their catalytic mechanism. These enzymes employ either a retaining or inverting mechanism (48). The inverting mechanism simultaneously utilizes the acid and base catalytic residues to cleave the glycosidic bond. Since the newly added hydroxyl group from the water attack exists in the opposite configuration of the original attacked β-glycosidic bond, the cleaved hemiacetal contains a 6C hydroxyl group in an α-configuration. Due to the inversion of the stereochemistry, this mechanism is called an inverting mechanism. The retaining mechanism employs two sequential attacks by both catalytic sites wherein a transition state is formed between attacks allowing for preservation of the β-configuration. Since each of the sequential attacks inverts the stereochemistry, the net result is the new hydroxyl group on the cleaved monomer retains the original β stereochemistry, hence a retaining mechanism.

Previous investigations have sought to characterize the laminarinases from a diverse set of organisms. The archaeon *Pyrococcus furiosus* contains a laminarinase that was determined most enzymatically active between pH 6.0 and 6.5 and at 100 °C (52). Additionally, the laminarinase was found to have maximal activity on laminarin as a substrate and approximately 90% lesser activity on lichenan and barley β-glucan (β-1,3-1,4 glucose). The *P. furiosus* laminarinase had no activity on other tested β-1,4 glucans. A laminarinase from the actinobacterium *Streptomyces sioyaensis* contained a laminarinase which was over-expressed and purified (53). This laminarinase was found to have optimal activity at 75 °C and pH 5.5. This enzyme was found to most active on the β-1,3 glucans laminarin, curdlan, and pachyman with 4-fold less activity on the β-1,3-1,4 glucan lichenan. The fungus *Phanerochaete chrysosporium* contains a laminarinase that was secreted and then purified (54). This laminarinase was found to have a substrate specificity for laminarin and lichenan (β-1,3-1,6 and β-1,3-1,4, respectively, linkages)

and no activity on cellulose (β-1,4 linkage). The laminarinase Bgn2115 from *Bacillus halodurans* was over-expressed and purified (55). Bgn2115 was found optimally active at pH 6 and 60 °C. The marine bacterium *Zobellia galactanivorans* contains a laminarinase *Zg*LamA that was over-expressed and purified (56). *Zg*LamA was found optimally active at pH 8.5 and demonstrated higher activity on laminarin than barley β-glucan.

1.8 Metabolic engineering of alginate and laminarin degrading pathways

In order to utilize macroalgae-based polysaccharides and glucans for microbial fermentation, the enzymes necessary for the degradation of these polymers must be expressed and then applied to the polymers to facilitate degradation. This method requires the expression of enzymes in a recombinant organisms, their purification, and then application to the biomass polymers and incubation at an optimal condition for degradation. The enzymes and incubation conditions must be experimental determined to allow for the most efficient degradation. The degraded monomers will then be fermented to produce any desired products. A schematic of this flow path can be seen in **Figure 1.3**.

The previously outlined schematic can take place as separate steps (enzymes for degradation expressed and purified by a recombinant organism followed by fermentation by a different metabolic organism) or as a single step (the fermentative organism expresses the degradation enzymes and then ferments the desired product). Each process will now be outlined.

There are many advantages to reproducing a metabolic pathway *in-vitro*. A reconstituted pathway would remove any cell toxicity issues that arise during fermentation. Additionally, a fermentative process does not need to include transporters to bring together metabolites or export products. Most importantly, one does not need to engineer a heterologous pathway to compete against native pathways, thus product yields can approach 100% of starting amounts.

"Cell-free metabolic engineering" has been employed to build a pathway to produce 2-deoxyribose 5-phosphate (57). In this study, the authors over-expressed the necessary enzymes from *Thermus thermophilus*. Since the necessary metabolites and cofactors for the pathway were found in the crude *Escherichia coli* lysate, purification was not needed. However, since the native *E. coli* proteins were then included with the heterologous *T. thermophilus* pathway, a means of deactivating the *E. coli* proteins was needed. The authors found that by incubating the crude lysate, containing both the native *E. coli* proteins and the heterologous *T. thermophilus* enzymes, at 70 °C they could deactivate the *E. coli* proteins only while leaving the *T. thermophilus* enzymes in their native state. The authors were then able to convert 55% of the starting reagent into their desired product within 4 hours. "Cell-free metabolic engineering" has also been shown to produce a chimeric pathway to produce lactate (58). In this work, a chimeric Embden-Meyerhof pathway was constructed that utilized the native *E. coli* enzymes with those from an archaeal Embden-Meyerhof pathway. The authors were then able to reach a conversion approaching 100% conversion. These works demonstrate a metabolic strategy to express enzymes and pathways outside the cell to facilitate fermentation under optimal conditions and conversions.

While "cell-free metabolic engineering" has many advantages compared to a consolidated bioprocess, an organism containing enzymes necessary for the degradation of feedstocks and those necessary for fermentation, also has advantages. This organism is easier to engineer since all the pieces are put together. We can use metabolic engineering techniques such as directed evolution to improve the engineered strain, again since all the enzymes are in-place in one organism. Additionally, since our combined bioprocess produces all enzymes, we do not need to produce enzymes nor lysates for each fermentation, as we would have to do with a "cell-free metabolic engineering" process.

The simplest approach to ferment macroalgae into a chemical product is to engineer an organism that can natively degrade macroalgae to produce a chemical product. An example of this has been accomplished in the bacterium *Sphingomonas* sp. A1. This bacterium natively degrades alginate and then metabolizes the degraded product. *Sphingomonas* sp. A1 possesses a novel transporter (59) for alginate wherein

the alginate polymer is transported across a "pit" on the cell surface. The polymer is then carried through the periplasm and inner membrane through alginate recognizing proteins and transporters. Alginate is then degraded by alginate lyases within the cytoplasm. Thus, alginate lyases are not secreted by *Sphingomonas* sp. A1 but rather the polysaccharide is physically imported into the cell and then degraded and metabolized. Having the machinery to degrade and metabolize alginate, *Sphingomonas* sp. A1 has been engineered to produce ethanol (60). This was accomplished by expressing the *Zymomonas mobilis* genes *pdc* and *adhB*. These genes allow for the conversion of pyruvate into acetaldehyde and ethanol. Through this expression and other strain engineering techniques, the authors were able to ferment 13 g/L ethanol in bacterium *Sphingomonas* sp. A1. While this strain is a non-optimal fermentation strain for the industrial product of ethanol, Takeda and coworkers demonstrate that alginate is a viable feedstock for the production of ethanol.

Another approach for a combined bioprocess is the expression of alginate degrading pathways in a host more amenable for industrial fermentation. This approach has been demonstrated for the production of ethanol by *E. coli* using brown seaweed as the sole carbon source (12). As the authors did not know of a pathway for alginate degradation that they could express in *E. coli*, they sought to identify this pathway by creating a fosmid library of an organism that could degrade alginate. The organism that the authors used to create this library is *Vibrio splendidus* 12B01. By shearing its genome into 40 kb fragments and expressing these fragments in *E. coli*, they could identify a contiguous region of the genome that possesses enzymes and transporters necessary for the degradation and metabolism of alginate and thus macroalgae. This *E. coli* strain was then engineered to produce ethanol by including the genes *pdc* and *adhB* from *Zymomonas mobilis*. During the preliminary engineering of *E. coli*, Wargacki and coworkers found that they needed to engineer a secreted alginate lyase to begin the degradation of alginate in the fermentation culture. The additional alginate lyase was engineered to be secreted at high levels by the *E. coli* host, presumably due to non-optimal secretion of the *V. splendidus* alginate lyases. Wargacki and coworkers have shown that a bioprocess can be engineered to express heterologous enzymes and

transporters from different organisms in a genetically amenable host for the consumption of alginate and macroalgae as the sole carbon source.

1.9 Conclusions

In order to build strains capable of utilizing alginate and laminarin as the sole carbon source for fermentative processes, a greater understanding of the degradation of macroalgae feedstocks is needed. Previous investigations of these enzymes sought to characterize single enzymes without context of industrial degradation. Since individual microorganisms might contain several alginate lyases or laminarinases, characterization of all the degrading enzymes is necessary to increase the space of available enzymes for strain engineering. Additionally, alginate- and laminarin-degrading microorganisms frequently exist as part of a consortia that forms complex ecosystems. These ecosystems frequently contain "cheaters" which cannot fully metabolize or degrade alginate or laminarin on their own (61). Thus, if these "cheaters" were to be cultured and tested, they would be predicted to have no alginate- or laminarin-degrading enzymes. However, when paired with other microorganisms, their enzymes allow for alginate metabolism. One must consider a microorganism's ecosystem when analyzing their enzymes to allow for an even richer field of enzymes for metabolic engineering.

To allow for a wide variety of enzymes to be analyzed, a pipeline for the characterization of enzymes has been developed in this work **Figure 1.4**. This procedure allows for the methodical analysis of marine bacteria to harness their enzymes for the industrial degradation of alginate or laminarin. Once a large set of enzymes are identified and characterized, a combinatorial strain engineering strategy can be employed to generate potential alginate- and laminarin-degrading pathways in a fermentative host. By understanding the enzyme kinetics and properties of many different alginate and laminarin degrading enzymes, an engineering strategy can be developed wherein enzymes are "mixed-and-matched" to produce candidate pathways, **Figure 1.5**. These are then screened for desirable traits, such as yield and productivity. The process can be iterated using directed evolution to create new proteins to further improve strain performance. The evolved enzymes can also be analyzed in the pipeline outlined above, allowing for total feedback in the metabolic engineering. Overall, this strategy will

couple preliminary enzyme characterization with powerful metabolic engineering techniques to create strains that ferment macroalgae to produce a desired product.

Chapter 1 Figures and Tables

Table 1.1. Composition of brown algae (16).

Brown Algae	Alginate %	Laminarin %	Fucoidan %	Mannitol %
Laminaria digitata	16-45	0-18	2-4	4-22
Saccharina latissima	21-46	0-26	-	6-22
Laminaria hyperborean	22-35	0-24	2-4	6-18
Ascophyllum nodosum	15-30	0-10	5-10	5-10
Fucus vesiculoses	14-17	2-5	-	8-16

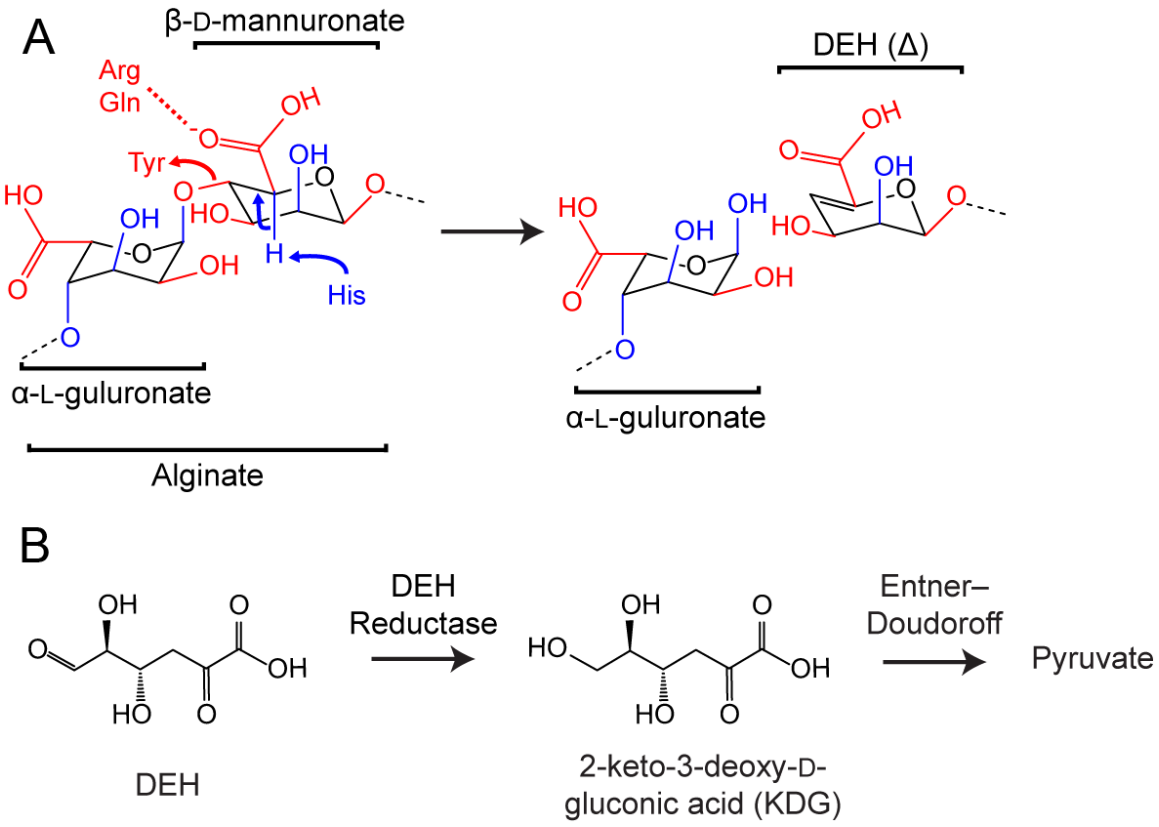

Figure 1.1. A) Structure of alginate. The indicated arrows are the catalytic attacks that lead of the degradation of alginate to form DEH. B) Following cleavage by an alginate lyase, DEH is converted to KDG by DEH reductase which directly enters the Enter-Doudoroff pathway.

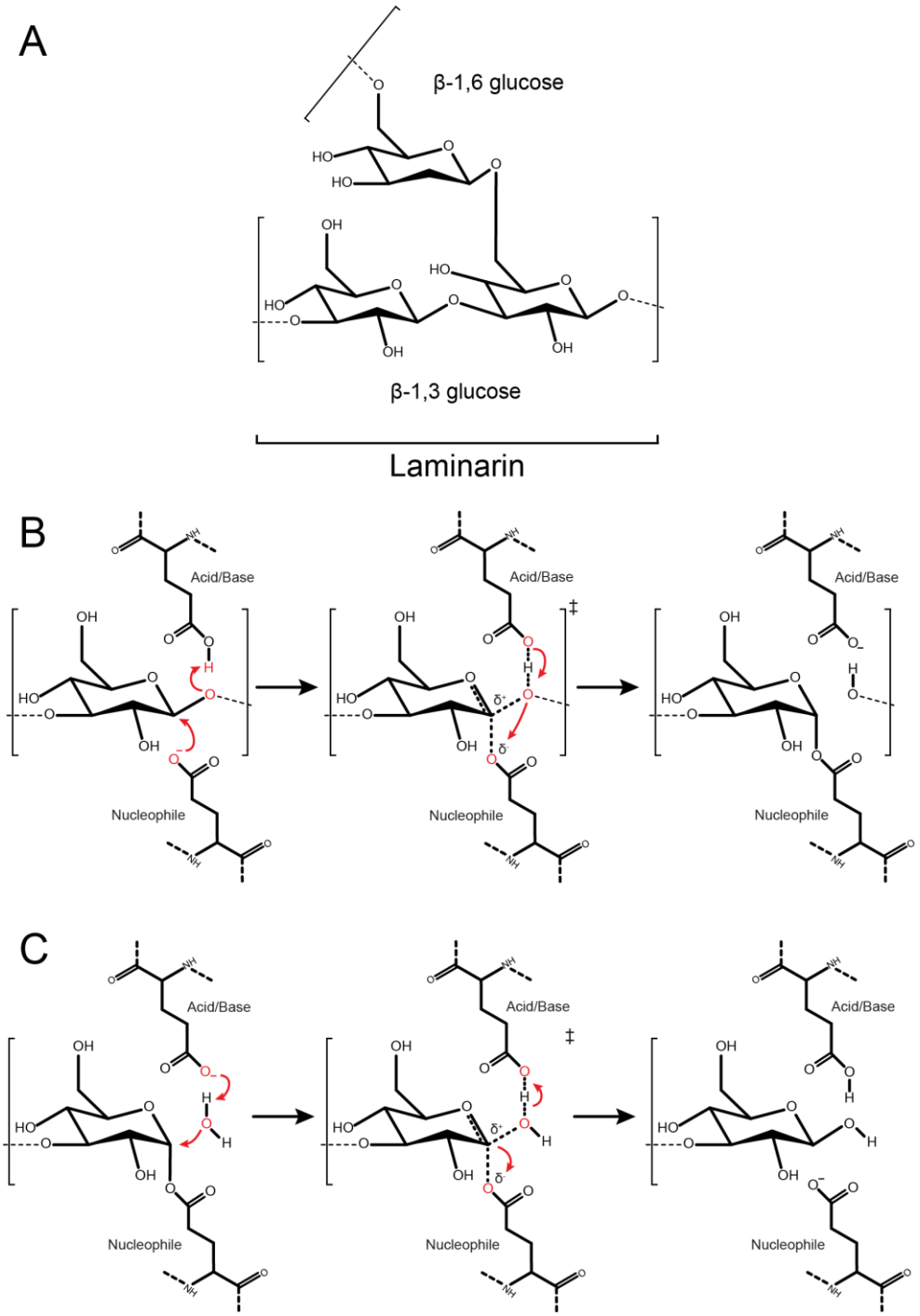

Figure 1.2. A) Structure of laminarin. B) Glycosylation of laminarin occurs when the catalytic amino acid residues attach the glycosidic bond in laminarin. An intermediate state is formed which is then followed by cleavage of the bond with one of the glucose monomers bound to the nucleophile residue. C) To liberate the bound nucleophile, water attacks the complex, which forms another intermediate state. Following donation of electrons, the catalytic amino acids are regenerated, thus forming a new reducing end of laminarin and a net cleavage of the glycosidic bond.

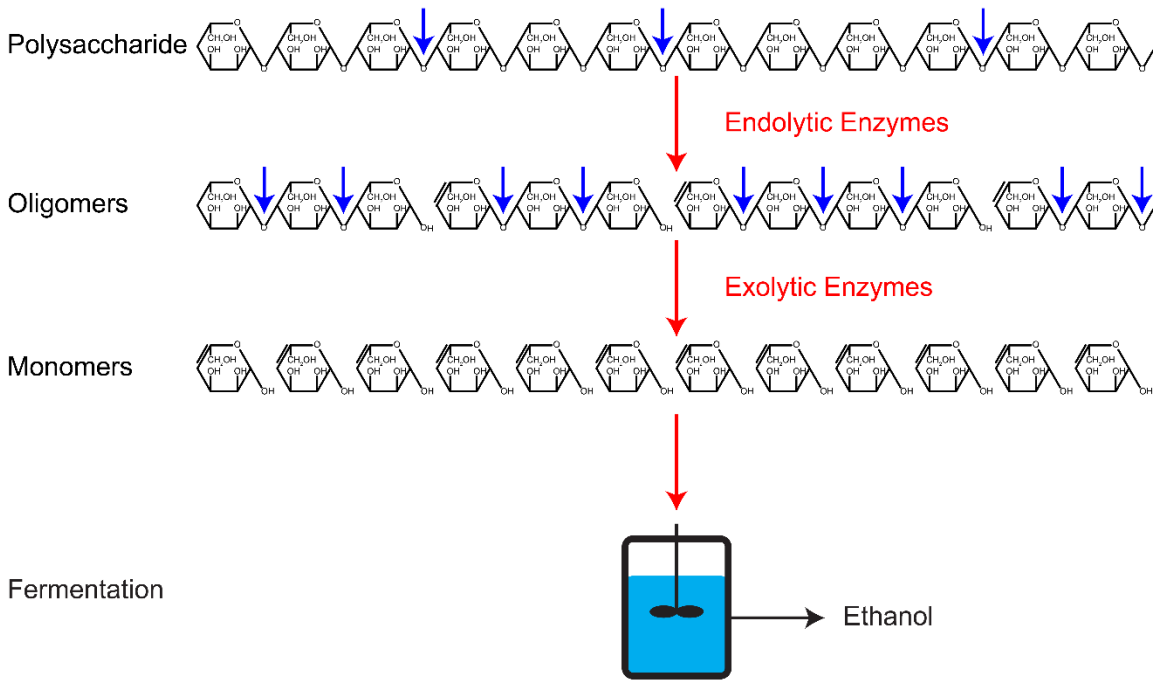

Figure 1.3. Degradation of polysaccharide (or glucan) by endolytic and exolytic enzymes for the fermentation of macroalgae to produce ethanol or other fermentative products. Blue arrows indicate the location of attack of either endolytic or exolytic enzymes.

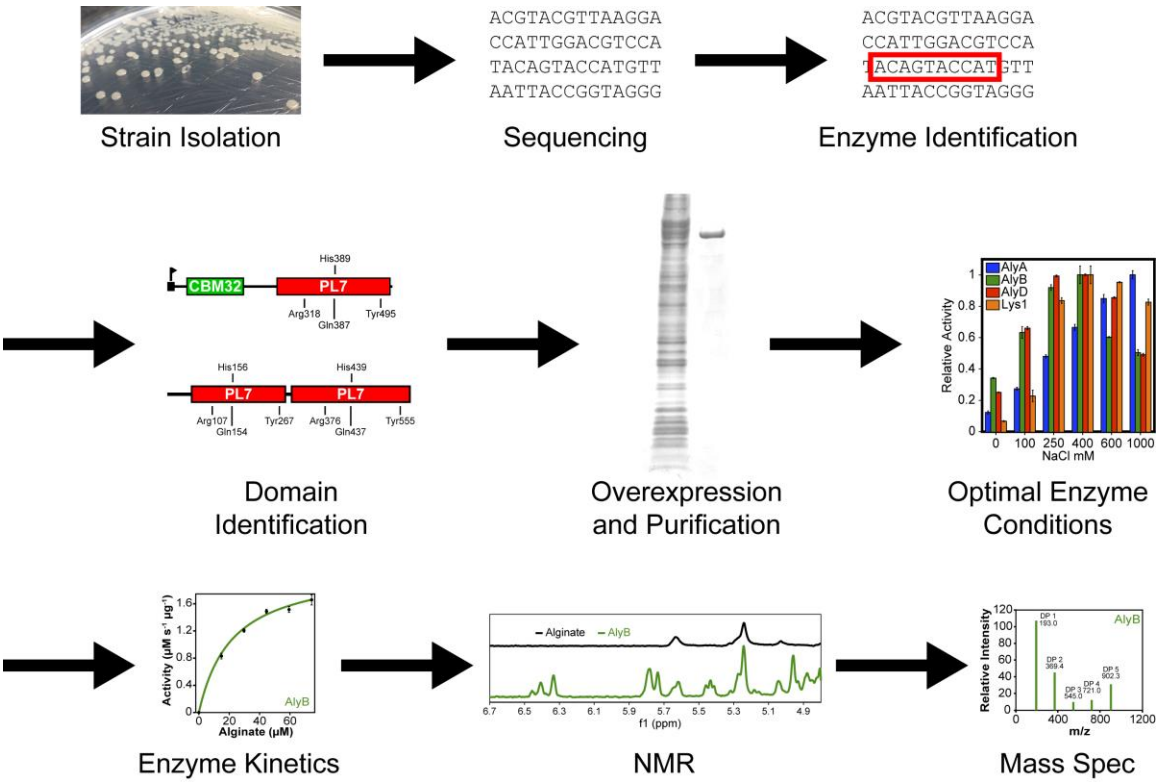

Figure 1.4. Pipeline for the characterization of enzymes for the production of biofuels from alginate and laminarin. Strains are identified that have desirable alginate or laminarin degrading abilities. These strains are then sequenced in Next-Generation Sequencing and bioinformatics is done to identify candidate enzymes for downstream analysis. These enzymes are further analyzed for identify their domains and catalytic residues. Enzymes are then over-expressed and purified and the optimal environmental conditions are determined by varying conditions sequentially. Enzyme kinetic parameters can then by determined at the optimal environmental conditions for each enzymes. NMR and ESI-MS analysis are then performed to identify the mechanism of enzyme action. These steps are performed for each enzyme under consideration which will give a complete picture as to how the candidate organism utilizes alginate or laminarin.

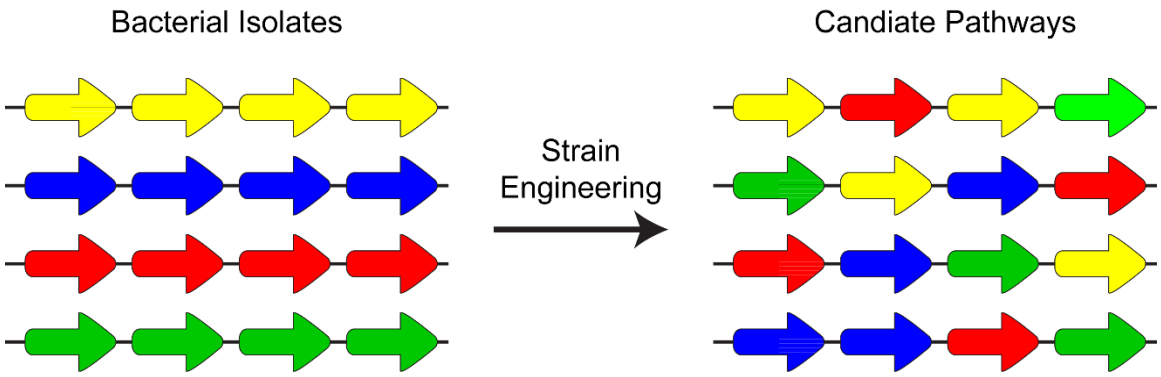

Figure 1.5. Metabolic engineering strategy to develop fermentative strains capable of degrading alginate and laminarin to produce ethanol and other products. Different bacterial isolates with distinct enzymes are shown on the left. Following strain engineering and rational design to pair enzymes, transporters, and regulators from different isolates, these candidate pathways are screened for better alginate and laminarin utilization and conversion.

Chapter 2 Materials and methods

2.1 Media

To culture *Escherichia coli* in rich media conditions, Luria-Bertani (LB) medium (10 g/L tryptone, 10 g/L NaCl, and 5 g/L yeast extract) was used. Milli-Q Type 1 water was used exclusively for this medium. Kanamycin was typically added at a concentration of 40 µg/mL. Chloramphenicol was typically added at a concentration of 20 µg/mL. Ampicillin was typically added at a concentration of 100 µg/mL.

To culture *Vibrio sp*. in rich media conditions, three media formulations were utilized. Luria-Bertani-salt (LBS) medium (per liter tap water: 10 g tryptone, 5 g yeast extract, 0.34 M NaCl, 20 mM glycerol, 20 mM Tris hydrochloride, pH 7.5) (62) was found to have excellent growth. Luria-Bertani 0.5 M NaCl (LB 0.5 M NaCl) medium (10 g/L tryptone, 29.22 g/L NaCl, and 5 g/L yeast extract) (63) was used in situations requiring no additional carbon sources. LB 0.5 M NaCl was made using Milli-Q Type 1 water. Growth on LB 0.5 M NaCl was slower than LBS medium. In conditions requiring lower NaCl concentrations, Tryptic Soy Broth (TSB, Difco) with 1% or 2% NaCl was employed. TSB is formulated as follows: 17 g/L tryptone, 3 g/L soytone, 5 g/L NaCl, 2.5 g/L K_2HPO_4, and 2.5 g/L glucose. TSB with 1% or 2% NaCl was formulated was TSB with an additional 5 g/L or 15 g/L NaCl, respectively. All TSB formulations were made with Milli-Q Type 1 water. Kanamycin was added at a concentration of 125 µg/mL. Chloramphenicol was added at a concentration of 5 or 10 µg/mL.

To culture *Vibrio sp.* in minimal conditions, two media formulations were employed. The first is a variation on the *E. coli* M9 minimal medium. This medium, M9-salt medium (M9S) (64), is formulated as follows: 11.28 g M9 minimal salts (Sigma-Aldrich M6030), 2 g casamino acids, and 18 g NaCl per liter tap water. M9S was then supplemented with 1 mM $MgSO_4$ and 0.1 mM $CaCl_2$ and the necessary carbon sources.

The other minimal medium used for the culture of *Vibrio sp.* was Tibbles and Rawlings nitrogen-deficient glucose (NDG) minimal medium (65) with modification. NDG was formulated in two portions which were separately prepared and autoclaved.

Salt Medium I was prepared by dissolving the following in 400 mL of Milli-Q Type 1 water: 25.95 g NaCl, 3 g $MgSO_4 \cdot 7H_2O$, 2 g $MgCl_2 \cdot 6H_2O$, 0.12 g $CaCl_2 \cdot 2H_2O$, 25 mL 1 M Tris (pH 8.0), 2.7 mL 0.5 M Na_2EDTA, and 10 mL 1 M NH_4Cl. Salt Medium I was then adjusted to pH 7.8 and the volume was made up to 500 mL. Salt Medium II was prepared by dissolving the following in 400 mL of Milli-Q Type 1 water: 0.8 g K_2HPO_4, 0.2 KH_2PO_4, and the carbon source. The pH was adjusted to 8.0 and the volume is made up to 500 mL. The carbon source in Salt Medium II was either alginate (Sigma-Aldrich W201502), laminarin (Sigma-Aldrich L9634), or glucose. Alginate was included in Salt Medium II before autoclaving, while laminarin or glucose were added to Salt medium II after autoclaving as was then filter sterilized. After autoclaving and cooling, Salt Medium I and II were mixed. NDG was then supplemented with 1 mL 1,000X Vitamin Mix (**Table 2.1**) (66), 1 mL 1,000X $FeSO_4$ solution (15 g/L, 0.22 µm filter sterilized and stored at -20 °C), 1 mL 1,000X Trace Element solution (**Table 2.2**) (65), and 1 mL 1,000X Na_2MoO_4 solution (5 g/L, 0.22 µm filter sterilized and stored in a dark container at 4 °C).

To culture *Photobacterium phosphoreum* (67) for maximal luminescence, PLBS medium was used (950 mL tap water, 50 mL 1 M Tris, pH 7.5, 3 mL glycerol, 10 g tryptone, 5 g yeast extract, 20 g NaCl). This bacterium also grows and luminesces on LBS medium.

2.2 Bacterial strains and growth conditions

Cloning of genes and genetic constructs was undertaken in the *E. coli* strains DH5α and Π3813 (63). Both strains were made competent using the method of Inoue and coworkers (68) and cells were recovered using Super Optimal with Catabolite repression (SOC) (69). DH5α was used with plasmids containing origins of replication colE1 and pBR322. Π3813 was used with plasmids containing the *pir*-dependent R6K origin of replication. Π3813 contains a thymidine auxotrophy due to the Δ*thyA*::(*erm–pir-116*) genotype, so thymidine was included in medium formulations at 0.3 mM. For conjugative transfer of mobilizable plasmids between hosts, the conjugative hosts β3914 (63) and WM3064 (70) were used. Both of these strains contain a diaminopimelate

auxotrophy due to the Δ*dapA* genotype, so diaminopimelate was included in medium formulations at 0.3 mM.

In order to over-expressed proteins for further purification, the *E. coli* strain BL21(DE3) was employed. This strain possesses a genotype advantageous of the production of recombinant proteins. All *E. coli* strains were incubated at 37 °C, unless otherwise noted.

Vibrio splendidus 12B01 and 13B01 and *Vibrio breoganii* 1C10 were gift from Martin Polz (Massachusetts Institute of Technology). These bacteria were cultured at 20 °C, unless otherwise noted. *P. phosphoreum* was cultured at 20 °C.

2.3 Homology modeling

Homology modeling was conducted using the SWISS-MODEL software package (http://swissmodel.expasy.org/) (71). Visualization was then done using the VMD software package (http://www.ks.uiuc.edu/Research/vmd/) (72).

2.4 Localization of alginate lyases

Localization of enzymes was predicted using PSORTb version 3.0.2 (73) software package and CELLO version 2.5 (74) software package.

2.5 Plasmid construction – *V. splendidus* 12B01 alginate lyases

AlyA, AlyB, AlyD, and AlyE were expressed from a T7 promoter using the plasmid pET-28(a). The *V. splendidus* 12B01 genomic sequence (accession number: AAMR01000016.1) and alginate lyase sequences were accessed from the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). The sequences of the oligonucleotide primers are based on the DNA sequence of the alginate lyases AlyA, AlyB, AlyD, and AlyE from *V. splendidus* 12B01 (GenBank accession number EAP94921.1, EAP94922.1, EAP94925.1, and EAP94396.1, respectively). The lyases were first analyzed for the presence of signal peptides using the SignalP 4.1 server

(http://www.cbs.dtu.dk/services/SignalP/) (75). Signal peptides were identified for AlyB, AlyD, and AlyE. These peptides were removed prior to cloning. The over-expression vectors were constructed first by amplifying the alginate lyase genes *alyA* (region 4103 to 5845), *alyB* (region 6319 to 7836), and *alyD* (region 11675 to 12649) and then cloning the fragments into pET-28(a) using the restriction enzymes NdeI and XhoI. The alginate lyase gene *alyE* (region 99593 to 100570) was cloned into pET28A using the restriction enzymes NheI and XhoI.

The two domains of AlyA (domain 1, region 4259 to 4960; domain 2, region 4973 to 5845) were amplified to include (5'-CTT TCC AGC-3') upstream and (5'-ACC ACC ACA-3') downstream of domain 1 and (5'-TCA AAC GAT-3') upstream and (5'-AAC TGA-3') downstream of domain 2. The additional nucleotides upstream and downstream were included to add hydrophobic amino acid residues flanking each domain. AlyA domains were then cloned into pET-28(a) using restriction sites NdeI and XhoI. Two additional stop codons were added to all genes. **Table 2.3** lists the primers used in these constructions.

2.6 Plasmid construction – *V. splendidus* 13B01 alginate lyases

AlyA, AlyB, AlyD, AlyE, AlyF, and AlyG were expressed from a T7 promoter using the over-expression plasmid pET-28(a). Each lyase was analyzed for the presence of a signal peptide using the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/) (75). The lyases AlyB, AlyD, AlyE, AlyF, and AlyG contained signal peptides. These peptides were removed before cloning. The nucleotide sequences of each lyase can be found in **Table 2.4**. The over-expression vectors pAlyA, pAlyB, pAlyD, and pAlyG were constructed by amplifying the alginate lyases genes *alyA, alyB, alyD,* and *alyG*, respectively, and then cloning these fragments into pET-28(a) using the restriction enzymes NcoI and XhoI. pAlyF was constructed by amplifying *alyF* and cloning the fragment into pET-28(a) using the restriction enzymes NcoI and EagI. pAlyE was constructed in two steps. First, pET-28(a) was digested with restriction enzymes NcoI and EcoRI, the overhangs were filled in with Klenow fragment, and the blunt ends were ligated together to create a modified pET-28(a) lacking the N-terminal His-tag, thrombin

cleavage site, and T7-tag. *alyE* was then amplified and cloned into the modified pET-28(a) using the restriction sites EcoRI and XhoI. Each of the over-expression vectors contains the alginate lyase gene with a C-terminal 6xHis-tag driven by an inducible T7 promoter.

The alginate lyase domains of AlyA (domain 1 domain 2) were independently amplified to include (5'-CTT TCC AGC-3') upstream and (5'-TGT GGT CGT-3') downstream of domain 1 and (5'-TCA AAC GAT-3') upstream and (5'-GTT-3') downstream of domain 2 and cloned into pET-28(a) using the restriction enzymes NcoI and XhoI. The domain of AlyB was amplified to include (5'-AGC GAA AAC-3') upstream and (5'-GTA TTG ATC-3') downstream of the domain and cloned into pET-28(a) using the restriction enzymes NcoI and XhoI. The domain of AlyG was amplified to include (5'-TCA GGC AAC -3') upstream and (5'-GTT-3') downstream of the domain and cloned into pET-28(a) using the restriction enzymes NcoI and XhoI. The additional nucleotides upstream and downstream of each domain were included to add hydrophobic amino acid residues flanking each domain.

When necessary, (5'-TT-3') was included upstream a cloned fragment to ensure the fragment was in-frame with the C-terminal 6xHis-tag. **Table 2.3** contains the primers used in this study.

2.7 Plasmid construction – *V. breoganii* 1C10 laminarinases

LamA, LamB, LamC, and LamD from *V. breoganii* 1C10 were expressed from a T7 promoter using the over-expression plasmid pGEX-6P-2. Each laminarinase was analyzed for the presence of a signal peptide using the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/) (75). The hydrolases LamA, LamB, LamC, and LamD contained signal peptides. These peptides were removed before cloning. The over-expression vectors pLamA, pLamB, pLamC, and pLamD were constructed by amplifying the laminarinase genes *lamA, lamB, lamC, and lamD* and then cloning these fragments into pGEX-6P-2. The restriction enzymes BamHI and SalI were used for *lamA* and *lamB*, and the restriction sites BamHI and XhoI were used for *lamC* and *lamD*. Each over-expression vector contains the laminarinase gene with an N-terminal GST-tag

driven by an inducible T7 promoter. A Precission Protease cleavage site lies between the GST-tag and laminarinase gene.

The hydrolase domain of LamC was independently amplified to include (5'- TCT GAG CCA GAA-3') upstream the domain into pGEX-6P-2 using the restriction enzymes NcoI and XhoI. The additional nucleotides upstream of the domain were included to add hydrophobic amino acid residues upstream the domain.

Table 2.3 contains the primers used in these constructions. The nucleotide sequences of each laminarinase can be found in **Table 2.5**.

2.8 Plasmid construction – *V. breoganii* 1C10 alginate lyases

AlyA, AlyB, AlyD, and AlyE, AlyH, AlyI, AlyJ, AlyK, AlyL, AlyM, AlyN, and AlyO were expressed from a T7 promoter in the plasmid pET-28(a). Each of the lyases were analyzed for the presence of a signal peptide using the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/) (75). Signal peptides were identified for AlyB, AlyD, AlyH, AlyI, AlyJ, AlyL, and AlyN. These peptides were removed prior to cloning. The over-expression vectors were constructed first by amplifying each alginate lyase using the *V. breoganii* genome as template. Then, each fragment was cloned into pET-28(a) using the restriction enzymes listed in **Table 2.3**. The sequences of each alginate lyase are listed in **Table 2.6**.

2.9 Plasmid construction – LCR

The construction of vectors without restriction digestion was accomplished using a Ligase Cycling Reaction (LCR) (73) with modifications. LCR allows for the cloning of arbitrary DNA sequences into an arbitrary vector by utilizing bridging oligomers which will allow joining of specific DNA sequences. This bridging oligomers contain the 3' sequence of the upstream DNA fragment and the 5' sequence of the downstream DNA fragment. Each upstream and downstream sequence was designed to have an identical 60 °C melting temperature and these sequences were joined and synthesized in a single oligomer. The assignment of an upstream and downstream sequence is arbitrary since the

bridging oligomer is single-stranded and will bind to only one strand of the joined DNA fragments. Once all the DNA fragments for LCR are synthesized via PCR or other means (including the vector backbone), all the DNA fragments were combined at a concentration of 55 ng/kb for each fragment. The fragments were then phosphorylated in a T4 Polynucleotide Kinase (PNK) reaction. This was accomplished by the following reaction mixture: 5 µL 20 mM ATP, 2 µL 10X Ampligase buffer (Epicentre), 1 µL PNK (10 U/µL, New England Biolabs), DNA fragments, and H_2O to 20 µL. The PNK reaction was then incubated for 1 hour at 37 °C followed by a deactivation step at 65 °C for 20 minutes.

The PNK reaction step phosphorylated all the DNA fragments, which was necessary for the LCR reaction step. The LCR reaction step accomplished the ligation of the DNA fragments by first denaturing the fragments, next each bridging oligomer was bound to their corresponding DNA fragments which effectively brought each neighboring fragment together. Since each DNA fragment was phosphorylated, a ligase can then ligate the bound DNA fragments together. Since this process is repeated many times, a completed and circular plasmid was formed. The LCR step was accomplished by the following reaction mixture: 0.83 µL 10X Ampligase buffer, 1 µL Ampligase (Epicentre), 16.7 µL of the phosphorylated DNA fragment mixture, 2 µL 100% DMSO, 2.25 µL Betaine, 0.5 µL each bridging oligomer at 1.5 µM, H_2O to 25 µL. The LCR reaction was then incubated in the following cycle: 94 °C for 2 minutes followed by 50 cycles of 94 °C for 10 seconds, 55 °C for 30 seconds, and 66 °C for 60 seconds. Five µL of the completed LCR mixture was then transformed into 50 µL of chemical competent cells. No salt cleanup of the LCR mixture is needed. The presence of each DNA fragment in the LCR construct was confirmed by colony PCR and DNA sequencing.

2.10 Conjugation between *E. coli* and *V. splendidus* 13B01

In order the transfer constructed suicide vectors into *V. splendidus* 13B01, conjugation was performed between *E. coli* and *V. splendidus* 13B01. Two methods were employed to conjugate vectors. Both of these methods transfer a plasmid containing an origin of transfer (*oriT*) to the new host. When *E. coli* and *V. splendidus*

13B01 are mixed and incubated in medium that sufficient for both organisms, the plasmid will be transferred to the recipient strain. Since the *E. coli* host responsible for the transfer contains an auxotrophy, in this case a Δ*dapA* genotype, when the mixture of cells is grown on medium without diaminopimelate, the *E. coli* donor cells will not grow, thus allowing selection of *V. splendidus* cells with the desired marker. Thus, we can select for *V. splendidus* 13B01 cells that contain the transferred plasmid and marker. Verification of the transfer was done using oligomers specific to the transferred plasmid in a PCR reaction.

The first conjugation method was the method of Le Roux and coworkers (63). In this method, a plasmid containing *oriT* was transformed into *E. coli* β3914. This strain contains a Δ*dapA*::(*erm-pir*) genotype which allows for diaminopimelate counter-selection to select against growth following conjugation of plasmids into *V. splendidus*. β3914 also contains the *pir* gene allowing $ori V_{R6K\gamma}$ origin of replication for plasmid maintenance. To conjugate to 13B01 from β3914 with the desired plasmid, both strains were diluted 1:100 in fresh medium without antibiotic and grown to an OD_{600} of 0.3. Diaminopimelate was included in the *E. coli* overnight and subculture. The strains were mixed at 10^9 *V. splendidus* 13B01 to 10^8 *E. coli* β3914 with the desired plasmid. The mixture was deposited on a 0.45 µm filter placed cell side up on a LB 0.5 M NaCl agar plate supplemented with 0.3 mM diaminopimelate. The plate was incubated at 30 °C overnight. The cells were suspended in 1 mL of LB 0.5 M NaCl liquid medium and plated on LB 0.5 M NaCl with appropriate antibiotic. The presence of a plasmid was confirmed using oligomers specific to the transferred plasmid in a PCR reaction.

The second conjugation method employed was the method of Cordero and coworkers (70) with modification. In this method, a plasmid containing *oriT* was transformed into *E. coli* WM3064 (76). Like β3914, WM3064 contains a Δ*dapA* genotype which allows for counter-selection to select against growth following conjugation. To conjugate to 13B01 from WM3064 with the desired plasmid, 13B01 was grown overnight in TSB with 2% NaCl and WM3064 with the desired plasmid was grown overnight in LB with antibiotic and diaminopimelate. A 100 µL aliquot of each overnight were mixed and washed once with TSB with 2% NaCl to remove residue antibiotic. The cell mixture was re-suspended in 10 µL of TSB with 2% NaCl and

spotted onto a TSB with 2% NaCl agar plated supplemented with diaminopimelate. The plate was incubated overnight at 30 °C. The cell mixture was harvested by dissolving in TSB with 2% NaCl and washing once. The cell mixture was then re-suspended in 1 mL TSB with 2% NaCl and plated on TSB with 2% NaCl with the desired antibiotic. Colonies were streaked several times and the presence of the plasmid was confirmed with a PCR reaction.

2.11 Single-crossover recombination in *V. splendidus* 13B01

In order to edit the *V. splendidus* 13B01 genome, a suicide vector was constructed that will allow for the deletion of the gene *alyG*. The suicide vector was constructed in two steps. First, LCR was performed to construct a vector containing the 1kb regions immediately upstream and downstream *alyG* situated adjacent to one another. The suicide vector pSW4426T (63) was circularized in a "Round-the-World" PCR reaction with primers AB478F and AB478R. This reaction will result in circularized pSW4426T around its EcoRI site. The upstream 1kb region was generated using the primers AB476F and AB476R and the 13B01 genome. Similarly, the downstream 1kb region was generated using the primers AB477F and AB477R and the 13B01 genome. These three pieces were then ligated together in a LCR reaction with bridging oligomers AB487F, AB490F, and AB510F to result in plasmid pCon6.2.

The *alyG* 1kb upstream-downstream region construct was then amplified from pCon6.2 using primers AB513F and AB513R. This fragment contains SpeI sites flanking the upstream-downstream construct. This fragment was then cloned into the suicide vector pJC4 (70) using the restriction enzyme SpeI. The resulting vector, pAlyG KO, was used to knockout *alyG*, as described below.

pJC4 is a derivative of plasmid pWM91 (76) and contains a chloramphenicol resistance replacing the original ampicillin resistance. Both plasmids contain an *oriT* transfer region, an $oriV_{R6K\gamma}$ origin of replication, and the *sacB* gene for sucrose counter-selection. As previously described, the *oriT* allows the transfer of the suicide vector from *E. coli* into *V. splendidus*. The $oriV_{R6K\gamma}$ origin of replication is maintained in hosts containing the *pir* gene, such as β3914 and WM3064. When found in hosts without the

pir gene, this origin will not replicate, so the plasmid will be lost following cell division. The *sacB* gene is toxic to gram-negative bacterium when grown in the presence of sucrose (77). Thus, we can screen for the presence of *sacB* in a cell by growing these cells in the presence of sucrose.

The *alyG* knockout was constructed using the plasmid pAlyG KO by conjugating the plasmid from WM3064 into *V. splendidus* 13B01. This method was adapted from Cordero and coworkers (70). Since the knockout plasmid will not replicate in 13B01 due to the $ori V_{R6K\gamma}$ origin of replication, we can plate the cell mixture on TSB with 2% NaCl supplemented with chloramphenicol. Since pAlyG KO contains 1kb homology upstream and downstream of *alyG*, 13B01 colonies that were chloramphenicol resistant were colonies in which the suicide vector was inserted into the genome. Following three serial streaks on TSB with 2% NaCl supplemented with chloramphenicol, the successful integrants were verified by PCR reaction with primers on the genome outside the integration site and within the integrated plasmid. At this point, positive colonies contained the knockout plasmid within the genome recombined at either the upstream or downstream region.

In order to excise the integrated plasmid from the genome, a sucrose counter-selection was employed to select for colonies that naturally lost the integrated vector through recombination of either of the duplicate upstream or downstream regions within the genome. This second round of recombination was conducted by growing the integrated strain on TSB with 2% NaCl to allow for the excising of the integrated plasmid. Then, the cells were plated on TSB with 1% NaCl and 5% sucrose. The lower amount of salt was used to allow for more effective sucrose counter-selection. Colonies that appeared were then screened for loss of chloramphenicol resistance and then verified for loss of *alyG* by PCR reaction. The loss to *alyG* resulted in a smaller PCR product in the PCR reaction.

2.12 Protein purification – 12B01 and 1C10 alginate lyases

Cells were grown overnight in LB medium supplemented with 1% glucose. They were then subcultured 1:33.3 into fresh medium and gown to an OD_{600} of 0.6. Protein

expression was then induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Cells were then grown an additional four hours and harvested by centrifugation at 5,000 x *g* for 15 minutes. The pellets were lysed by resuspending in Buffer B (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-Cl, pH 8.0) and inverting the tube for 1 hour at 20 °C. Supernatants were clarified by two serial centrifugations (7,000 x *g*, 10 minutes; 40,000 x *g*, 60 minutes) and then passed through a 0.45 µm filter. The lysates for each protein were individually loaded onto three 5 mL HiTrap Chelating HP columns (charged with 0.1 M $NiSO_4$) installed on AKTA prime or AKTA 25L FPLC systems (GE Healthcare). Lysates were loading onto the column at 1 mL/min.

The columns were then washed with 5 column volumes Buffer B. This was accomplished by a 1 mL/min flow rate for 1 column volume, then followed by a 5 mL/min flow rate for 4 column volumes. Next, 5 column volumes Buffer C (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-Cl, pH 6.3) were applied at 5 mL/min to further remove non-tagged proteins. Elution was then preformed with 5 column volumes Buffer E (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-Cl, pH 4.5) at a 5 mL/min flow rate. Fractions containing the purified proteins were identified with SDS-PAGE.

The fractions containing the purified protein were pooled and refolded by dialyzing three times against TKMD-G (50 mM Tris-Cl pH 8.0, 5 mM $MgCl_2$, 50 mM KCl, 0.1 mM dithiothreitol, 10 % glycerol, pH 8.0) at 4 °C for 2 hours followed by an overnight charge at 4 °C (78). Gel filtration was employed to further purify the refolded lyases using a 16 mm diameter HiPrep Sephacryl S-200 HR (GE Healthcare) gel filtration column charged with TKMD-G. Each sample was loaded at 0.5 mL/min and then fractions were collected using TKMD-G at 0.5 mL/min to elute the purified protein.

The size of purified proteins was determined by size exclusion high-performance liquid chromatography using a Shimadzu high-performance liquid chromatography system containing an SPD-10A UV-VIS detector set at 280 nm (Shimadzu, Kyoto, Japan). A Bio-Rad Bio-Sil SEC-250 column (300 by 7.8 mm) was used to determine protein size using a mobile phase of 0.05 M Na_2HPO_4, 0.05 M NaH_2PO4, and 0.15 M NaCl, pH 6.8. The molecular weight was calculated by comparing the retention times to those of protein molecular weight standards. The quaternary structures were determined

based on the molecular weight observed by HPLC, and the molecular weights of monomeric subunits were determined by SDS-PAGE analysis.

2.13 Protein purification – 13B01 alginate lyases

Cells were grown overnight in LB medium and were supplemented with 1% glucose. Subcultures were then started in fresh medium and grown to an OD_{600} of 0.5. Cells were cooled to 25 °C and protein over-expression was then induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a concentration of 1 mM. AlyD and AlyE were grown for an additional 21 and 19 hours, respectively, at 25 °C. AlyA, AlyB, AlyF, and AlyG were grown for an additional 24 hours at 16 °C. AlyF containing cells were lysed by suspending the cell pellet in native binding buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0), and then lysozyme was added at 1 mg/mL and followed by incubation on ice for 30 min. The lysate was then sonicated six times for 10 seconds on ice. The AlyF lysate was clarified by centrifugation at 10,000 x *g* for 20 minutes and then passed through a 0.45 µm filter. AlyF was purified by loading the lysate on two 5 mL HiTrap Chelating HP columns which were charged with 100 mM $NiSO_4$ installed on an AKTA prime FPLC system (GE Healthcare). The columns were washed with 5 column volumes wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0), and then AlyF was eluted from the columns with 5 column volumes elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0). AlyA, AlyB, AlyD, AlyE, and AlyG containing cells were lysed by suspension in Buffer B (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-Cl, pH 8.0) and inverting the tubes at 25 °C for 1 hour. The lysates were clarified by centrifugation at 9,000 x *g* for 30 minutes and then passage through a 0.45 µm filter. The lyases were then loaded on two 5 mL HiTrap Chelating HP columns charged with 100 mM $NiSO_4$ installed on an AKTA prime FPLC system. The columns were then washed with 5 column volumes Buffer C (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-Cl, pH 6.3). Each protein was then eluted with 5 column volumes Buffer D (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-Cl, pH 5.3). AlyF was dialyzed three times against TKMD-G (78) (50 mM Tris-Cl pH 8.0, 5 mM $MgCl_2$, 50 mM KCl, 0.1 mM dithiothreitol, 10 % glycerol, pH 8.0) at 4 °C for 2 hours followed by

an overnight charge at 4 °C. AlyA, AlyB, AlyD, AlyE, and AlyG were refolded via dialysis against TKMD-G three times at 4 °C for 2 hours followed by an overnight charge at 4 °C.

2.14 Protein purification – laminarinases

Cells were grown overnight in LB medium and were supplemented with 1% glucose. Subcultures were then started in fresh medium and grown to an OD_{600} of 0.5. Cells were cooled to 25 °C and protein over-expression was then induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a concentration of 1 mM. LamA, LamB, LamC, and LamD were grown for an additional 16 hours at 20 °C. LamA, LamB, LamC, and LamD containing cells were lysed by resuspending in 3 ml/g pellet of TBS (6.1 g/L Tris, 8.8 g/L NaCl, pH 7.4), and then adding 1 M dithiothreitol at 1 µL/g pellet and Triton X-100 at 30 µL/g pellet. The resuspension was sonicated six times for 10 secs on ice. The lysate was clarified by centrifugation at 9,000 x *g* for 10 minutes followed by 40,000 x *g* for 60 minutes and then passed through a 0.45 µm filter.

The lysates were then passed through three 5 mL GSTrap HP (GE Healthcare) columns installed on an AKTA Pure 25 L FPLC system (GE Healthcare) at 1 mL/min. The columns were washed with 1 column volume TBS at 1 mL/min and then 4 column volumes of TBS at 5 mL/min. Each laminarinase was then eluted from the columns with 5 column volumes GEB (3g/L glutathione, 6.1g/L Tris, pH 8.0) at 5 mL/min and concentrated in an Amicon Ultra-15 centrifugal filter (EMD Millipore).

To cleave the GST-tag from the purified laminarinase, Precission Protease was added to each laminarinase and the mixture was dialyzed three times against PPB (6.1 g/L Tris, 5.84 g/L NaCl, 0.37 g/L disodium ethylenediaminetetraacetic acid, 154 mg/L dithiothreitol, pH 8.0) at 4 °C for 2 hours followed by an overnight charge at 4 °C. The laminarinase and cleaved GST-tag mixture was then run over the GSTrap columns at 1 mL/min and the flow through was collected. The cleaved laminarinases were then dialyzed three times against TKMD-G (78) (50 mM Tris-Cl pH 8.0, 5 mM MgCl, 50 mM KCl, 0.1 mM dithiothreitol, 10 % glycerol, pH 8.0) at 4 °C for 2 hours followed by an overnight charge at 4 °C.

The native sizes of each protein were determined by size exclusion high-performance liquid chromatography using a Shimadzu high-performance liquid chromatography system that utilized an SPD-10A UV-VIS detector set at 280 nm. A Bio-Sil SEC-250 column (300 by 7.8 mm) was then used to determine the native protein size using a mobile phase (0.05 M Na_2HPO_4, 0.05 M NaH_2PO4, and 0.15 M NaCl, pH 6.8).

2.15 Alginate lyase activity assay

Enzymatic activity was determined using the thiobarbituric assay (79-81). Enzymatic reactions were performed in 100 µL of APT buffer (20 mM sodium acetate, 20 mM monosodium phosphate, and 20 mM Tris base) containing 0.33 µg of protein and 0.05% sodium alginate, unless noted otherwise. The reactions were incubated for 20 minutes at the designated temperature, pH, and NaCl concentrations. Reactions were placed on ice following incubation if activity was not immediately determined. Activity was determined by adding 0.125 mL of 0.025 N H_2IO_6 in 0.125 N H_2SO_4 to the reaction mixture and then incubating for 20 minutes at 20 °C. Next, 0.25 mL of 2% sodium arsenite in 0.5 N HCl was added and the mixture was incubated for two minutes. Last, 1 mL thiobarbituric acid (0.3%, pH 2) was added and the mixture was heated at 100 °C for 10 minutes. The increase in absorbance at 548 nm was measured using a Shimadzu BioSpec-1601 spectrophotometer. 2-Deoxy-D-glucose was used as a standard because it reacts with thiobarbituric acid (82). Activities were reported as 2-deoxy-D-glucose equivalent concentrations. Kinetic parameters were determined by fitting the alginate dose response data to the Michaelis-Menten kinetic model. In order to determine the K_m of alginate, an average chain length of 70, as determined by ^{1}H-NMR analysis, was used to evaluate the concentration in solution.

2.16 Laminarinase activity assay

Laminarinase reaction conditions for were as follows: 1.65 µg of enzyme was added to 100 µL AMT buffer (20 mM sodium acetate, 20 mM 3-(N-

morpholino)propanesulfonic acid (MOPS), and 20 mM Tris base) containing 0.05% laminarin or 0.05% β-D-glucan from barley (Sigma-Aldrich), unless noted otherwise. Laminarin was reduced prior to use in enzymatic assays according to Labourel and coworkers (56). Each reaction was incubated for 2 hours at the reported pH, temperature, and NaCl concentrations.

For pH, temperature, NaCl experiments, and substrate comparison experiments, the 3-Methyl-2-benzothiazolinonehydrazone (MBTH) assay (83) was used to determine enzymatic activity. To the laminarin or β-D-glucan reactions, 100 µL of 0.5 N NaOH was added followed by addition of 100 µL of freshly mixed MBTH reagent (equal volumes of 3 mg/mL MBTH and 1 mg/mL dithiothreitol). Samples were then heated for 15 minutes at 80 °C. Upon removal from heat, 200 µL of developing solution (0.5% $(FeNH_4(SO_4)_2)\cdot 12\ H_2O$, 0.5% sulfamic acid, and 0.25 N HCl) was added to each sample. Activity was determined by measuring the increase in absorbance at 620 nm and is reported as equivalent concentrations of D-glucose.

For divalent cation and dose response experiments, the p-Hydroxy benzoic acid hydrazide (PAHBAH) assay (84) with modification was used to determine enzymatic activity. To the laminarin reaction, 1 mL of freshly mixed PAHBAH working reagent was added. The PAHBAH working reagent was prepared by mixing 1 part of reagent A (5% PAHBAH in 0.6 N HCl) with 9 parts of reagent B (48.2 mM trisodium citrate, 9.9 mM $CaCl_2$, 0.5 N NaOH). Samples were heated at 100 °C for 6 minutes. Activity was determined by measuring the increase in absorbance at 410 nm and is reported as equivalent concentrations of D-glucose. Kinetic parameters were found by fitting the dose response activities to a Michaelis-Menten model. The K_m toward laminarin was determined by using a laminarin average chain length of 30 (46).

2.17 Stripping of cations from alginate lyases and laminarinase samples

Divalent cations were removed from enzyme stored in TKMD-G by adding 5 mg of Chelex 100 resin (Bio-Rad) to 100 µL of the solution. The resin/enzyme mixture was inverted for 1 hour at 20 °C and then spun for 5 minutes at 18,000 *x* g to remove the stripped enzyme solution from the resin.

2.18 Preparation of enriched polyG and polyM fractions

Enriched polyG and polyM were produced using the method of Chhatbar and coworkers (85). Briefly, 4 g of sodium alginate dissolved in 0.25 M H_2SO_4 were microwaved for 5 minutes. The polyG and polyM fractions were then separated based on their pH solubility, lyophilized, and then dissolved in APT buffer.

2.19 Gene expression of alginate lyases and laminarinases

V. splendidus 12B01 and 13B01 were grown overnight in M9S medium and then subcultured 1:50 in fresh medium. *V. breoganii* 1C10 was grown overnight in NDG medium and subcultured 1:50 in fresh medium. Samples were harvested at an OD_{600} of 1.0. Total RNA was isolated with the RNeasy Mini Kit (Qiagen) using the manufacturer's suggested protocol. cDNA was generated using the QuantiTect Reverse Transcription Kit (Qiagen) following the manufacturer's protocol. Quantitative PCR (qPCR) was performed in triplicate using the primers listed in **Table 2.3**, which were designed using Primer3Plus software *(*www.bioinformatics.nl/primer3plus*)* (86). Reactions were carried out using HotStart-IT SYBR Green qPCR Master Mix with UDG (Affymetrix) and a Bio-Rad MiniOpticon Real-Time PCR System**.** To quantify the amount of mRNA, standards were constructed using serial diluted *V. splendidus* 12B01, 13B01, or 1C10 genomic DNA. The gene *rpoA* was used in *V. splendidus* 12B01 and 13B01 was used to control for differences in total mRNA quantities (87). The gene *rpoS* was used in *V. breoganii* 1C10 to control for differences in total mRNA (88).

2.20 Phylogenetic analysis

For the phylogenetic analysis, a structure based alignment approach was used with the pdb structures 2CWS (89) and 1J1T guiding the structural alignment performed with ClustalW/T-coffee (90) as part of Strap (91). After the alignment, we removed long inserts, N-terminal extensions such as the CBM32 domains present in sequences of AlyB

and AlyG, and also the part containing the N-terminal signal peptide and the C-terminal His-tag of the sequences 2CWS and 1J1T. This alignment without the sequence 2CWS was used for the phylogenetic tree construction and the sequence of 1J1T, a PL7 alginate lyase from *Alteromonas* sp. 272, was used to root the tree. The phylogenetic tree was calculated in Mega6 using the Maximum Likelihood method based on the JTT matrix-based model. The structural alignment, used for the phylogenetic tree construction, was plotted with Endscript to show the secondary structural elements of the associated PDBs (92).

2.21 Enzyme secretion screening

To compare the amount of alginate lyase secretion in 12B01 and 13B01, the cells precultured for 24 hours in 2216 marine medium (Difco) were plated in triplicate onto 2216 marine broth agarose plates containing 0.25% (wt/wt) low viscosity alginate (Sigma). The colonies were grown for 36 hours and imaged for colony size measurements. The colonies were removed by scraping the plate surface and washing with MilliQ water. The secreted alginate lyase activity was revealed by overlaying the agarose plate with 10% (wt/wt) cetylpyridinium chloride (Sigma) in water for 20 min. The cetylpyridinium chloride solution was decanted and the plate was washed twice with MilliQ water for a total of 20 min at 20 °C. The water was decanted and alginate lyase activity was visible as a cleared halos on an opaque background.

Chapter 2 Figures and Tables

Table 2.1. 1,000X Vitamin Mix for NDG minimal medium. Solution was prepared and then 0.22 µm filter sterilized and store in a dark container at 4 °C.

	Amount (mg/L)
Aminobenzoate	40
Biotin	10
Calcium pantothenate	50
Folate	30
Lipote	10
Niacin	100
Pyridoxamine dihydrochloride	100
Thiamine hydrochloride	100
Vitamin B_{12}	50

Table 2.2. Preparation of 1,000X Trace Element solution for NDG minimal medium. Solution was prepared and then 0.22 µm filter sterilized.

	Amount (g/L)
$Co(NO_3)_2 \cdot 6H_2O$	0.49
$CuSO_4 \cdot 5H_2O$	0.79
H_3BO_3	2.86
$MnCl_2 \cdot 4H_2O$	1.81
$NiCl_2 \cdot 6H_2O$	0.005
$ZnSO_4 \cdot 7H_2O$	0.24

Table 2.3. Oligonucleotides used in the preparation of over-expression vectors and qPCR experiments. Underlined portions indicate restriction sites used in cloning.

Primer	Sequence	Characteristic
AB241F	5'-ACG TCA TAT GAA TAA GCC AAT CTT TGT CGT CGT ACT CG-3'	AlyA (12B01), NdeI
AB241R	5'-ACG TCT CGA GTT ATT ATC AGT TAT GCT CTG CTC TTA GAG AAC TAA AG-3'	AlyA (12B01), XhoI
AB305F	5'-ACG TCA TAT GCT TTC CAG CTC AGA TCT TCA GGT G-3'	AlyA (12B01) domain 1, NdeI
AB305R	5'-ACG TCT CGA GTT ATT ATG TGG TGG TGT ATC GCA GTG C-3'	AlyA (12B01) domain 1, XhoI
AB306F	5'-ACG TCA TAT GTC AAA CGA TTG GGA TAT TAA TGA TTG GAA GTT G-3'	AlyA (12B01) domain 2, NdeI
AB306R	5'-ACG TCT CGA GTT ATT ATC AGT TAT GCT CTG CTC TTA GAG AAC-3'	AlyA (12B01) domain 2, XhoI
AB272F	5'-ACG TCA TAT GGT TGG TTG TGC GAG CAC GAG-3'	AlyB (12B01), NdeI
AB238R	5'-ACG TCT CGA GTT ATT ATT ACT TTT TGT ATT GAT CGT GCG ATA CAT CTA GC-3'	AlyB (12B01), XhoI
AB273F	5'-ACG TCA TAT GAA TAA CGG TGT TTC TTA CCC CGT ACC-3'	AlyD (12B01), NdeI
AB239R	5'-ACG TCT CGA GTT ATT ACT ATT TAC CGT TTA ACT TAA GCG CAG AGA AAG TC-3'	AlyD (12B01), XhoI
AB274F	5'-ACG TGC TAG CTC TAA TCA CGA TAT TGG TCA ACA GTT CAA TC-3'	AlyE (12B01), NheI
AB240R	5'-ACG TCT CGA GTT ATT ATT ATT GAT TAA GAA CTA ACT GGT AGA AGC TTG CTT G-3'	AlyE (12B01), XhoI
AB324F	5'-CGA ATC GAG CAA AGA CCT TC-3'	AlyA (12B01), RT-PCR
AB324R	5'-CTT CAT CGC TGG TGC TAC AA-3'	AlyA (12B01), RT-PCR
AB321F	5'-TAA CTC GCC AAG CGA AAA CT-3'	AlyB (12B01), RT-PCR
AB321R	5'-GCC GTC ATC AGC TGT GTA GA-3'	AlyB (12B01), RT-PCR
AB322F	5'-TGG CGT TAT TAG CGA CTG TG-3'	AlyD (12B01), RT-PCR
AB322R	5'-AAT CGC TTG GTT CTG CAC TT-3'	AlyD (12B01), RT-PCR
AB323F	5'-CCA TGG GTC GGA TAA TGA AC-3'	AlyE (12B01), RT-PCR
AB323R	5'-GGC GAA TGT CTT TAC GAA GC-3'	AlyE (12B01), RT-PCR
AB326F	5'-CCG GTT GAT AAA ATC GCC TA-3'	RpoA (12B01), RT-PCR
AB326R	5'-CGC ATC CAG TTG TTC AGC TA-3'	RpoA (12B01), RT-PCR

Table 2.3 (cont.)

MP007F	5’-GAC GCC ATG GTT AAT AAG CCA ATC TTT GTC GTC-3’	AlyA, NcoI
MP008R	5’-CTG ACT CGA GGT TAT GCT CTG CTC TTA GAG AAC-3’	AlyA, XhoI
AB446F	5’-ACG TCC ATG GTT CTT TCC AGC TCA GAT CTT CAG GTA TC-3’	AlyA domain 1, NcoI
AB446R	5’-ACG TCT CGA GTG TGG TCG TGT ATC GCA GTG C-3’	AlyA domain 1, XhoI
AB447F	5’-ACG TCC ATG GTT TCA AAC GAT TGG GAT ATT AAT GAT TGG AAG TTG-3’	AlyA domain 2, NcoI
AB447R	5’-ACG TCT CGA GGT TAT GCT CTG CTC TTA GAG AAC TAA AGC-3’	AlyA domain 2, XhoI
MP006F	5’-TAT ACC ATG GTT AGC ACG CCT ACT GCT GAT TTT C-3’	AlyB, NcoI
MP005R	5’-GTC ACT CGA GCT TTT GGT ATT GAT CGT GCG ATA CGT CTA GC-3’	AlyB, XhoI
MP003F	5’-CAG TCC ATG GCG AAT AAC GGT GTT TCT TAC-3’	AlyD, NcoI
MP002R	5’-TCA GCT CGA GTT TAC CGT TCA ACT TAA GCG CAG AGA-3’	AlyD, XhoI
MP015F2	5’-GCG GAA TTC TAA TCA CGA TAT TGG TCA AC-3’	AlyE, EcoRI
MP015R	5’-TCA GCT CGA GTT GAT TAA GAA CTA ACT GG-3’	AlyE, XhoI
MP014F	5’-TAA TCC ATG GTG ACA GGC TGT ACG ACA C-3’	AlyF, NcoI
MP013R	5’-CTA GCG GCC GCC TTC TTG GTA TTT TCA AGC AC-3’	AlyF, EagI
MP011F	5’-GCC GCC ATG GTT GAA ACA CTG AAC ATT CAA TC-3’	AlyG, NcoI
MP010R	5’-GCC GCT CGA GGT TGT GAT TGT TGT TTA ACT GAT AG-3’	AlyG, XhoI
AB354R	5’-ATT CAC AGC GCT CAG GTT CT-3’	AlyA (13B01), RT-PCR
AB355F	5’-TAA CTC GCC AAG CGA AAA CT-3’	AlyB (13B01), RT-PCR
AB355R	5’-GCC GTC ATC AGC TGT GTA GA-3’	AlyB (13B01), RT-PCR
AB356F	5’-TGG CGT TAT TAG CGA CTG TG-3’	AlyD (13B01), RT-PCR
AB356R	5’-AAT CGC TTG GTT CTG CAC TT-3’	AlyD (13B01), RT-PCR
AB357F	5’-CCA TGG GTC GGA TAA TGA AC-3’	AlyE (13B01), RT-PCR
AB357R	5’-GGC GAA TGT CTT TAC GAA GC-3’	AlyE (13B01), RT-PCR
AB358F	5’-AAG GGC AAA GAG CTG AAC AA-3’	AlyF (13B01), RT-PCR
AB358R	5’-AAT GGG TAA TCT TGC GTT GC-3’	AlyF (13B01), RT-PCR
AB359F	5’-TGT TCA AGA CGT CGC GAT AG-3’	AlyG (13B01), RT-PCR
AB359R	5’-TTG AAC ATC CGT CAC ATC GT-3’	AlyG (13B01), RT-PCR
AB360F	5’-CCG GTT GAT AAA ATC GCC TA-3’	RpoA (13B01), RT-PCR
AB360R	5’-CGC ATC CAG TTG TTC AGC TA-3’	RpoA (13B01), RT-PCR

Table 2.3 (cont.)

AB478F	5’-CTG CAG CCC GGG GGA TC-3’	pSW4426T, RTW
AB478R	5’-GAT ATC AAG CTT ATC GAT ACC GTC GAC G-3’	pSW4426T, RTW
AB476F	5’- TGT CTA AAT CTG TCG TCA GTC AGC AC-3’	1kb *alyG* upstream
AB476F	5’- ACA AGT CTC CAT GAT ATG TTT CAC GTA TAT TAT TTA TTC C-3’	1kb *alyG* upstream
AB477F	5’-TCA CGC TGT AAA TAG TGC GTG AC-3’	1kb *alyG* downstream
AB477R	5’- TTT TGG TTG GAT TAA ATC GGT GTA GGT G-3’	1kb *alyG* downstream
AB487F	5’-CGT CGA CGG TAT CGA TAA GCT TGA TAT CTG TCT AAA TCT GTC GTC AGT CAG CAC-3’	pCon6.2 LCR, Bridge 1
AB490F	5’- CAC CTA CAC CGA TTT AAT CCA ACC AAA ACT GCA GCC CGG GGG ATC-3’	pCon6.2 LCR, Bridge 3
AB510R	5’-ATA AAT AAT ATA CGT GAA ACA TAT CAT GGA GAC TTG TTC ACG CTG TAA ATA GTG CGT GAC-3’	pCon6.2 LCR, Bridge 2
AB513F	5’-ACG TAC TAG TTG TCT AAA TCT GTC GTC AGT CAG CAC-3’	pAlyG KO, SpeI
AB513R	5’-ACG TAC TAG TTT TTG GTT GGA TTA AAT CGG TGT AGG TG-3’	pAlyG KO, SpeI
AB329F	5’- ACG TGG ATC CCC AGT GCA GAT TAC TCC AGA CC-3’	LamA, BamHI
AB329R	5’-ACG TGT CGA CTT ATT ATT AGC GTT CAA AAC GCA CGT TAT CC-3’	LamA, SalI
AB330F	5’-ACG TGG ATC CGG ATG GGA AGT CCA GTG GAT AGA TTC-3’	LamB, BamHI
AB330R	5’-ACG TGT CGA CTT ATT ACT AAT TCA AGA TTT TGC AGA AAC TAT GAC GTG-3’	LamB, SalI
AB331F	5’-ACG TGG ATC CAT GAT GGG ATG TGG CGA TGA ATC C-3’	LamC, BamHI
AB331R	5’-ACG TCT CGA GTT ATT ATT ATT TAA TCT CTA ATC GAA CAT TAT CAA ACG ACA CTT G-3’	LamC, XhoI
AB332F	5’-ACG TGG ATC CGA TGA GCA GCT GAG TGT TGG TG-3’	LamD, BamHI

Table 2.3 (cont.)

AB332R	5’-ACG TCT CGA GTT ATT ACT ACT CAT ACC AAG AGA TGT TTC TAA CCT C-3’	LamD, XhoI
AB445F	5’-ACG TGG ATC CTC TGA GCC AGA AAA CCC TTC CGA C-3’	LamC GH16 Domain, BamHI
AB445R	5’-ACG TCT CGA GTT ATT AGG TAA TGA CGG CTG GGG CAG-3’	LamC, GH16 Domain, XhoI
AB341F	5’-TCT GCA CGC CTT AGA ACC TT-3’	LamA, RT-PCR
AB341R	5’-GGT TTT CAA CCT CAC CCT CA-3’	LamA, RT-PCR
AB342F	5’-AAC AAG CCA CGA TGG AAA TC-3’	LamB, RT-PCR
AB342R	5’-GCC TTC TGC TCG TCC AAT AG-3’	LamB, RT-PCR
AB343F	5’-AGA ACC ACC GGT AGA CGA TG-3’	LamC, RT-PCR
AB343R	5’-CTT GCC AAG CTG AAA CAT CA-3’	LamC, RT-PCR
AB344F	5’-GCA GAG GTT CCT TCT GTT GC-3’	LamD, RT-PCR
AB344R	5’-CCA AGA GTT CAG TGC GTC AA-3’	LamD, RT-PCR
AB346F	5’-ATC GGC TTC TCC CCA CTA CT-3’	rpoS, RT-PCR
AB346R	5’-AGC TAA GCC ACG ATT GCT GT-3’	rpoS, RT-PCR
AB314F1	5’-ACG TCA TAT GAT GGA AAT GAA TAA ATC AAT TTT GGC ATT GAT GC	AlyA (1C10), NdeI
AB314R2	5’-ACG TCT CGA GTT ATT ATT AAT AGT GAT CTA TGT TCA AAG AAC TGA AGC TAA CG	AlyA (1C10), XhoI
AB313F1	5’-ACG TCA TAT GAC TTC TGT ATT ACT AGC GGT TGG CTG	AlyB (1C10), NdeI
AB313R	5’-ACG TCT CGA GTT ATT ATT ACT TAG CTG TGT AAG TAT CGT GAG ATA CGT C	AlyB (1C10), XhoI
AB309F	5’-ACG TCA TAT GGA TGT AGC CAA CAA CGG AGT TTC G	AlyD (1C10), NdeI
AB309R	5’-ACG TCT CGA GTT ATT ATT ATT TAC CGT TCA ATT TTA GTG CTG AGA ATG TC	AlyD (1C10), XhoI
AB308F1	5’-ACG TCA TAT GAT GGG TAT TGT TCC CGT TTC TCT TG	AlyE (1C10), NdeI

Table 2.3 (cont.)

AB308R	5’-ACG TCT CGA GTT ATT ATT ATT GAT CTA GGC TGA ACT TGA AGA AGC	AlyE (1C10), XhoI
AB312F	5’-ACG TCA TAT GGC GGC ACC TAC GCC AAA C	AlyH (1C10), NdeI
AB312R	5’-ACG TCT CGA GTT ATT ATT AGT CGT GAG TCA CTT TAA GAT CAT AGA TGG	AlyH (1C10), XhoI
AB315F	5’-ACG TCA TAT GGA GAC AAA AGC ACC TTC TGA CAA ATT TG	AlyI (1C10), NdeI
AB315R	5’-ACG TCT CGA GTT ATT ATT AGT GAG TAA CTT TCA AGT CGT AGA AAG TCG	AlyI (1C10), XhoI
AB317F1	5’-ACG TCA TAT GAA CGA GCT CGA GCT AAC CAA TCC	AlyJ (1C10), NdeI
AB317R	5’-ACG TGA ATT CTT ATT ATT AGT GAG CAT ACC CTG TAT GGC TG	AlyJ (1C10), EcoRI
AB318F	5’-ACG TCA TAT GAT GGA GGT GTC TGC CGC TG	AlyK (1C10), NdeI
AB318R	5’-ACG TGA ATT CTT ATT ATT ATT TAT GGT CGA GTT CCA GGT GG	AlyK (1C10), EcoRI
AB316F	5’-ACG TCA TAT GAT CAC CTT TGA TGA CGC GGG C	AlyL (1C10), NdeI
AB316R	5’-ACG TCT CGA GTT ATT ATT ACT TAT GCT CTA CTT GAT ACT CAA GCT GG	AlyL (1C10), XhoI
AB310F	5’-ACG TCA TAT GAT GAT GAA AAT AAA CGC TAT TAC CAC AAC AGT TTT G	AlyM (1C10), NdeI
AB310R	5’-ACG TCT CGA GTT ATT ATT ATT GGT GGG TGA TTT TTA TTT CAC TGA AGC	AlyM (1C10), XhoI
AB311F	5’-ACG TGC TAG CCA ACC AGA AGA GCC TAT GAC CG	AlyN (1C10), NheI
AB311R	5’-ACG TCT CGA GTT ATT ATT ATT GGA TTA CGT AGT CTG GTC CTG C	AlyN (1C10), XhoI
AB320F1	5’-ACG TCA TAT GAT GAA TAA ACA TAT ACT AGC AGT CCT AAT TGG ATT AAC	AlyO (1C10), NdeI
AB320R	5’-ACG TGG ATC CTT ATT ATT AGT TTT GGT CAT TTC GAG TTG CCA AAC	AlyO (1C10), BamHI

Table 2.4. Nucleotide sequences of 13B01 alginate lyases. Signal peptides are underlined.

Alginate Lyase	Sequence (5'-3')
AlyA	GTGAATAAGCCAATCTTTGTCGTCGTACTCGCTTCGTTTACGTATGGCTGCGGT GGAAGCAGCTCCAGTGACTCTAGTGACCCTTCTGATACCAATAACCCAGGAGC ATCTTATGGTGTTGTTGCTCCCTATGATATTGCCAAGTATCAAAACATCCTTTCC AGCTCAGATCTTCAGGTATCTGACCCTAATGGGGAGGAGGGCAATAAAACCTC TGAAGTCAAAGATGGTAACTTCGATGGTTATGTCAGTGATTATTTTTATGCTGA CGAAGAGACGGAAAATCTTATCTTCAAAATGGCGAACTACAAGATGCGCTCTG AAGTTCGTGAAGGAGAAAACTTCGATATCAATGAAGCAGGCGTAAGACGCAG TCTACATGCGGAAATAAGTCTGCCTGATATTGAGCATGTAATGGCGAGTTCTCC CGCAGATCACGATGAAGTGACCGTGCTACAGATCCACAATAAAGGTACAGACG AGAGTGGTACGGGTTATATCCCTCATCCGCTATTGCGTGTGGTTTGGGAGCAAG AACGAGATGGCCTCACAGGTCACTACTGGGCAGTCATGAAAAACAATGCCATT GACTGTAGCAGCGCTGCTGACTCTTCGGATTGTTACGCCACTTCATATAATCGT TATGATTTAGGAGAGGCGGATCTTGATAGCTTCACTAAGTTTGATCTTTCTGTT TATGAAAACACCCTTTCGATCAAAGTGAACGATGAAGTTAAAGTCGACGAAGA CATCACCTACTGGCAGCACCTACTGAGTTACTTTAAAGCGGGTATCTATAATCA ATTTGAAAATGGTGAAGCCACGGCTCACTTTCAGGCACTGCGATACACGACCA CACAGGTCAACGGCTCAAACGATTGGGATATTAATGATTGGAAGTTGACGATT CCTGCGAGTAAAGACACTTGGTATGGAAGTGGGGGTGACAGTGCGGCTGAACT AGAACCTGAGCGCTGTGAATCGAGTAAAGACCTTCTCGCTAATGATAGCGATG TCTATCATAGCGATATTGACCTTTCTTATTTCAATACCGATGAAGGGAGAGTGC ACTTTAGAGCGGATATGGGATATGGCACCTCTACCGAAAATTCTAGCTATATTC GCTCTGAGCTCAGGGAGCTGTATCAAAGCAGTGTTCAACCGGATTGTAGCACC AGCGATGAAGATACTAGTTGGTATTTGGACGACACTAGAACGAACGCTACCAG TCACGAGTTAACCGCCAGCTTACGAATTGAAGATTACCCGAACATCAATAATC AAGATCCAAAAGTGGTAGTTGGGCAAATCCACGGTTGGAAGATCAATCAAGCA TTGGTGAAGCTGTTATGGGAAGGCGAGAGTAAGCCAGTAAGAGTAATACTGAA CTCCGATTTTGAACGCAACAACCAAGACTGTAACCATTGTGACCCGTTCAGTGT CGAGTTAGGTACTTATTCGGCAAGTGAAGAGTGGCGATATACGATTCGAGCCA ATCAAGACGGTGTCTATTTGGCGACTCATGATTTAGATGGAACTAATACAGTCT CTCACCTAATCCCGTGGGGAAAGGATTACTCAGATAAAAATGGGGACACGGTC TCGTTGACGTCAGATTGGACATCGACAGACATCGCTTTCTATTTCAAAGCGGGC ATCTACCCACAATTTAAGCCTGATAGCGACTATGCGGGTGAAGTGTTTGATGTG AGCTTTAGTTCTCTAAGAGCAGAGCATAACTGA

Table 2.4 (cont.)

AlyB	ATGAAACAAATTACTCTAAAAACTTTACTCGCTTCTTCTATTCTACTTGCGGTTGG TTGTGCGAGCACGAGCACGCCTACTGCTGATTTTCCAAATAACAAAGAAACTGG TGAAGCGCTTCTGACGCCAGTTGCTGTTTCCGCTAGTAGCCATGATGGTAACGG ACCTGATCGTCTCGTTGACCAAGACCTAACTACACGTTGGTCATCTGCGGGTGA CGGCGAGTGGGCAACGCTAGACTATGGTTCAGTACAGGAGTTTGACGCGGTTCA GGCATCTTTCAGTAAAGGTAATCAGCGCCAATCTAAATTTGATATCCAAGTGAGT GTTGATGGCGAAAGCTGGACAACGGTACTAGAAAACCAACTAAGCTCAGGTAA AGCGATCGGCCTAGAGCGTTTCCAATTTGAGCCAGCAGTGCAAGCACGCTACGT AAGATACGTTGGTCACGGTAACACCAAAAACGGTTGGAACAGTGTGACTGGAT TAGCGGCGGTTAACTGTAGCATTAACGCATGTCCTGCTAGCCATATCATCACTTC AGACGTGGTAGCAGCAGAAGCCGTGATTATCGCGGAAATGAAAGCGGCAGAAA AAGCACGTAAAGATGCGCGCAAAGATCTACGCTCTGGTAACTTCGGCGTAGCAG CGGTTTACCCTTGCGATACGACCGTTGAATGCGACACACGCAGTGCACTGCCAG TTCCGACAGGCCTGCCAGCGACACCAGTTGCAGGTAACTCGCCAAGCGAAAAC TTTGACATGACGCATTGGTACCTATCTCAACCATTTGACCATGATAAAAACGGCA AGCCTGATGATGTGTCTGAGTGGAACCTTGCAAACGGTTACCAGCACCCAGAA ATCTTCTACACAGCTGATGACGGCGGCCTAGTATTCAAAGCTTACGTGAAAGGT GTACGTACCTCTAAAAACACTAAGTACGCGCGTACAGAGCTTCGTGAAATGATG CGTCGTGGTGATCAGTCTATTAGCACTAAAGGTGTTAACAAGAATAACTGGGTAT TCTCAAGCGCTCCTGAATCTGACTTAGAGTCGGCAGCGGGTATTGACGGCGCTC TAGAAGCGACGTTGAAAATCGACCATGCAACAACGACGGGTAATGCGAATGAA GTAGGTCGCTTTATCATTGGTCAGATTCACGATCAAAACGATGAACCAATTCGTT TGTACTACCGTAAATTGCCAAACCAAGCTACGGGCGCAGTTTACTTTGCACATG AAAGCCAAGACGCAACTAAAGAGGACTTCTACCCTCTCGTGGGCGACATGACG GCTGAAGTGGGTGATGATGGTATCGCGCTTGGCGAAGTGTTCAGCTACCGTATT GACGTTAAAGGCAACACGATGACTGTAACGCTAATGCGTGAAGGCAAAGACGA TGTTGTACAAGTGGTTGATATGAGCAACAGTGGCTACGATGCAGGCGGCAAGTA CATGTACTTCAAGGCCGGTGTTTACAACCAAAACATCAGCGGCGACCTAGACGA TTACTCACAAGCGACTTTCTACCAGCTAGACGTATCGCACGATCAATACCAAAA GTAA
AlyD	ATGTTTAAGAAAAACATATTAGCAGTGGCGTTATTAGCGACTGTGCCAATGGTTA CTTTCGCAAATAACGGTGTTTCTTACCCCGTACCTGCCGATAAATTCGATATGCAT AATTGGAAAATAACCATACCTTCAGATATTAATGAAGATGGTCGCGTTGATGAAA TAGAAGGGGTCGCTATGATGAGCTACTCACATAGTGATTTCTTCCATCTTGATAA AGACGGCAACCTTGTATTTGAAGTGCAGAACCAAGCGATTACGACGAAAAACT CGAAGAATGCGCGTTCTGAGTTACGCCAGATGCCAAGAGGCGCAGATTTCTCTA TCGATACGGCTGATAAAGGAAACCAGTGGGCACTGTCGAGTCACCCAGCGGCT AGTGAATACAGTGCTGTTGGCGGAACATTAGAAGCGACATTAAAAGTGAATCAC GTCTCAGTTAACGCCAAGTTCCCAGAAAAATACCCAGCTCATTCTGTTGTGGTT GGTCAGATTCATGCTAAAAAACACAACGAGCTAATCAAAGCTGGAACCGGTTAT GGGCATGGTAATGAACCACTAAAGATTTTCTATAAGAAGTTTCCTGACCAAGAA ATGGGTTCAGTATTCTGGAACTATGAACGTAACCTAGAGAAAAAAGATCCTAAC CGTGCCGATATCGCTTATCCAGTGTGGGGTAACACGTGGGAAAACCCTGCAGAG CCGGGTGAAGCGGGCATTGCTCTTGGTGAAGAGTTTAGTTACAAAGTGGAAGT GAAAGGCACCATGATGTACCTAACGTTTGAAACCGAGCGTCACGATACCGTTAA GTATGAAATCGACCTGAGTAAGGGCATCGATGAACTTGATTCACCAACGGGCTA TGCTGAAGATGATTTTTACTACAAAGCGGGCGCATACGGCCAATGTAGCGTGAG CGATTCTCACCCTGTATGGGGGCCTGGTTGTGGCGGTACTGGCGATTTCGCTGTC GATAAAAAGAATGGCGATTACAACAGTGTGACTTTCTCTGCGCTTAAGTTGAAC GGTAAATAG

Table 2.4 (cont.)

AlyE	ATGAAGCTGTCTTACCTTAGCCTACTGACTGCCAGCCTTTTAGCCGCTCCCGCTC TTGCTTCTAATCACGATATTGGTCAACAGTTCAATCTTGACCCTGCAAAAGCACC AGCACAAAACTTCGATTTATCTAAATGGAAAATTAACTTACCGGAGCTGACGAC TGAAGGGTCTAGAAAAGGTAAAACGCTGGAGATTGGTAAAAAGGAACTGTCGA ATGTAGACACGCCTTACGTTCACCCTAAATGGTTCTACACGGATGCCGAGTCTGG TGCGATGGTGTTTGTGGCTCCGAATACAGCTCCAACGACGCCAAACAGTAAAAA CACGCGTAGTGAGCTAAGAGCGATGCTAGCGGATAGCTACTCTGCGCCGAGCAA CAACTTCGCGATTTCAAGCCATAAGAACGCGGAAGAGTTTGGCTCTATCGGTGG GCAGATGACGGCAACACTTTCTGTTGATCAGGTGAGCACCAGCGGTAACTATAA GAAAACCGGTGCATTCTCTGTGGTTATTGGTCAAATCCATGGGTCGGATAATGAA CCTCTGAAAATTGTTTACCGCAAATTGCCAGAGCATGAGCATGGTTCGTTAACGT GGAACTATGAATTAAATCCGCCGACAGAGATGAAAAATGCGAAGGATGAAAATG GTAAGAAGCTTCGTAAAGACATTCGCCATGATGTATTTGGCCAATACAATCTGAA AAAAGGCAGCTCGGATCCTACTGATGGTATCAAGCTAGGTGAAGTGTTCTCATAT GATGTGAATATCAAAGATAACATCATGCACTTAACCTTTACTAAAAATCCAAACT CAGCCGAACCTGTTGTGAAGACCTATGATGTTGACTTGGCAAAGGGTAAATATC AGGGACACGATATCGATCTTGGTTACGGTCAAGACTGGATGTACTTCAAAGCGG GTGCGTATAACCAGTGTAATACTAAGAAGTCGAGTTCTGCCTGTGAGTGGCGTG GTATGGAAGCGGGTGACTACACCCAAGCAAGCTTCTACCAGTTAGTTCTTAATC AATAA
AlyF	ATGAACAAAAAACTATTATCGGTAAGCATTATTAGCGCGTTCACGCTCGCTTTTG CAACAGGCTGTACGACACAAGAGAAAACAGCACCCGTAGTCAGTGTCGCTGAA CAAGCTGCGCCTGCTATAAGCGAAATCGACCGTAGTTACCTATTGAGCAGTGAC CGCTTAACTGAGGTCGATGGCGACACTTTAGCCGTTGCTTCAGAAGAACAAGTG GCTGCGCTTAAAGCACAATTTGAAAACCTAAAAGATGGCGACGAAGTGGTGAT CCCTGCTGGTAAATACGCGAACTTAGGTCAAGTGACGATCACGGCGAATGACAT CACTATCAAGGCTGAGCAAGCAGGCTCTGCGTGGATCACTGGTTTGATTCAGTT TGAACTGAAAGGCGATGACATCACGCTTGATGGCTTGGTATTTACTGAGGGCGG TCCTAACGAACGCTTTGGTGCAGTACGTATGATGGGTAATGGCAACACGCTGCA AAACTCAACATTCTACTACTTCAACCATGATTACACGTACGAGCCAGACGAGCG TCGTTCTGAGTATCCAAAGTACCTATGGGTTTCTCTATGGGGTAAAGATGGCAAA GTCATCAACAACCGTTTCGAAGGTAAGCAAAAACGCGGCACTTTGATCGGTGTA CAAAAAGATGACACAGCCGACAACCACCTGATCGCAAATAATATCTTCATGGAT CAAAAGCCTAACCAATTCAATGAATTCGATATCGAAGAAGCGATTCGTTACAAC GGCAACAGCTGGGAAGCGATCCGTATTGGTGATTCTAAAGCTTCACAGTGGGAT TCAAGTTCTAAGTTCGTGAACAACCTGATGATCGATATGGATGGTGAGCGTGAG CTTATCTCGATTAAGTCGGGCGACAACACGATCTCAGGTAATACTATTTTCCAAA GCACCGCGCTTATTTCACTGCGTCACGGAAAAGGCAACACGGTTGAGAACAAC ATGATTCTTGGTAATGGAAAGCGCCTAACCGGCGGCGTCCGTATCTACGATGAA GACCATGTGATCCGTAATAACTACATTGCAAACACTCGAGGCCGCGATGGTTTG ATCGAAGGCAACGCTGACCTACGTGGCGGCATTGTTATTAATACCGGCATTATCG ACGTAGCGAATGGTGAGCAGCTAGATCAATCGGTGAAGGGCAAAGAGCTGAAC AAGCAGTGGACGCCGAAAAACATCACCATCGAGAACAACTCATTGGTTGATAC ACAGTGGGGCATTATCTATGGTAATCAAACTCACCGTGTGAGCCTGTTTAATAAT GCGGAAGTTGAAAAGATTTATGCAGGTGTTGATGTGGCGTTCAAACACAACGTG GTTGATAACTCGCAATCTCCTGAGTTTGTCAGTGTGCGTGCAACGCAAGATTAC CCATTAGTCGGCGCAACCTACACAGACGAAACTTACGTAGGTCAAGTAACGGAT TCTGAGTTGATTGAAAGCTACTCGGTTGAACTGCCAAAAATGACGGTTGAGAAC GGCATCAACGCTTATCAAGGCGAAGGTGCTGACGTGTCTAAACTTGCAGTGGTA ACAGCTGAAACAGCGGGTCCAAATTACGTGCTTGAAAATACCAAGAAGTAA

Table 2.4 (cont.)

AlyG	ATGACCTTTAAACCTTGCAAACTCACTTCTTGCAAACCTCGAACTCACCAATTTA AACCTTACAAACAATTGTCGTGCGCGGTTCTTTTAGCGATGGCTACTTATGGCGT ATCGGCTGAAACACTGAACATTCAATCCGCATCAGACTGGGGCGGAGCGCACA GCTCTTACCCAGCATCGAATACGATTGATGGCAGTACTGATTGGTCATCACGTTG GGCAGCTCAGAATGCGCCCGTTAATTTAGTGCTCGACCTGGGATCAGTACAAAA TGTTCAAGACGTCGCGATAGCTTGGGGTAAAGGTGAAGAGCAAACTTATAAATT CGAGATCAGAGCGCTAGCTGATGAAAGTTCAAGTAGCTGGGACAAGGTTTACTA TGGATATAGTAGTGGCAGCACATCTGGTTTTGAAACCTACGATGTGACGGATGTT CAAGCTCGTTGGATTCGTATCAAAGTCTTTGAAAACAGCGCGGAAAGTGTTTGG ACGAATATTACTGAAGTCGAAATCAGTGGCAACGATAGCCCTGACTACGGACTC GATCCAAACCTACCGCCATCAGGCAACTTCGACTTACTGGATTGGTATGTGAGC ATTCCCGTTGATGAAGGTGATGGCTACGCGACTTCTATCAAAGAGAACACGCTT GATGCGGGTTATGAAGACCAATTCTTCTATACCGGTAGTGATGGTGGCTTGGTGT TCTACACACCTGTCGAAGGTGTGACAACATCTAGCGGAACAAAATATGTTCGTA CCGAATTAAGAGAAATGCTTCGTCGCGGTGATACCTCTTACTCCACCTCGGGTAA AGACAATAACTGGGCGTTTTCGTCTATTCCATCGAGTGACCAGTCTGACTTTGGT GGTATCGACGGGACGTTAAATGCGACGTTAGCTATCAATCATGTGACGACAACTA CTTCGAACACTGAACAAGTGGGTCGAATCGTGATTGGCCAGATCCACGCTGAG AAAAACGAACCTATTCGTCTGTACTACCATAAACTGCCAGGTAACGACAAAGGC GCAATCTACTTCGCTCATGAAACCTCGAAATCGAGCGGTGGCGATGAAACTTGG CATAACCTTTTAGGTAACATGGTAACTTCTGACGGTGACTTAAACAGCACTAGCA ACCCAAGTGGCGGAATTGCACTCGATGAAACCTTCTCGTATTCCATTGTTGTTGA AGGTGACAAGTTGATTACTACCATTAGTCAAAATGGCTCAGAATTGGCGGCAAA AGAAGTGGATATGAGTAACAGCGGTTATGACGATGCAGATAACTACATGTACTTC AAAGCAGGTATCTATTTGCAAGATAACTCAAGTGACGACAGCGATTATGCGCAA GTGACTTTCTATCAGTTAAACAACAATCACAACTGA

Table 2.5. Nucleotide sequences of *V. breoganii* 1C10 laminarinases. Signal peptides are underlined.

Laminarinase	Sequence (5'-3')
LamA	ATGGAGTTAGAAATCGTGAGCAAAACTATAAACCGAACAATGTTGGCAAG TTTGATTCCACTTATATTAGCAGGCTGTAGCTCAAGCAGTGCTCCAGTGCAG ATTACTCCAGACCAACTGGTAGATACATCAAAAACACCAGATACAGGCTGG CAGATGGTTTGGAATGATGAATTCTCAGGCACCGAAATCGATAAATCTAAG TGGAGCTTTGAAGAAAACTGCTGGGGTGGCGGTAACAATGAGCAGCAATG TTATACCGACCGTAAAGTGAATGCGTTTGTTGATAGTGGTGTTCTGCATATT GTTGCGAAGAAAGGCAGTTTTACTGGTCCAAACAATCCCGATGGCGACAT GCGCGCTAAAGCGACCCTTCCTTATACGTCTGCACGCCTTAGAACCTTAAA CAAGGGTGACTGGAAGTACGGTCGCTTTGAAATTCGAGCTCTCATGCCTTC CGGTCAAGGAACCTGGCCTGCGATTTGGATGCTTCCAAGTGATTGGAAATA TGGCACGTGGGCAGCATCGGGCGAGATAGACATCATGGAAGCGGTCAACC TCAAGACACCAACCGATAAAAAGGATGCGGCTGAGGGTGAGGTTGAAAA CCGCATTTATGGTTCTCTACATTACGGCCGAGCTTGGCCTGAGAATGTACAC ACTGGTACCGAATATGTTTTACCAAACAACGCAAGCCCTGCGGATGCTTTT CATACCTATGCCATTGAATGGGAAGAAGGCGAAATCCGTTGGTACGTCGAT GATGTTCACTACGCTACCCAAACACAAGACGGCTGGTATGGCCAATATAAA GAGAATGGACAGTTAGTAACGGCGCCGGATTCTGCGCCTTTTAACGAGCGT TTTCACCTGCTACTTAACCTTGCGGTAGGGGGTGCTTGGTCTGGGAATACC AATGAAGGTGGCATTGATGCCTCAGCCTTCCCACAAACTTTAGCCGTTGAT TATGTTCGAGTTTATCAATGCTCAAACGACGTAGAAACAGGTCGTGGATGT GCAACGAGTGATGAGAGCATTCAAATCACCAAAGGCCATGAAACGCCGAA AATCTTAGTGGCTGATGCCAACTACGGAAAAGGAAATTCGCTCGAAGTCTT TTCTGAGCAGATTAACCCTCTTCTTGTATTAAGCGGATATGATCCTGACGCC AACATCAATGCGAGCTTTGTTGATGATGCGAGCAAAGGAAAGGTATTGCGC ATTGAAAAATCGGGTGCCAGTGGTAACACCTACTTCAAAACACCAGAAGT GGATCTGAGTGATTGGCTAGCAAGTGGTGAGTTGGTATTCGACCTGCTTGT TGAATCAAACCCTGGTGATTCTGAACTGCTTGTGAAGATAGACAGCGGCTG GCCAAAGGTCAGCGACCTAGGCGTGTCACTACCTTCTCAAGGACAGTGGC AAGAAGTGCGAATTAAGTTGTCAGATCTTGCGAACGGAGGCAACCGCTTC GCTGCAGGAAACAAAGTAGATATGAGCAAAATCATAAACTTGCTGGTTATT GAGCCTCAAGGTGAGATGACGCTAATGATGGATAACGTGCGTTTTGAACGC TAA

Table 2.5 (cont.)

LamB	ATGCTAGATTCGCTTATTAACAGAAAAAAAACCTCCTCAACTATGTTGCTAGCAC TTGGACTTACATTAGGTTTCTCTGCACCAGCAAGCGCCGGATGGGAAGTCCAGT GGATAGATTCTTTTGAAGGTGACGGGGTTGATTGGCGTAATTGGACTGCACAAA CTCAAGCCAATTATAACAATGAAGTCCAGTGCTACACCGCTGACGACTATTCATC CGAACGAAATTATGACGTGTCTGATGGCACATTAAAAATCATAGCTCGAAAAAA ACTGCACAGTTGCCCAACACTTGGAGGCCAAACTAAGTCTTGGACCTCCGGAC GATTAAATAGTAAAGACAAACGAGAATTTTTATATGGTCGTATTGAGTCAAGAAT ACGCTTCCATAATCTAGAACGAGGCACTTGGCCTGCGTTTTGGATGCTAGAAAA CCGCATTGCAGAGCAGCCTATTAAAGGGGATGGTGATAATGTCGGTTGGCCTAAT CCAGGAGCAGGAGAAATTGATGTTTGGGAATGGTTCTCTAATCAACCAACCAGC TACATTACTAATTTTTATAATGCTTCAGGTTGCGGCAGTGAATACCGTCATCCATA CCCTCAAGGTGCAGCTGATGTCCTAGAGTGGCAAAAATATGCAGTGGAGTGGAC ACCCGATAAAATTGACTTCTATATGAATGATACATTGGTTGTCAGCCAAAATATTC AAGGTTGTCGTCAGTACCAAGAGCCAATGTTCGTGCTACTTAATGTCGCCATGG GTGGTAATTTAGGTGGGAGTATTGATCCCAACCTACAACAAGCCACGATGGAAA TCGATTACATAGCGCATTGCCAATTTTCGGAAGGTAATGATGCAAAGTTCTGTGA TGAACAAACTCCTAAATCTGATGAGTTAATTCCGGCTATACCCCTACCAGAACTT CAGCTTACTTTAACTCAAGGTAATGGAAGCGTTGATGTGATAGAGCCTTTTGCAG GCGAAGCTTCAGTGACCGTTGATATAGATCTATTGGACGAGCAGAAGGCAGATT ATAGCCTTTACTGGCAAGTAGACGATATTCCTAGCCCTGTAATGGAGATGCAAAC CTTGACATTTGACCCTTCATCGATGCTCGATGGCGACTACGAAATTGGAGTATTG CTAGAGCATGTTACGGATGAGTCATTAAACAGCAGAGCCGTAATGATTGTCCGA GTTCGAAGTAAAGAAGATGCAACAAGCCCTCCTATTGAGCCGACACCTAGCAGT GGCAGCTCAGGAGGTAGCTCTAGCTTACCGTTCATTTTCGGCTTATTTCTTTTGG CAGTTTCACGTCATAGTTTCTGCAAAATCTTGAATTAG

Table 2.5 (cont.)

LamC	ATGGAAGTGAGTAATCGACGTTTTAAAGTCGCAAATCTAGCTCTCGTTACCGCTA TAGCATTAGCTATGATGGGATGTGGCGATGAATCCCTTAGTTCACAAGCAGGAGC ATCCGCAGATAGTCCTGATGGTTCTGGAACACCAACATTACCAACACCAACGCC GCCATCAGGTAGTGATATGGTCGTATTTGAAAACTCCGTGAATGCCAAGTGGCCT GCATGGGATTGTTGCGGTGGAACAACGCCAATTTATCCGATGGAGCTTGATGAG TACGGGTTAACGACAGAGTTTAGAGTCGGTGAGCAACCAGCAGTATTAGGGTTT GTTCGTGCTGAAGGACTTGCGGGTGTCGATGTCAGTGACATGGAATCTGCAGGC ATGCTTGAATTTGAGATCAAATTACGAAACAGCCCTGGTGACAATGTAAATTGG ATTGTGAAACTAGAAAGTAACGGCGGAGATCAAGAAAGTAATGGCGAAGCCGT TGAGTTATCTATCCCTGCACCAAAAATTGGTATGTGGACCAAGGTTCAAGTTTCT CTTGCGGATTTGTCTAATCAAGGCCTTGATTTAACCAATATCGATAAGATTCTGAT TAACCCTGCATGGGAAGAGGGTGAGGGGGCAGTATACCGAGTTGATAATGTTAA ATTTGTTGACGGTGATGCGTCTGAGCCAGAAAACCCTTCCGACCCGATTATTCCA AATGAGTCTGATGAAGGCTACAAGCCAGATCTAGTGGTTGCAATGGAAGATTTT GGTGGTGCAGCATCAGAGCAAGCGGCTGCCAATCCATCGAGTGAAACATTACCT GCAGGAACCTTGATAAAAACAATCAAACCAGCAGGTGCAGAGATATGGGCTGG AACGACTCTTGGTGATATTACTGTTCTTGACATAACACCTGAACGAAGCATTGTT TCCGTATGGGTGTTCTCTGCAGAAGCAGGTATACCGGTGTTATTCAAAGTCGAA AACTCAGTAGTACCAGAGCAATTAGCTGAGGTGTTAGTTCAAACTTCGCTTGTA AACGAGTGGGAAAAGCTTGAGTTTGATTTTAATGATTTAAATGGCGGTGTATTAG ACCCAAACTATAGCTTTGACAAAAAGTCCATATTCTTTGATTTCTTGGCAGAGGC TGCGAACAAGGAATTTTACTGGGCCGATATGACATTTGTTGAAGTCGACGAAGT TGTTGATCCAGAAGAGCCGCCTGTTGATCCAGAAGAACCACCGGTAGACGATG GAGAGTCTTTTGTCATTTTCAATGATGTTGCTAATCCTGCGTGGTTAGCATGGGA TTGCTGTGGCGGCACTGCCCCAGCCGTCATTACCGATAGTGACGACAATTATGGC GCTGTGACTGAATTCAATATTGTAGGGGCTACAGTCGTGGGCTTTACTTCGCGTG AAGCCGAAGATCATGGAGCAGTAGGCGGTGTGCCAGTTGATGTTTCAGCTTGGC AAGATACAGGGACAGTGTCATTCGATCTGAAATTAACCAATGACAATGGTGCAA TGGATTGGAAGTTTAAGGTTGAATCCGCTGGTGGTGGAGCCTTGGAGCTTACTC TAGCCGATGTACCAGAATTGGATGTTTGGAAACGTTATACCTTTAACTTAAGTGA CTTAGCCGATGGCGGCGTGAACCTAAGCGCAATAGATCTCGTTATGATGTTCCCT GCATGGGGCTCGGGTGATGGTGCATCTTATGTCGTTGACAATGTAATCTTCTCAT CGGCAGGCAGCTCTGCTCCAGAAGAACCAGAACAGCCAGAAGGGCCGGTATTA CCTCCAGTAGATGTTGAAGGTAATGTCGTTGTGAATGGTGATTTTGAAACCGGTT CTATTGACCCTTGGTATTCGATTGGCGGCGGAAGTGTATCTATCGATAGCGGTGC CGCGCGTTTACAAGCAGGAAATGGCGCTGAATCTAGGATTAAAGCTAATGGAAT TGGAGCGGGCGACATTAATCCAAACCAAACCATTACTGTTTCGTTCTCTTATCGT GGTGAAGCCGTTGATGGTGGTGTAGCGAATGCAATTATCCACTTTATTGGTGATG GAGTTGTTGGTACTGAGGTCATTGATATGCCTGCGCCTACTACTGAATGGCAATC ATACTCTCAAGAAATGGTCGTTAGTGCAGGAACATCTGCAGGCTTAGATTTCACT ATTGGGGGAGTTTGTGGTGCGGTAGCAACCTGTAGTGTCGACCTTTTCTTAGAC AATATTGCTGTAGTGCCCGAGGAAGGAACCGGGGAAACACCAGATAACCCAGA AGAAGGCCCAATTCTGCCACCTGATGATGGAACCGGTAATCTCATTGTGAATGG TGATTTTACTGATGACCTGTTAACCCCTTGGTTCCAAGTTGGTGGTGGTAGTGTT GTTGTTCAAGACCAGTCCGTTACTGTTGCCGCTGCGACGGGAAATGAAGCGAG GATTAAAGCTGAAAAAGTCGGTCAGGGCGTAGTGACCGCAGGCCAGACAATTA CTTTGTCTTTCCAAGCTAAAGGCTTCACTGCAAATGGTAGTGTTGCCAATGGGTT GCTGTATACGACAAGCTCAGCCGGTGTTTCTAAGACAGATATCTTTGATATCCCT AATTTGACGACAGTATGGAGCGAATATAGCTACGACTTTGTTGTTGGTGACAATC

Table 2.5 (cont.)

LamC (cont.)	CTGAGTGGGGACTAGACCTAGCTATTGGTGGTGTGTGTGGTGCCGTAGATGGAT GCCAAACGCAAGTGTCGTTTGATAATGTTCGATTAGAGATTAAATAA
LamD	ATGAGACGGTCTAATTTTAAGATTTTCGCAACCACAGTACTGTCCTGCCTACTCA TTGCCTGTGGCGATGAGCAGCTGAGTGTTGGTGGTAATGCTCCGGATGCTGGCG GTGATAATGATATTGCTGAACTGGCCCCGCAGCCAGTCCCTACCTCATTCCCAAC AGCGAGAAATGGTGAGCCTCTGCTAGGAAACCCTGAATACCTAGCGGTTTCTTA TGGTGCTTGGCGCACAACCACTCGCGAGTCAGGAGCAGAGGTTCCTTCTGTTG CTGAACATAAAGAAGATATGAAAATATTGTCCGCAATGGGTATAAAAGTATTGCG TACATATAACACCCAAGGCTTCACTGGGCTAGATGGAAAGAGCAATACTGAAAA CTTATTGCAAGCCATTGATGAGTTACAACAAGAGGATTCATCATTTGAAATGTAC GTGATGCTAGGGGTGTGGATTGACGCACTGAACTCTTGGACGGGTAATGAAGTA ATACACGATCAAGAAAACCCTGTTAATGCGGATGAAATTGCCAAAGCGAAGGA ATTAGCTCTAGCGTATCCAGATATTATCAAGGTAATAGCGGTAGGTAACGAAGCC ATGGTGGCGTGGGCTGAATATCATGTTGTGCCTAGCATTATCCTTGACCACGTCA ATGATTTGCAATCTTGGAAGATGCAAAGTGGTGATACAGATGATTTGTGGATCAC AAGCTCTGATAATTTTGCTGTTTGGGCGGGTGATGATGCCAATGGTAACAACAA CGATCAGACCGATATTAAAGCGCTGATTCAAGCGGTTGATTATGTTTCTCTGCAT ACGTACGCGCATCATGACACGCATTATGACCCAATCTTTAAAGAGGAATGGAAG GTCCCTCTTAGTGAACAATCTTTATCTAAAGAGGAGCAGATTGCTTCTGCGATGA CCAAAGCGCACAGCCGCAGTATTTCTCAACTAGCTCAAGCACAAACATTTATTA ATAGTGTGGATGCTACCAAGCCGATTCATATTGGCGAGACTGGCTGGTCTACTGT TTCTTCAGAAATGTTTGGAGAAGGTGGAACTCAAGCTGCAGACGAATATAAGCA GAAGCTGTTCTATGATGATATGCGAGAGTTTACCAATGACTTCGGTGCTTCACTG TTCTTTTTCCAAGCTTTTGATGAGCCGTGGAAAGGTGACCCTAATAATCCTACGC ACTCAGAAAAACACTTTGGCCTAATTGATATTGATGGCAACGTTAAATATGTAGC GTGGGACAAAGTCGACACGCTAAATAATCTAGGTCTAACTCGTGGTGATGTTTC GTCTTTCCAAGCTAGCTTTTCTGGCGATGAAATTACTTTAATGGATACCGTTCTAC CACCTCCGTTTCAAGCTGCTGTTGCGCCGCCAGAAGAAGGTGAGTTTCAGGTAT TGGGCGCTGCCTTGTACTCAGGAGCTGCCGCTTATGGTTGGGATAACCCAATCA CGGCATGGGCGGGTGTAGATGAAACGACAGGAGTTCTAACGGTTGCGGCAGAC CCAGCATTGGCAGCAGTCTGGGGATGGGGCGCAGGTGTTGGTATTGCAGGTCAA ACGAGTAACCTTTCTGGATATCAATCAATGAGTTTCCAAATTAGAGGCGTAGAGA GCGGTGAAAGTGTATTAGCTCAATTTGGTTTCTATTTGGGTTTCCAAACAGAGGG GGGTGGCAATCACTGGATTCGCTTTAATGCAGGTCAAGGTTATTCCTTAACTACC GACTGGCAAACCATCAACGTCAATCTTAGTGAGTTTTCTGGCACACCTGACCTT AACCAAGTGATTTCGCCATTTACTATCTCTGATCTCTATGAAACATCGGGAGGAA GCGCGCCAACCAAGAGCAATATAGAGGTTAGAAACATCTCTTGGTATGAGTAG

Table 2.6. Nucleotide sequences of *V. breoganii* 1C10 alginate lyases. Signal peptides are underlined.

Alginate Lyase	Sequence (5'-3')
AlyA	ATGGAAATGAATAAATCAATTTTGGCATTGATGCTAGCAACCGCACTGACTGG TTGTGGAGGCAGTGATAGCCAAGGCAATAACAATAATCCCAATGACTCAGATC CCGGCACGAGTGAACCTGTTGAGCCGGGCGATCCTATCGCTCCTTATAGTATTA CTAAATATCAAGATATCCTTTCAAATTCGGACTTACAGCAGTCTGATCCTTACG GTAACGCATCCAATAAAGAGAGCGTTGTGACCAATGGGGACTTTTATGGGTAC TACGATGAGTACTTTTATGCCCAAGAGAACACGCAGTATCTTGTGTTTAAGATG TCGAACTACTCTATGCGCAATGAAGTTCGCGAAAGAGACAATTTCGATATTAA TGTTGAAGATGAAAAACGTACCCTACTCGCTGAGGTGAAACTGCCAGGTATCG AAGATGCCATGAGCAACTCTTCTTCAAGTAGAGACCAAGTTACGTTTTTGCAG ATTCACAATAAGGGAACATCAAGTGATGGTACGGGATACATCCCTCACCCACT TTTGCGAGTTGTCTACGAACTTGAGCGTGACGGTAAATACGGTCACTACTGGG CTGTTGTAAAAAATAACGCCGTAGATTGTAAGGGTGACGGTTCAGACCCAGAC TCTGCAGAGTGTCAGAATGCCTATGACCGATACGACCTTGGCGAAGCGGATAC AGATGCATTCACTAAGTTTGAATTAATCGTTCAAGAAAATAACCTATCCATTGA AGTGAATGACATTGTCAAAGCTGATCTGAATATCGCTTACTGGGAACACCTGC TGAGCTACTTTAAAGCGGGCGTTTACAACCAATTTGAAAATGGTGAATCAGAA GCTCACTTCCGAGCACTCAAAGTTCTAACTGGTGATTCAAACACTGCTTACGAT TGGGATATCGATAGCTGGAAGCTCACTATTCCTGCGAGCAAAGATGATTACTA TTTCTCGGGCGTTGATAGTGCAGCTGAGCTTACTCCAGAACGCTGTGGTTATTC AGACAAGGATACACTGGCAAACGATGAAGACGTCGTAGATAAAGACCCAGAC AACAATACCACCTACTTCAGTGTAGAAGATGGACGAATGCACTTTAGAGCTGA CATGGGTTACGGTTCTTCTACTGCCAACTCCAACTACATCCGCTCAGAGCTTAG AGAACTGTTTATTAGCAACAATAACCCTGACTGTAGCACCAGTGATGAAGACA CTAGCTGGTATCTAGACGATTCAAGAACGGGTGATTCTACTCATACATTAAAC GCAACTCTTAAAGTCGAACAGCACCCAAACATCAGTCAACCTAAAGTGGTTTT AGGTCAGATACATGGTTGGAAGATCAATCAAGCGCTAGTTAAGCTGCTTTGGG AAGGCGATAGCAGACCCGTTCGAGTCATCCTAAATGATGATTACGAAATCAAT AACCAAGACTGTGGTCACTGTGAGCCATTTAGCGTTGAATTAGGCACCTATGCT GCCGGTGAGGAATGGAGTTATACCATTCGTGCCGATGAAGATGGTATCTACCT TGCGACCTATGACACTGATGGCAAAAACCGAGTCGCGCACTCAATACCTTGGG GTCAAAATTACACCGATAACGATGGTGACACTGTGGTTTTGTCCTCGGATTGGG CCTCTCCAGATATCGCTTTTTACTTCAAAGCAGGGATCTATCCTCAGTTTAAAC CTGAATATCCTGGACAAATTTTCGACGTTAGCTTCAGTTCTTTGAACATAGATC ACTATTAA

Table 2.6 (cont.)

AlyB	ATGAAACATATATATCTAAAAAGCTTATTAGCTACTTCTGTATTACTAGCGGTTGG CTGTACTTCTACTCCTGTTCCACAGTTCGACAACAACAAAGAAACGGGTGAAGC TCTTCTGACTCCTGTTGCTATTACAGCAAGCAGCCACGATGGTAATGGCCCTGAC CGCCTTTTTGACCAAGATCTAACTACGCGTTGGTCTGCAGCTGGTGACGGCGAG TGGGCAACATTGGACTACGGTTCAGTACAAGAATTTGACGCTGTTCAAGCAGCA TTCAGCAAAGGTAATGAGCGTCAGTCTAAATTTGATATTCAAGTAAGTGTTGACG GTGAAACATGGACTACAGTACTTGCAGACCAAATGAGCTCAGGTAAAGCAATCG GCCTAGAGCGCTTCCAGTTTGAGCCTGCGGTTAAAGCTCGTTACGTTAAATACGT TGGTCACGGTAACACGAAAAGCGGTTGGAACAGTGTCACTGAACTAGCAGCTG TTAACTGTAACGTTAACGCTTGTCCAGCTAGCCACATCATCACTTCAGACGTTGT AGCTGCAGAAGCCGTAATGATCGCTGAAATGAAAGCAGCAGAACAAGCATTTA AAGATTCGCGCAAAGACCTACGTTCTGGTGACTTCGGTGCGCCGGCAGTTTACC CTTGTGATACTACAGTAAAATGTGACACTCGCAGCGCACTACCAGCAGTAACTG GTCTACCAGCAACTCCACTTGCAGGTAACGCACCAAGCGAAAACTTTGACATG ACGCATTGGTACCTATCTCAGCCATTCGACCATGACAAAAATGGCAAGCCAGAT GACGTTTCTGAGTGGAACCTTGCAAACGGTTACCAACATCCAGAAATCTTCTAC ACTGCTGACGACGGCGGCCTAGTATTTAAGTCTTACGTGAAAGGTGTTCGTACC TCTAAAAACACTAAGTACGCACGTACAGAGCTTCGTGAAATGATGCGCCGTGGC GACCAGTCTATTCGCACTCAAGGTGTTAACAAGAACAACTGGGTATTCTCAAGC GCTCCTGAGTCAGACCTAAAAGCAGCAGCTGGTATTGACGGCGTTCTAGAAGCA ACGTTGAAGATCGACCACGCAACAACTACGGGTAACGCGAACGAAGTAGGTCG CTTTATCATCGGTCAGATTCACGACCAAAACGATGAGCCAATTCGTCTGTACTAC CGTAAACTGCCTAACCAAGCAACAGGTGCTGTTTACTTTGCACACGAAAGCCAA GACGCAACTAAAGAAGATTTCTACCCTCTAGTGGGTGACTTGACTGCTGAAGTT GGCGAAGATGGTATCGCTCTTGGCGAGAAATTTAGCTACCGCATTGAAGTTAAA GGCAACACTATGACTGTGTCTGTAATGCGTGAAGGTCACGACGACGTAGTTCAA GTTGTAGACATGAGCGACAGTGGCTACGACGTTGGCGGCAAGTACATGTACTTC AAAGCGGGTGTTTACAACCAAAACATCTCTGGCGACCTAGACGACTACTCACAA GCAACTTTCTACCAGCTAGACGTATCTCACGATACTTACACAGCTAAGTAA
AlyD	ATGAAACAAACTCTTGTTCTTGCGGGTTCACTCTTACTTGCTCTACCGGCTGCTG CTGATGTAGCCAACAACGGAGTTTCGTACCCTGTTCCTGCAGACAAATTCGATAT GCGTAACTGGAAGATCACTATCCCTTCAGACATCAATGAAGATGGTAAAGTGGA TGAGATTGAAGGCGTTGCAATGCTAAGTTACTCTCACGAAGATTTTTTCCACTTA GATAAAGATGGCAACCTTGTATTTGAAGTACAAAACAAAGCTATCACCACTAAG AACTCAAAGAATGCCCGCTCTGAGCTGCGTCAAATGCCTCGCGGTGCAAACTTC GACAACATTCTTACAGACTCTAAGATGAACCAGTGGGCACTATCAAGCCACCCT GAAGCGGATCAATACAGCGCTGTTGGCGGTACTTTAGAAGCAACACTGAAGGTT AACCACGTTTCTCTGCACGCTAAATTCCCTGAAAAATACCCAGCACACTCAGTT GTGGTTGGTCAGATCCATGCGAAGAAACACAACGCTCTGATTGAAGCGAAAAC CGGCTACGGTCACGGCAACGAACCACTGAAAATCTTCTACAAGAAATTCCCAG GTCACGAATATGGTTCAGTTTTCTGGAACTACGAGCGTAACCTAGCGAAGAAAG ATCCAAACCGTGCAGATATCGCTTACCCAGTATGGGGTAACACTTGGGAAAACC AAGCGGAACCAGGTAAAGCGGGCATCGCTTTAGGTGAAGAATTTAGCTACAAA GTTGAAGTTAAGGGCACAATCATGCACCTAACCTTCACAACAGAAAGACACGAT ACAGTGACTTATGACATCGACCTAAGCAAAGGTATCGATGCGAAAGACCACCCA ACTGGCTATGCAGAAGATGACTTCTACTTCAAAGCAGGCGCTTACGGTCAATGT AGTGTTCAAGAGTCTCACCCTGTATGGGGCCCAGGCTGTCAAGGTACGGGTGAC TTCACTATCGATAAAAAGAATGGCGACTACAATAGCGTGACATTCTCAGCACTAA AATTGAACGGTAAATAA

Table 2.6 (cont.)

AlyE	ATGGGTATTGTTCCCGTTTCTCTTGGCACTAAAAATAAATACCTAGAGGTTAATAT GCATACAAAGAACAAGATTGCTGCTGTTGTTATTTCAGCACTCGTTGCTGGCCTA GCAGGCTGCTCAACAGCTTCTTCCCCCGATACTGCATCTGCGACACAACAAGCT TCGTCTGCAACGCAAGCTCCGTCAGCGAATAATGATCCGCAACTGGCTGTGCAG TACAACTTAGACGTGAACAAGGTTCCAGCTGAAAACTTCGATCTATCTAAGTTTA AGCTAAATACGCCAAGAGACGATAACAAGCCAGAGCGTGCGGGTAAGGTAATG GAAATCTACAAGCCTATGTTGAATGACAAGGCTAACCCATTCTCTGATGCTGAGT GGTTCTACACTGATTCTATAACTGGCGCTATGGTGTTTGCTTCTCCAAACAAAGC AATGACAACACCAAACTCTAAGAATGCTCGTAGTGAACTGCGTGCGATGCTTTC TGATGACTACTCATCTCCAAAGAACAACTTCACTATTGCTTCTACAGATAATGCT GAAGCATACGGTGCGATTGGCGGTCACATGAGTGCGACCATGTCGGTTGACTGG GTAAGTACCAGTGGTGATTACAAGAAAAATGGTGCATTTGCTACCGTAATCGGTC AAATCCACGGTTCGAAGAACGAGCCTCTAAAAATCATGTACCGTAAACTGCCAG AGCATGAATACGGCTCTATTTACTGGAACTACGAAACTAATGCTCTAGGTGACGA TTACAGCAAGCGTCGTGATATTCGCCATGAAGTGTTCGGTCAAAGCGGTCTGCG CCAAGGTTCAGAAGATCCTGTAACGGGTATCAAACTGGGCGAGATCTTCTCGTA CGACGTAAATGTAGAAGGTGATATCATGCACCTAACGTTCACTAAGAACCCAGG CAAACCAAACCAAGAAGTTAAGACGTATGATATTGACCTAGCAAAAGGTGAATA CCAAGGCGACAAATACGACCAAGGCTACGCAAACGACTGGATGTACTTCAAAG CAGGTACTTACAACCAATGTAATACTGGCTCAAGCGGTTGTGCAAACAACGGCA TCGAAGCTGGCGATTACTCTAAAGTGAGCTTCTTCAAGTTCAGCCTAGATCAATA A
AlyH	ATGTCTGCGTTCAGCAAAATTGCTATTGGCGTTCTATCTACTTTTGCCGTTTCTTC GGCTATTGCGGCACCTACGCCAAACGAGGTTCTGGACCTGAGTTGTTGGAAAAC GACATTGCCGGTATCACTAACTAACGGTGACAAACCAACTGAGTTCAGCGAGCA AGAGATCGCACAAGGCGCTCAGCATCCAGAATTCTTTTATGTAAATGAAGCTGG CGACGGTGTTGTTTTCCGTTCTCCTGTTTCTGGCGTAAAGACTTCTGAGAACAC CAAGTATATTCGTTCTGAACTACGTCAGATGCTTCGCTGTGGCGATACCTCTATTA GCACTCGCGGCACAAATAAAAACAACTGGGTATTCGGTTCTGCGCCTGAAGGTG ACCACGCTAAAGTAGGTGGTGTGAATGGTCGTATGGAAGTCACTATGTCAGTTG ATGAAGTGACAACTACTGGTGTTGATTGGCAACAAGGTCGTGTAATCATTGGCC AAATCCACGCACCTAACGATGAGCCAATTAAGGTTTACTACCGCAAAATGCCAA ACCACAAAACTGGCTCTATTTGGTTCAACGTAGAACCCGATCGTCGCTCTGGTC TGCGTGACGGCGACATCACTTTCCCAGTTCTAGGCTCAACTAAGCCTAACTTCT GGCGTCAAGGTGAAAAGAACACTCCTGAATCATTCGATGGCGGTATCGCTCTAG GTGAGAAGTTCTCTTACGCGATTGATGTAAACGACAGCATCCTAACCTTCGAGC TATACCAAGAAGGCAAAGACCCACGTAAAGTTTCTGTAGACCTAGACATCTACG GCGTGAGCCTACAAGACAGCTGGAACTACTTTAAAGCTGGCGTTTATGTACAAA ACCGTACTGGTGAAGCGAACGATATGACCGCTGCTACCATCTATGATCTTAAAGT GACTCACGACTAA

Table 2.6 (cont.)

AlyI	ATGATGAATACTAAGACCCTTAGTCTTGCTACTAGTAAGAAGTTTGCACTTAGCA CAGTTGCTACTTTCGCTGCTGCTCTAACCATTAGCGCAGGCGCACAAGCTGAGA CAAAAGCACCTTCTGACAAATTTGACCTTCTTGGCTGGACGATTAGTGTCCCGG TTGACTCAAACGGTGATGGCAAGTCAGACCAAATCAAAGAAAATGAGCTGGCT GGTGGCTATTCAGACGCTGACTTCTTCTACCTAGATCAAGATGGCGGTATGGTAT TTAAGGCTCCTATTAAAGGCGCTAAAACGTCAAAGAACACTACCTATACTCGCTC GGAACTGCGTGAAATGATGCGTCGTGGCAACACTTCTCATAAAACCAAGGGCGT TAATGGTAACAACTGGGTTTTTTCTAGTGCACCTGAAGCAGACCTAAAAGCGGC CGCTGGTGTTGACGGCACTCTAGAAGCAACTTTGAAAGTTGACCACGTAACCA CTACTGGTGAAAACTGGCAGGTAGGTCGTGTGATCGTAGGCCAGATCCATGCAA ACAACGACGAGCCAATTCGTCTTTACTACAGAAAGCTTCCTCAGCATGAAACAG GTTCCATCTACTTTGCACATGAACCACGCGAAGACTTTGGTGATGAAACTTGGC ACGACATGATTGGTAACTCACTACCAAACTATTGGAATCAAGACGCGACTCCAG CAGAGCCTAAAGACGGTATCAAACTTGGTGAAGTGTTTAGCTACCGCATTAACG TAACAGGTAACGAGCTAACGGCGACTATCATGCGTGAAGGTAAAGAGGACATCG TTAAAACAGTAGACATGTCTAAGAGTGGCTACGACGAAGGCGGTCAGTACATGT ACTTCAAAGCAGGCGTATACAACCAAAACAAATCGGGTAAGCCTGATGATTACG TGCAAGCGACTTTCTACGACTTGAAAGTTACTCACTAA
AlyJ	ATGAGTAGCAAAATCGGCAAAAACCTTTTGATGTGTAGCATGCTAAGTACACTT CCTTTCTATGCCAATGCAAACGAGCTCGAGCTAACCAATCCAGGTTTTGAAGCG GGAAATTGGGACGGTTGGCAAGATACTGACCCTTCCTCTATTTCAGGAGACACC TATCAAGGTTCTCATTCAGCAAAGATTACTGGCTCAAGTGGCTTATTCAGTCAAA GCGTTGCTGTAACCCCTCAAACCAGCTACACCTTATCTGCGTACATTAAGGGATC AGGAGCGATCTTCGCCGACATTGATGGACAGCGTATAGAACAAAGCACTAGCAA CAGTGACTGGTCTAAAGTGGAATTAACCTTTGATTCTGCGAATCAGACGGAAAT TGGATTGGGTGGTAAATATCATTCGGGAGAAGGACGCTTTGATACCTTTGTGCTA GTACAAAACGGATCAAGTGAAGGGGGCAACGGTGGCGAGGAAGGCGAGATAT GCACTAATGAATCCCTATCGATTTTATCCGCAAGCGATGACGGCAGTCATGATGG ACACGCGCCAAACAACACCATAGACAATAACCTAGCTGACGAATCTCGCTGGTC TAGTCTGGGCATCGGCAAAAGTATCACTTACGACCTCGGCACTTCATCAACCGTT GGACAAATTGATGTTGCTTGGTACAAGGGCAATAGTCGAAGCTCTTACTTCAGC GTTGATGTTTCTAGCGACCAACAAAATTGGCAACGCGTCATTGCTAACTCCACTT CAAGCGGCAACATTGCAGGTTTTGAGAGCTACCCTTTTGATGAAGTTAACGCTC GCTATGTGCGTATCGTTGGCGAAGGCAACTCATCGAACAATTGGAACAGCATCT TAGAAGTCGATTTATATGGCTGTGGCACAGACTCAGGTGAAGAACCCGAGACTC CGCCCGAGACAGGACTGGATCCTAACTTGCCACCGTCTGGCAATTTCGATCTTC TTGATTGGACCCTAAGTATTCCAGTAGATAACGATGGCGATGGCAAAGCGGATAC CATTAAAGAAAACGCGCTGTCTGCAAGCTACGAAAACTCATCATTTTTCTATACG TCAGCTGATGGAGGCATGACCTTTAAAGCTCCTGTAGATGGTGCAAAAACATCC ACAAATACTAGCTATACCCGCTCGGAACTTCGTGAAATGTTACGCCGTGGCGATA CTTCTCACGACACTAAGGGAGTTGGCAAAAATAACTGGGTATTCTCAAGTGCAC CGTCTGATGATAGATCTGCTGCAGGGGGTGTAGACGGGGTACTGTCCGCGCAGC TTAAGGTTGACCATGTAACAACAACAGGCGACAGCTCTCAGATTGGTCGTGTAA TCGTTGGGCAAATTCACGCCAATGATGACGAACCTGTGCGCCTATACTATCGTAA GCTTCCTGGAAATTCGAAGGGCTCAATCTATATTGCTCACGAACCCAATGGCGG CTCAGACTCATGGTATGAAATGATCGGAAGTCTCTCTAGTAGCGCGAGTAATCCA TCAGATGGTATCGCTCTTGGGGAAGTGTTTAGCTATCAAATCGAGGTACAGGGC AACTCTCTAATTGTGACCATAATGAGAGAGGGCAAAGCCAATGTTCGTCAAACG GTAGACATGAGCAATAGTGACTATGACGTTGGTGGTCAGTACATGTACTTTAAAG

Table 2.6 (cont.)

AlyJ (cont.)	CGGGTGTCTACAATCAAAACAACACCGGTGACAGTGACGACTACGTACAGGCG ACTTTCTACAAAGTAGAAAACAGCCATACAGGGTATGCTCACTAA
AlyK	ATGGAGGTGTCTGCCGCTGAACTTAACGAGGGTTACACTCATCCAGAATGGTTC TACACCGACCCGCAAAGCGGAGCGCTAGTATTTATTGCACCGAACAAAGCGATG ACTACGCCAAACTCTAGTAACGCTCGCTCTGAGCTGCACGCCATGCTGGCCGAC AATCCAGAGGTGGGTACCTATGAGCCAGCAAATAACTTTGCTGTAGCCTCAAAC AAAAATGCTGAAGACTATGCGGCGGTTGGCGGAAAGCTGAGCGCTGAACTTGC GGTCAATCATGTAAGCTTAAGTGGCGATCACAGACACAATGATTCTTTCTCAGTG GTAATAGGACAAATACATGCTCGCCACAACGAACCACTAAAGATCTTTTATCGTA AGCTACCGGATCACGAATACGGCTCGGTTTACTGGAACTACGAAAACAATGCAT TGGGAGATGATTACCACAAACGTCTCGATATTTCTCACAATGTATTCGGCAAAGC TAAGTTACGTTTTGGCCAAGAAGACCCGCAAGATGGGGTTAAGCTGGATGAGA AGATCTCTTACACGGTCAATGTAGAAGGCGACATCATGCACCTCGAGTTTATTAA GGATGCAGATTCCGATAATCCGGTGAAAAAGACGTTCGCAATGAACATTGCTGA AGGGAACTACCTAGGCAATAAATATGATGCCGGTTATGCACAATCACTCTTCTTC TACAAAGCCGGTGCATATAACCAATGCAGTACTGGGGTAGTGCGTTGTACTAAC AATGGTATGGAAGCGGGCGATTATACCAAGGTGAGCTTTTACCACCTGGAACTC GACCATAAATAA
AlyL	ATGACACTTCGTAAAACTAAAATACTCGGCCTAGCCACTACCCTACTCTTATCTT CTCAAGCGTTCGCCATCACCTTTGATGACGCGGGCGACAACCGTGGTATTCCTG CTGATTATGCGCAATACCGTCCGATTCTGTCTGAATCAGAATTACAGATCTCTGAT CCTGCAGGCAAGAAAGGCAACAAGCTTTACATCGCAACCAACACCGACTTTTC TGGTGTTGTGAACGAAGTATTTTATGTGGACAAAGAATCAGAAGCTTTCGTCTTT AAAATGACGGGCGATCACAAAAGAAACGAGTTGCGTGTTCATAAGAACTTTAA CACCTCGCTGCCACATACCTTCTACCATCTAAACGCAAGCATTGAGCCGGTAAG CCCAGAAGCTTCCATGAAAGACTCTACGTCTAAGCAAAATGAAATTACCTATCTT CAAGTACACAACAAAGGCGTCACTGTTGATGGTAAAGACAATATTCCTCACCCG CTTCTACGCGTAGTATGGCGTGAAGGCGCTGGTGAAACTGCTGGACACTACTGG GCAGTAATTAAAGACAATGCGCTTATCTGTAAAGGTAAGAAAGGCAAAGAAAA CATGGGTAAGCCTGCTTGTAAGTCAGAAAATGCTTACAAACAATACGACCTTGG CAAAGCAAAGACTGGCGCAACAGATTTCAATATTATCGTTGGTAACAGCACTCT TATTGTGAATGTGGATGGTGAGCAAAAAGTGAACCACAACATCGATTACTGGAG TCACCTACTCAGCTACTTCAAAGCGGGTGTATACAACCAGTTTACCAATGGCGA AAGCGAAGCTCACTTCTACCAGCTTGAGTATCAAGTAGAGCATAAGTAA
AlyM	ATGATGAAAATAAACGCTATTACCACAACAGTTTTGCTATCAATCATGCTAGCTG GATGCGATTCAGGTGGCGATAATCCACCGAACCCAGAGATTACTGAACCCGGAA ACGGTTGGAACATCAACCAATGGAAAATCACACTTCCCGTAAGTGAATCCTATT ACAAAGAACACTTTGGCGTGAGCTATGGTTTAAATGATCGCGACAGCGCTGTCG AATTGCTACCCAGTAAATGCAGTGGCAAGGATGTATTCTCATTAGAAACATCCCT CCCCTACTTTTATGTTGCTGATGATGATGACGTTCACTTCATTGTTGATTTAGGTG ACGCCGGAATATCCACTACGACCAACACTAAGTATGCACGTTCGGAGTTAAGAG AGCTGTATAACTATAATTCTTCTAGCATCTGCTCATCATCCACGCAAAACTGGAC GGTAGACGACGACGCAAACCACCAACTGCAAGCACAGTTGCAGATTGAGGATT ATCCGAACATCTCAGGGCAAGACCCAAAGGTCATTGTGGGACAGGTGCACGGC TATAAAATCAAGCAAGCACTGATTAAGTTACAATGGGAAGGTGGGAATAAGCCT ATTAGGGCAATCTTAAACAATACTTTCTTACCTGACGATCAATCTTGTAGCTATTG TAAATCGTTCAGTGTGGATTTAGGCACAGCTCAAGCAAACTCAGACTGGAGATA CAATATCGAAGTGAATGAAAATGGTGTTGTATTAGAGGCTGCAGGAGTAAGTAA ATCGTTCGCTTGGGGCGAAAAGATAGAAAATACCGGTTACACACTAGACCCAAA

Table 2.6 (cont.)

AlyM (cont.)	CTGGGCGCATAGCGATAATAGTTTCTACTTCAAAGCAGGTATCTACCCGCAAATA GAGCCTAGCTCATCGCTTTCTGGTCAAATCTTCGACGTAAGCTTCAGTGAAATAA AAATCACCCACCAATAA
AlyN	ATGAAAAAAACTGCCATTGCCTTAGCTGCTACGTTAGCTTTGTCTGGATGTAGCC TTTTCAACAACTCTCAACCAGAAGAGCCTATGACCGTTGCGGATGTAAACCGCA GCATGCTTTTAGAAAGTGATCGTTTAACGGTTATTGAAAAAGCGTCTATGGAAG ATGCCTATAAAGGTGAAGTTGATGCACTTCGTGAGAAGATCCTCAACGCCAAAA ACGGTGACGTGATTGAAATCGAACCAGGCAAGTATCGCAACCTTGGTCAACTGA CTATTGAAGCGAACAACGTAACGATTAAAGCGAGAAAGGCAGGTACTGCTTGG ATCACTGGCCTTGTTCAGTTTGAACTGAACGGTGATGGCATTACTTTAGATGGAT TGGTATTCACTGAAGGCGGTCCAAACGAGCGCTTTGGTGGCGTACGTATGATGG GTGACCGAATCACGCTGAAGAATTCAACATTCTACTACTTCAATGATGATTACCC ATATGAACCAGACGAGCGCCGCTCAGAGTATCCAAAGTACCTTTGGGTTTCTCT ATGGGGTAAAGATGGTCAAGTGATCAATAACCGTTTTGAAGGCAAGCAAAAAC GCGGTACTTTGATCGGTGTTCAGAAAAATGAAACGCCTGATAACCACATTATCA AGCACAACATTTTCTTGGACCAAAAACCAAACCAATTCAATGAGTTCGACATTA AAGAAGCGATCCGCTACAACGGCAACAGCTGGGAAGCGATTCGAATTGGTGAC TCTAAAGCATCACAATGGCCATCAAACAGCCAGTTTGTTGAGAACCTAATGATTA ACATGGACGGTGAGCGTGAGCTTATCTCCATTAAATCAGGTGGCAACATTATTGG TGGCAACACTATTTTTGAAAGCACAGCATTGATTTCTCTTCGCCACGGTAAAGCC AACGTCGTTGAAGACAACGTAATTCTAGGCAATGGCAAGCGTCTCACTGGCGGC ATGCGTATCTATGATGAAGACCATGTAATCCGTAATAACTACATTGCTAACACTCG TGGCCGTGATGGTCTGATTGAAGGTAATGCGGATCTTCGTGGTGGTATTGTTATC AATACTGGCATTATCGATGTAGCGAATGGCGAAGAGCTTGACCAATCTGTGAAA GGCAAAGAGCTAAACAAGCAGTGGACGCCAAAGAACATCGCGATTGAAAACA ACACTTTAGTTGATACAGAGTGGGGCATTGTCTATGGCAACCAGACTCACCGTG TAAGCCTGTTCGACAACAAAGAAGTGGAAAATATCTTTGGTGGTGTAGATGTAC ACTTTAGCAAGAACCTAGTGGACAACTCTGCTAACCCTGAGTTTGTTGCTGTTC GTGCTACTGCTGACTTCCCGCTTGATGCTGCAACTTATGCTGATGAAGTGTACGT TGGTCAAGTGACAGAAGCAGAGCAAGTTTCTAACTACTCTACGGAATTGCCAGT AATCACAAACGTGAATGGCTTTGAATCTGCAGAAAACGTCGGTGCTGATGCTTC TAAACTGAAGATCATCACCGCTGAGGTTGCAGGACCAGACTACGTAATCCAATA A
AlyO	ATGAATAAACATATACTAGCAGTCCTAATTGGATTAACTCTCGTTGGGTGTAATGG GGGAAGTGACGACGTTAACTTACCGTCCGATAGTCCTGAAGTACCCGAAGACTC GACACCTGACGTTCCAAATCCGCCAGATCTACCATCACCTGAAAGTCCAGATCC AGACTCACCTTTACCCCCTTTTAATGGCGATCTAACTTTCCTATCAAGTGGCATTC CTGCAGCAGTAGTAAATGTACCTGATGTAAATTGTTCTGAAGTGTTTGATAGTAC TAAGGCATTGGAATCTGCGGTGTCAGATGACATGGAGCCGGGCACTACGCTTTG TTTAGCCGATGGTGAGTATAGTGATGGCCTGTACTTAAAGTTCGGCGGACAAGG TAGTGTTGATGCTCCAGTCAAAGTAGCAGCAGAGAACCCAGGCAAGGCGATTAT TAACGGCGGAACTGTTGCTGTAAAAATGGCAGGTAGCCATGTGCAGGTTCAGGG CTTTGTTTTTGACGGCGTGCAATATAGCAGCAGCCTTATTGAAACACGCCACGGT ACTCATAATTTGTGCACAGATTGTCGAATTACCGAGATTTCGGCAATTGATACAA AAGCGAGCGGCAGCTCTGGCATACTTGTACACATCTATGGTCAAGGTAACTGGC TAGATCACAGTGTTCTAAGCGGTAAAATGGTGAAAAACCCAATGATTTCATTTAA TCGCTGGGTGAGCGATGATTGGGATGAAGAAACTAAAACCAATGAGTTGGCCC GTGACATTGTGATTTACAAAAATTACATTGGAAATAGACCACCTACTGACGGAA AACTCTACGCAGACTCAAGTGATAATGATTACGAAGCGATTCGAACTGGCCTAA GTGCTACTCACCATTACCCTGGTGATTCATTCATTGTGGGGAATTTGTTTGAACAT ATTCAAGCGGAAGCTGAAGTGATCTCGAATAAGGGAACCAACAATGTTATTAGT CACAATACCATTCGTAATAGCAATGGTTCTCTGACTACTCGACATGGTAGCAACG CAAAAATAAACAACAACTTTATTATGGGTGACGGCTATCCTCTTGCTGGTGGCAT

Table 2.6 (cont.)

AlyO (cont.)	ATGAATAAACATATACTAGCAGTCCTAATTGGATTAACTCTCGTTGGGTGTAATGG GGGAAGTGACGACGTTAACTTACCGTCCGATAGTCCTGAAGTACCCGAAGACTC GACACCTGACGTTCCAAATCCGCCAGATCTACCATCACCTGAAAGTCCAGATCC AGACTCACCTTTACCCCCTTTTAATGGCGATCTAACTTTCCTATCAAGTGGCATTC CTGCAGCAGTAGTAAATGTACCTGATGTAAATTGTTCTGAAGTGTTTGATAGTAC TAAGGCATTGGAATCTGCGGTGTCAGATGACATGGAGCCGGGCACTACGCTTTG TTTAGCCGATGGTGAGTATAGTGATGGCCTGTACTTAAAGTTCGGCGGACAAGG TAGTGTTGATGCTCCAGTCAAAGTAGCAGCAGAGAACCCAGGCAAGGCGATTAT TAACGGCGGAACTGTTGCTGTAAAAATGGCAGGTAGCCATGTGCAGGTTCAGGG CTTTGTTTTTGACGGCGTGCAATATAGCAGCAGCCTTATTGAAACACGCCACGGT ACTCATAATTTGTGCACAGATTGTCGAATTACCGAGATTTCGGCAATTGATACAA AAGCGAGCGGCAGCTCTGGCATACTTGTACACATCTATGGTCAAGGTAACTGGC TAGATCACAGTGTTCTAAGCGGTAAAATGGTGAAAAACCCAATGATTTCATTTAA TCGCTGGGTGAGCGATGATTGGGATGAAGAAACTAAAACCAATGAGTTGGCCC GTGACATTGTGATTTACAAAAATTACATTGGAAATAGACCACCTACTGACGGAA AACTCTACGCAGACTCAAGTGATAATGATTACGAAGCGATTCGAACTGGCCTAA GTGCTACTCACCATTACCCTGGTGATTCATTCATTGTGGGGAATTTGTTTGAACAT ATTCAAGCGGAAGCTGAAGTGATCTCGAATAAGGGAACCAACAATGTTATTAGT CACAATACCATTCGTAATAGCAATGGTTCTCTGACTACTCGACATGGTAGCAACG CAAAAATAAACAACAACTTTATTATGGGTGACGGCTATCCTCTTGCTGGTGGCAT TCGGATTGTTGATGGTGACCATGAGGTTACTAATAACTACATCGAAGGTGCACGT TACCTCAATACAACTCACCATGGTGGCATTGTTTTATTGGGCTCTGACGGTTCAG GTGATGGTGGTAACGGTTACCAACAAGTAGAGAATGTGCATTTAGCTCATAACA CCATAGTAGACAGTGTGAACAGCTTGAATTTAGATGGTGGAGGCAAGAAGACTC AGCCTCGCGAGGTAATTATCGCCAATAATTTGGTGGATAAAGCTATAGGGCCTAT TTTCCGCAGTTCAGATCGCGGTGTACCCACCAACTCAACGATAACTAACAACAT TGTTTCTGGCCAATCAGTGGCTGATAGTGACTCGATTACTTATTATGAAGAGGGT TTTGAGTTTATATCATCTGAGCTTAAGCGCCACAGTGAGGATAACTTATTCCGAC CTTCAACAGACTCCCCGAATCTCGATGCCATTGATTATTGGTTTGATGAAGTTTC TAAAGTGCTGACAGATATGGATGGCCAGACACGAAGTTCATTGACTATGGTGGG TGCCGATGAAGCCTCTGTAATGGACCGAAAGCTAAAGCCGTTATCTTATGAAGA AGTTGGCCCAGTTCATTATCGTATTGAGAAACCAGAACCGACTATTGTGGTTTCA GATATTGCCAATAATGACTTTGCAAATGGTATTGATGACTGGTTTAGTTTTGGTGC TTCAGCGGTAACAGGTGTTGAAGCATTCTCATACTCTGGTAGCGTTTCAATCAGT AACAATGGCTATGTAAGTCAGAATGTGACACTACTGCCAAATCACGACTACGAA CTCAGTGCCTTTGTTAAAGGGGCGTACAGATTAAGTGTCGACGGGTTGGTTCAA GAGCAAGGTCTAGCATCAGACAGCGAGTACCGCTGGGTGCGTGTACCGTTCAA CTCTGGAAGTCACACAATTGCAGACGTTACCTTGGGTATCCCTACCGAAGTGAC ACTGCCAGTAGAGGTTCATGACGCACAGTTCGTGGATTTCCGTGCAAATAGCGG GACAAGTGATGTTTGGGTTCAGCATGAGGGCAGCAGTGCAGGATTTGGTGACGT AGGTAGCTCTGGAGACAACGCTTTCGGTGATGGTGGCTCGGCTCGAGTTCGCTT CAAGAAAGATGTATTCAATCACGACTTTTCAGCCTTGCCAGGTGTTAGCCAAGT AGTCGAGGGACTACCGTTCAATACAGATGTGACTTACTCTTTGTATTACTGTGAT AACAAGAAAGACGATTCACTATCTACCTTGTACTTCGGTGCCAGAGATATCAATG GTAATGCGATAACTGAAGAATATGCACACGTCAAAGATTTGAGTAATGCTCCTCA AGGTACTAATAAAACATGTTTCAAAAAGGTATCTACAACATTTAATACTGGGAAT AATGGTAGCGTAGAGCTATTTGCGTTGATGGCTATCGATGTCAATGGAACGATGA CGGAAGAAGAGATCTATGCAAGTAGCCAGTTTACGAGCAACGAACTTGAAGTG CGCTTGGACGAATTCTCGTTAACCTATAAGGGCGAAGCGAGTGATGAACTAGTT GGGTATTTCGATGAGGTTCGTTTGGCAACTCGAAATGACCAAAACTAA

Chapter 3 Over-expression and characterization of four alginate lyases from Vibrio splendidus 12B01[1]

3.1 Introduction

The marine bacterium *Vibrio splendidus* (93) is a member of both planktonic and animal associated microbial communities and is the dominant *Vibrio* species in the temperate oceans (94). *V. splendidus* has been associated with infection and death of commercial oyster stocks (95, 96) and can causes vibriosis in humans through ingestion of infected mollusks. A sequence search of *Vibrio splendidus* has found seven putative alginate lyases. In this study, characterization of the seven alginate lyases has been undertaken in order to determine the activity and substrate specificity of the alginate lyases.

Multiple studies have investigated alginate lyases from diverse organisms (32, 97-104). In this study, we investigated the alginate lyases from *V. splendidus* 12B01 (93). This bacterium is the dominant *Vibrio* species in the temperate oceans and bivalves (94). It can also grow on alginate as its sole carbon source (12). In one notable study, *Escherichia coli* was engineered to produce ethanol from alginate by expressing the requisite metabolic, transporter, and lyase genes from *Vibrio splendidus* 12B01 (12). However, the native alginate lyases from *V. splendidus* 12B01 were not characterized in that study nor was their individual contribution to alginate degradation explored. In this work, we cloned, purified, and characterized the four PL7 alginates lyases in *V. splendidus* 12B01. We found that these lyases have optimal activity between pH 7.5-8.5 and at 20-25 °C, consistent with their use in a marine environment (105). Additionally, we found that divalent cation Ca^{2+} is necessary for optimal enzyme activity. Finally, we found that the four tested alginate lyases to be polyG or polyMG specific and employ an endolytic mechanism. The significance of this work is that it provides a comprehensive investigation of multiple alginate lyases within a single marine bacterium, thus providing

[1]Portions of this chapter were reprinted from "Alginate Lyases from Alginate-Degrading Vibrio splendidus 12B01 Are Endolytic" in the journal Applied and Environmental Microbiology, Mar 2015, vol. 81, no. 5, pp 1865-1873, by Badur, *et al*., with kind permission from American Society for Microbiology. NMR and ESI-MS analysis were performed by Geethika Yalamanchili.

a further insight regarding how this organism is able to efficiently use alginate as its sole carbon source and potentially informing the design of other organisms capable of producing fuels and chemicals from alginate.

3.2 Results

3.2.1 Identification of four alginate lyases within *V. splendidus* 12B01

V. splendidus 12B01 possesses four putative alginate lyases: AlyA (V12B01_24254), AlyB (V12B01_24259), AlyD (V12B01_24274), and AlyE (V12B01_09446). An additional alginate lyase within *V. splendidus* 12B01, AlyC (V12B01_24264), was annotated as an alginate lyase but found not to be active on alginate (data not shown). AlyB, AlyD, and AlyE contain one polysaccharide lyase family 7 (PL7) alginate lyase domain whereas AlyA contains two PL7 domains (30, 31). Catalytic sites were then identified through comparison to other PL7 domains **(Figure 3.1**). Arginine and glutamine have been proposed as neutralizing the negative charge on the carboxyl group. Histidine is then used to remove the C-5 proton, while tyrosine donates a proton to cleave the 1,4-linkage. Finally, a double bond is formed (30, 31, 89, 106-108). These residues all lie within the anti-parallel β-sheets of the PL7 domain cleft as determined using a homology model of AlyD derived from the structure of the alginate lyase AlyA5 from *Zobellia galactanivorans* (36) (**Figure 3.1**). AlyB also contains a carbohydrate-binding module family 32 domain (CBM32) in addition to the PL7 alginate lyase domain. The role of this CBM32 domain has not yet been determined.

3.2.2 Expression of alginate lyases

To determine whether the four putative lyases were expressed during growth on alginate, we measured gene expression using quantitative PCR. Briefly, the cells were grown in M9 minimal salt medium supplemented with either alginate or glucose. As shown in **Figure 3.2**, the expression of all four lyases was strongly induced when grown on alginate as compared to glucose. Moreover, expression increased with the alginate

concentration. The effect was most pronounced for *alyA*, *alyD*, and *alyE*, where expression increased 2-fold when the alginate concentration was increased from 0.1% (wt/wt) to 1% (wt/wt). However, expression of *alyB* increased only moderately as it was already strongly expressed at low alginate concentrations. These results demonstrate the alginate lyases are not constitutively expressed but rather conditionally expressed in response to alginate. In addition, these results suggest that AlyB may be employed at low alginate concentration whereas AlyA, AlyD, and AlyE at high concentrations.

3.2.3 Purification of alginate lyases

AlyA, AlyB, AlyD, and AlyE were cloned with an N-terminal 6xHis-tag and expressed in *E. coli* from a T7 promoter. The lyases were then purified under denaturing conditions and re-folded to yield a functional enzyme **(Figure 3.3)**. The native molecular weights were determined using size exclusion chromatography, and each protein was found to exist as a monomer (**Figure 3.4**). AlyA, AlyB, AlyD, and AlyE genes encode a polypeptide of 580, 505, 324, and 325 amino acids, with a calculated molecular mass of 67.4 kDa, 57.5 kDa, 38.3 kDa, and 38.6 kDa, respectively. The molecular masses of AlyA, AlyB, AlyD, and AlyE were approximately 70, 62, 38, and 38 kDa as determined by SDS PAGE (**Figure 3.3**). Using gel filtration chromatography on a Bio-Sil SEC-250 column, AlyA, AlyB, and AlyD, AlyE was eluted as a symmetrical peak between ovalbumin and γ-globulin, and myoglobin and ovalbumin, respectively. AlyA, AlyB, AlyD, and AlyE corresponding to a molecular weight of 68.2 kDa, 59.0 kDa, 36.5 kDa, and 35.2 kDa, respectively (**Figure 3.4**). These results indicated that the enzymes migrate as monomers in gel filtration and are active as a monomer in solution.

3.2.4 Determination of optimal enzymatic conditions

To determine optimal conditions for each lyase, a universal buffer was formulated that allows a wide range of pH concentrations to be tested. The optimal pH, temperature, and NaCl concentrations are listed in **Table 3.1** and associated data in **Figure 3.5**. The optimal pH for all tested enzymes was found to lie within pH 7.5-8.5, while the optimal

temperature was found to lie between 20 and 25 °C. AlyB, AlyD, and AlyE were found to have optimal activity at 400 mM NaCl, while AlyA had optimal activity at 1000 mM NaCl.

To determine whether divalent cations are necessary for enzymatic function, each protein was stripped of divalent cations using Chelex 100 resin and then individual divalent cations were added to the reaction mixture at a concentration of 1 mM (**Figure 3.5)**. $CaCl_2$ was found to moderately increase enzymatic activity for all four lyase. All other cations tested were found to either decrease or not change enzymatic activity.

3.2.5 Activity of AlyA domains 1 and 2

AlyA possesses two PL7 domains. To determine if these individual domains have lyase activity, both domains were over-expressed independently and tested on alginate (**Figure 3.6**). AlyA domain 1 was found to have no lyase activity whereas AlyA domain 2 was found to possess approximately 1.4-fold greater activity than the full enzyme. When accounting for the gram loading of each protein, AlyA domain 2 has almost 3-fold greater activity per µg of protein than the full enzyme.

3.2.6 Determination of enzyme kinetics

We next determined the kinetic parameters for each enzyme by measuring the initial velocities at different substrate concentrations (**Figure 3.7)**. This was accomplished by dosing alginate and measuring enzymatic activity at the optimal environmental conditions listed in **Table 3.1** and 1 mM $CaCl_2$. All four enzymes exhibited Michaelis-Menten type kinetics. The associated K_m, V_{max}, and turnover number are found in **Table 3.1**. All four enzymes have micromolar affinity for alginate. AlyB had the highest apparent affinity, with a K_m equal to 20 µM. This result is consistent with our gene expression results, where *alyB* was found to be strongly expressed at low alginate concentrations. However, the apparent affinities for the other three enzymes were not significantly lower, with K_m's ranging from 35 to 123 µM.

3.2.7 Determination of substrate specificity

The substrate specificity was first evaluated by comparing the relative activity on polyG-enriched and polyM-enriched substrates (**Figure 3.8**). AlyB, AlyD, and AlyE were found to have greater activity under polyG-enriched substrate than polyM-enriched substrate. AlyA was found to have similar activity under both polyG-enriched and polyM-enriched substrates, pointing to specificity of polyMG/GM substrate.

Substrate specificity was next evaluated using ^{1}H NMR **(Figure 3.9)**. ^{1}H NMR of AlyA degraded alginate produced a doublet corresponding to the anomeric proton of the G-reducing end residue. Hence, AlyA is expected to be acting on GM or GG dyads. The ratio of M units to G units in the degraded alginate was calculated and compared to the ratio of the M units to G units of the undegraded alginate. A decrease in the ratio was observed. Therefore M units were converted to the non-reducing Δ residue. This implies that AlyA is a GM-specific lyase that degrades alginate to fragments an average degree of polymerization (DP_n) of approximately 11. AlyB degraded alginate produced a doublet corresponding to the anomeric proton of the G-reducing end residue and a lesser intensity signal corresponding to the M-reducing end residue. Also, a decrease in ratio was observed. Therefore, M units are being converted to the non-reducing Δ residue. This implies AlyB is a GM-specific lyase with mild MM-specificity and degrades alginate to fragments with an average DP_n of approximately 4. AlyD degraded alginate produced a doublet corresponding to the anomeric proton of the G-reducing end residue and a lesser intensity signal corresponding to the M-reducing end residue. An increase in the ratio was observed, implying that it is a GG-specific lyase with mild GM-specificity. The DP_n of AlyD degraded alginate is approximately 16. The AlyE degraded alginate produced a doublet corresponding to the anomeric proton of the G-reducing end residues and an increase in the ratio is observed. This suggests AlyE is a GG-dyad specific lyase that degrades alginate to fragments with a DP_n of approximately 21.

Further, action of these enzymes on polyG- **(Figure 3.9)** and polyM-enriched alginate fractions **(Figure 3.9)** was also analyzed. AlyE showed very high degradation activity on polyG-enriched alginate fractions. Degrading activity of AlyD on this fraction also increased considerably. However, the activity of AlyA and AlyB decreased on polyG-enriched substrate. None of the enzymes showed any noticeable activity on

polyM-enriched alginate. Therefore, it leads us to conclude that lyase AlyB can be considered to be GM-specific lyase with almost negligible MM-specificity.

3.2.8 ESI-MS analysis

The ESI-MS spectrogram in the negative-ion mode of AlyA, AlyB, AlyD and AlyE degraded alginate showed *m/z* 369.4, 545.0, 721.0, 902.3 [M—H] corresponding to di-, tri-, tetra-, penta- and hexa-saccharide fragments, respectively (109) **(Figure 3.10)**. AlyE degraded alginate showed an additional peak at *m/z* 1073.1 corresponding to a septa-saccharide. Larger oligosaccharides could not be detected due to weaker signal. Though positive-ion mode was more sensitive, it is much more complex to analyze due to higher ion adduction.

3.3 Discussion

A recent study explored alginate metabolism in *V. splendidus* 12B01 (87). This study was motivated by the potential of producing fuels from alginate (4, 12). In the present study, we investigated the alginate lyases from *V. splendidus* 12B01. Four enzymes – AlyA, AlyB, AlyD, and AlyE – were purified and their properties characterized. An additional gene, *alyC*, is annotated as a putative alginate lyase; however, we did not detect any alginate lyase activity (data not shown). Unlike the other lyases, AlyC contains a PL6 domain whereas the other enzymes contain PL7 domains.

The PL7 domain contained within AlyA, AlyB, AlyD, and AlyE contains three adjacent β-strands within a structurally rigid cleft as determined through homology modeling. Through comparison to other PL7 family alginate lyases, we identified the catalytic residues within the cleft **(Figure 3.1)**. AlyB also contains a CBM32 domain; this domain has been demonstrated to bind galactose, N-acetylglucosamine, and the disaccharide N-acetyl-D-lactosamine (110). The presence of this domain within AlyB indicates the possibility of AlyB binding other carbohydrates. The CBM32 domain appears in a PL7 alginate lyase *from Zobellia* galactanivorans (104), in addition to a characterized homolog of AlyB found in *Pseduoalteromonas* sp. CY24 (111). The role

of CBM32 within these alginate lyases remains to be determined though we found that it is essential for lyase activity: no activity is observed when the CBM32 domain is deleted (results not shown).

The investigated alginate lyases were found to have an optimal pH for enzymatic activity between 7.5 and 8.5, an optimal temperature between 20 and 25 °C, and an optimal NaCl concentration of either 400 mM or 1000 mM NaCl **(Table 3.1** and **Figure 3.5)**; these conditions coincide with marine environments (105). The conditions for optimal enzymatic activity were investigated to further understand how *V. splendidus* 12B01 metabolizes alginate. Previous work sought to exploit these lyases (12), so an understanding of the activity of these lyases in different environmental situations is critical for their commercial use. The presence of signal peptides in AlyB, AlyD, and AlyE **(Figure 3.1)** indicates possible secretion. The investigation of divalent cations found that only Ca^{2+} increased enzymatic activity, while all other cations either decreased activity or had no effect **(Figure 3.5)**. The stimulatory effect of $CaCl_2$ has been shown in an alginate lyase from a *Littorina sp* sea mollusc. (112), along with a lyase from the bacterium *Azotobacter chroococcum* (38). Likely, Ca^{2+} increases activity by weakening ionic interactions between the enzyme and the alginate substrate (112).

The K_m values of other investigated alginate lyases from marine bacteria range from 6.8 µM to 6.18 mM (32, 87, 97, 101, 103, 104), however, the majority of these enzymes have K_m values less than 238 µM, in accordance with the 22 to 123 µM affinity of AlyA, AlyB, AlyD, and AlyE. AlyA, AlyB, AlyD, and AlyE were found to have turnover numbers less that those reported (32, 87, 101, 103, 104); the reported turnover numbers ranged from 0.052 to 164 s^{-1} with the majority of lyases having turnover numbers at least two-fold larger than those investigated in this study. AlyA, AlyB, AlyD, and AlyE were also found to be acting on either G-M or G-G **(Table 3.1** and **Figure 3.9)**. Alginate lyases from *Vibrio* sp. and other marine bacteria were found to have polyG, polyM, and polyMG specificity (87, 97-100, 102-104). Since full alginate utilization necessitates degradation of both G and M linkages, it is not expected that polyG, polyM, or polyMG will be over-represented in the characterized alginate lyases.

The oligoalginate lyases OalA, OalB, and OalC (lyases which degrade alginate exolytically) of *V. splendidus* 12B01 have been recently characterized (87). These lyases

were found to have different substrate specificity than the lyases investigated in this study: OalA and OalB were found to have polyM specificity, while OalC was found to have polyMG specificity. This lies in contrast with AlyA and AlyB (G-M dyad specificity) and AlyD and AlyE (G-G dyad specificity). The enzyme kinetics of OalA, OalB, and OalC were also found to be remarkably different than the lyases in this study. The K_m values were of similar magnitude, however turnover numbers were all greater for OalA, OalB, and OalC, indicating that these lyases process alginate at greater rates. Considering the substrate specificity and enzyme kinetics data, we find that each group of lyases serves a different purpose, in that each group targets different dyads for cleavage. The differing kinetic rates also indicates that exolytic and endolytic activity have different rates of degrading alginate in *V. splendidus* 12B01. This presents a picture of metabolism of alginate within *V. splendidus* 12B01 wherein AlyA, AlyB, AlyD, and AlyE slowly degrade alginate into longer chains of oligomers: as indicated by the presence of 4-, 5-, and 6-mers in the lyase degraded alginate **(Figure 3.10)**. The oligoalginate lyases are then employed to quickly degrade these smaller oligomers into the monomers needed for metabolism.

Chapter 3 Figures and Tables

Table 3.1. Optimal environmental conditions and enzyme kinetics of alginate lyases.

	AlyA	AlyB	AlyD	AlyE
pH	8.5	7.5	8.0	7.5
Temperature (°C)	25	20-25	20	25
NaCl (mM)	1000	400	400	400
K_m (μM alginate)	36 ± 7	22 ± 5	60 ± 2	123 ± 6
V_{max} (μM s^{-1})	0.13 ± 0.01	0.66 ± 0.06	0.52 ± 0.06	0.83 ± 0.02
Turnover (s^{-1})	0.60 ± 0.02	3.7 ± 0.3	4.5 ± 0.5	7.1 ± 0.2
Specificity	G-M	G-M	G-G	G-G

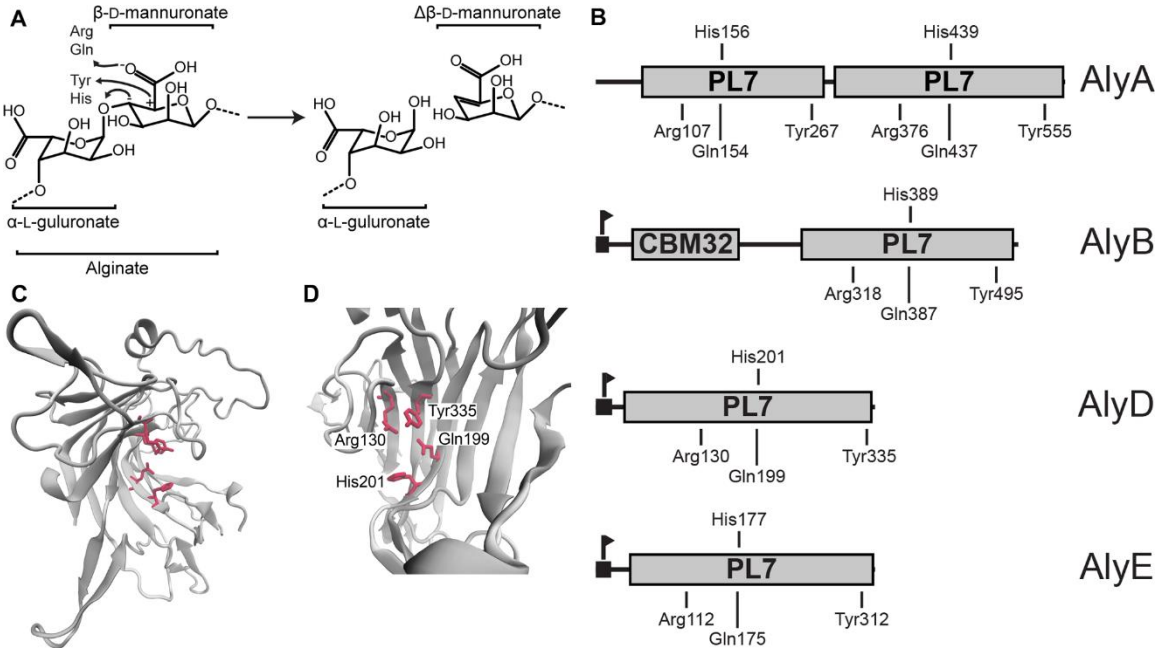

Figure 3.1. A) Mechanism for β-elimination of alginate to form α-L-guluronate and Δβ-D-mannuronate. B) Domain structure of alginate lyases AlyA, AlyB, AlyD, and AlyE, drawn to scale. The indicated amino acid residues are the hypothesized catalytic sites. PL7 is the polysaccharide lyase 7 domain and CBM32 is the carbohydrate-binding module family 32 domain. Signal peptides are indicated with a flag. C) Homology model of AlyD PL7 domain. The depicted amino acid residues are the proposed catalytic residues indicated in the PL7 domain structure. D) AlyD PL7 domain cleft. The catalytic residues lie within antiparallel, adjacent β-sheets within the cleft.

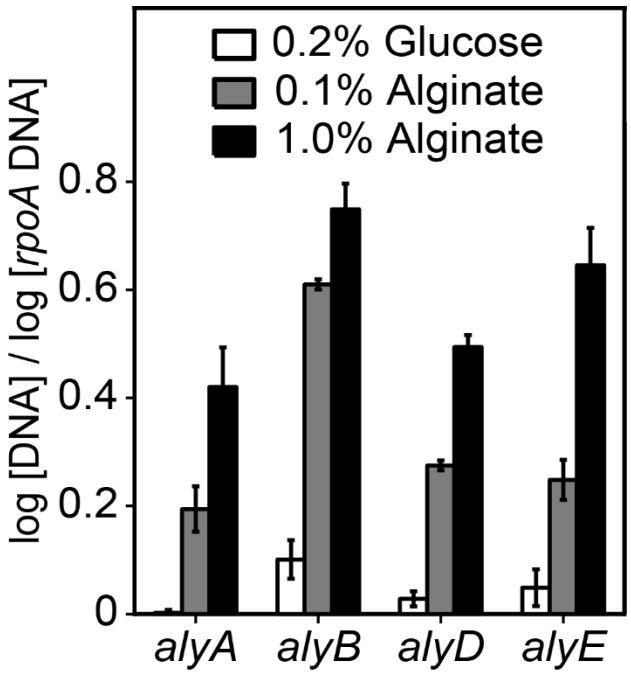

Figure 3.2. Expression of *alyA*, *alyB*, *alyD*, and *alyE* during growth on alginate or glucose. The mRNA levels of *alyA*, *alyB*, *alyD*, and *alyE* in *V. splendidus* 12B01 were determined using RT-PCR. The housekeeping gene *rpoA* was used as an internal control.

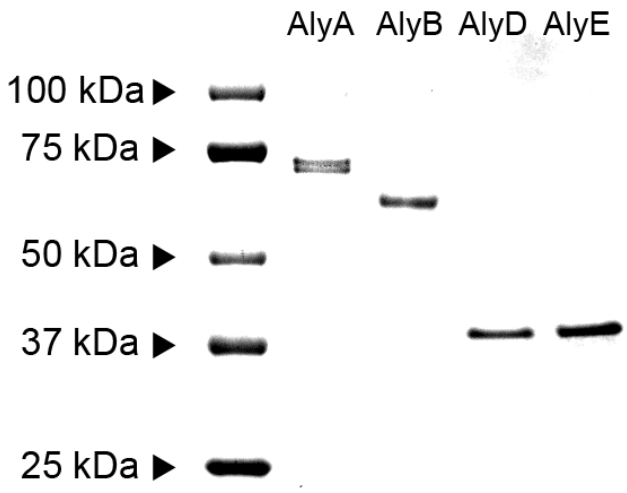

Figure 3.3. SDS-PAGE of purified AlyA, AlyB, AlyD, and AlyE. The first lane is a molecular marker with indicated molecular weights.

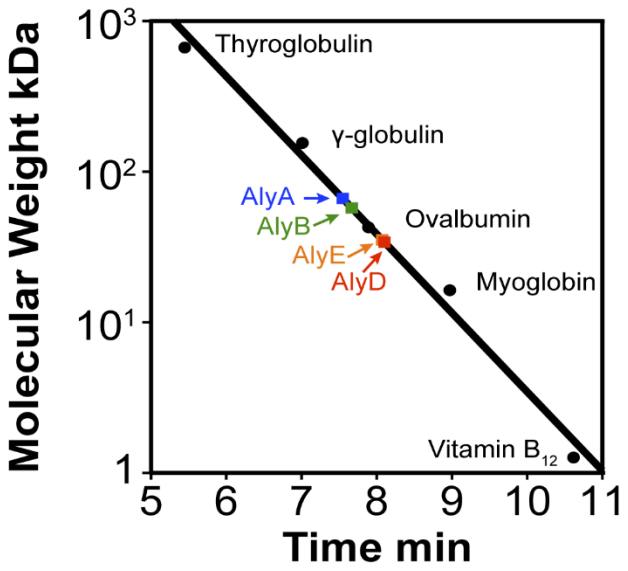

Figure 3.4. Size exclusion chromatography of native alginate lyases. The native size of AlyA, AlyB, AlyD, and AlyE was determined by comparison to standards thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B_{12} (1.35 kDa). Each protein is indicated with an arrow. AlyD and AlyE overlap due to their similar size. The standards are indicated with a solid circle.

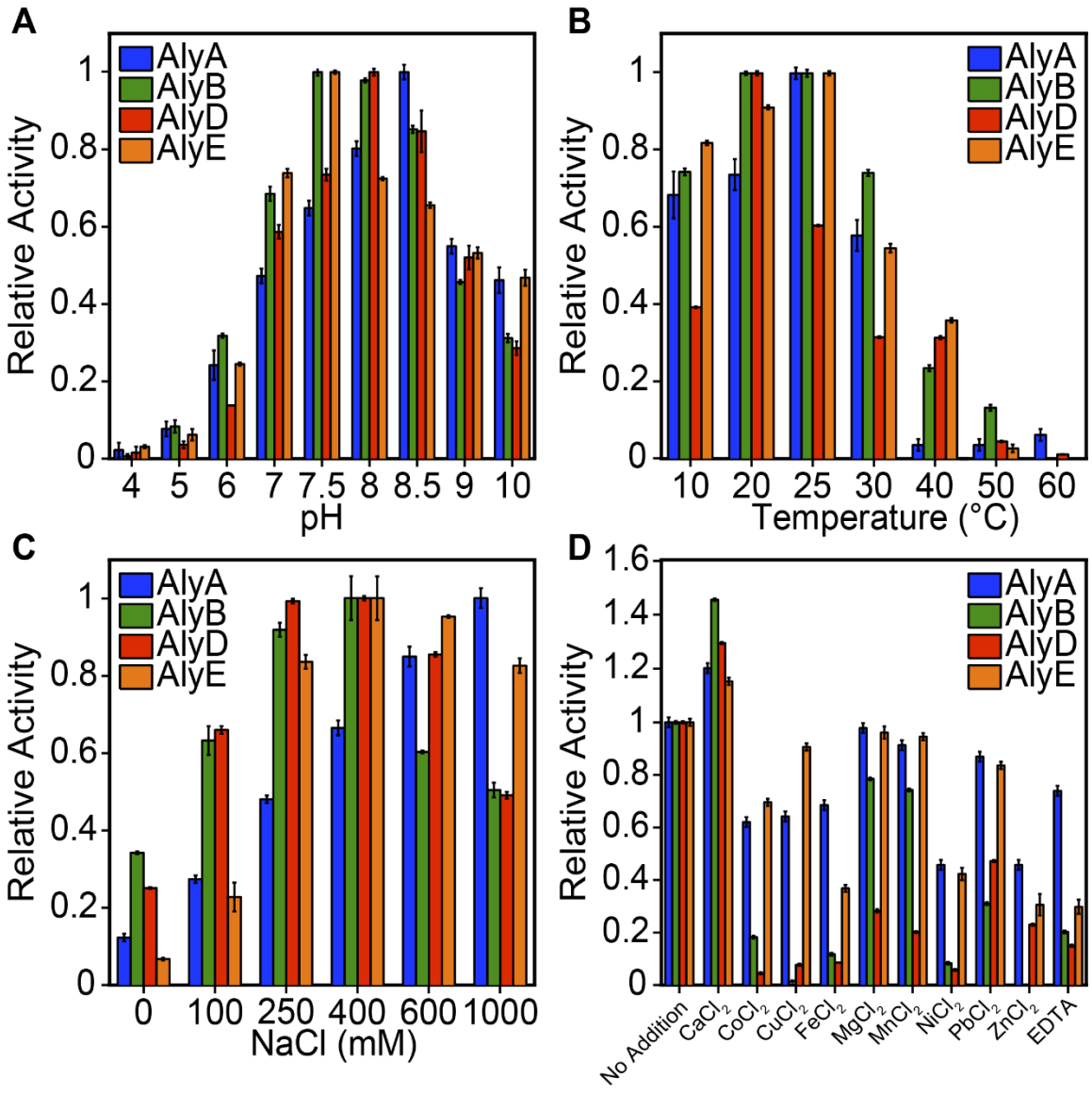

Figure 3.5. Determination of conditions for enzymatic activity. A) The optimal pH for enzymatic activity was determined in 0.05% alginate, 20 mM APT at indicated pH, and 250 mM NaCl incubated at 20 °C. Activity was normalized to the maximal pH. B) The optimal temperature for enzymatic activity was determined in 0.05% alginate, 20 mM APT at pH 7.5, and 250 mM NaCl incubated at indicated temperatures for 20 min. Activity was normalized to the optimal temperature. C) The optimal NaCl concentration for enzymatic activity was determined in 0.05% alginate, 20 mM APT at pH 7.5, and indicated NaCl concentration incubated at 20 °C for 20 min. Activity was normalized to the optimal NaCl concentration. D) The effect of divalent cations was determined in 0.05% alginate, 20 mM APT at pH 7.5, and 250 mM NaCl incubated at 20 °C for 20 min. 400 mM NaCl was used for AlyE. Divalent cations and EDTA were added at 1 mM. Activity was normalized to the no addition experiment.

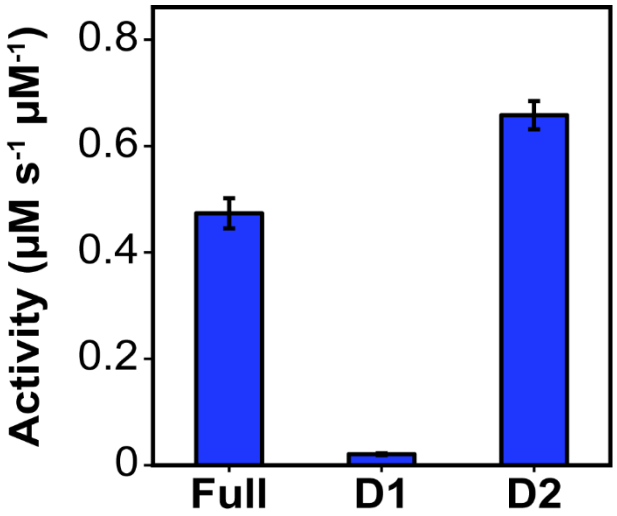

Figure 3.6. Activity of full AlyA alginate lyase and AlyA domains 1 (D1) and 2 (D2). The activity is defined as the production of 2-deoxy-D-glucose equivalent concentration per second per μM protein. The enzymatic activity of the full AlyA enzyme and each of the alginate lyase domains independently over-expressed was evaluated in 0.05% of alginate dissolved in 20 mM APT at pH 8.5 with 250 mM NaCl and incubated for 20 minutes at 20 °C. When considering the gram loading, domain 2 was found to have an almost 3-fold greater activity per μg enzyme than the full form.

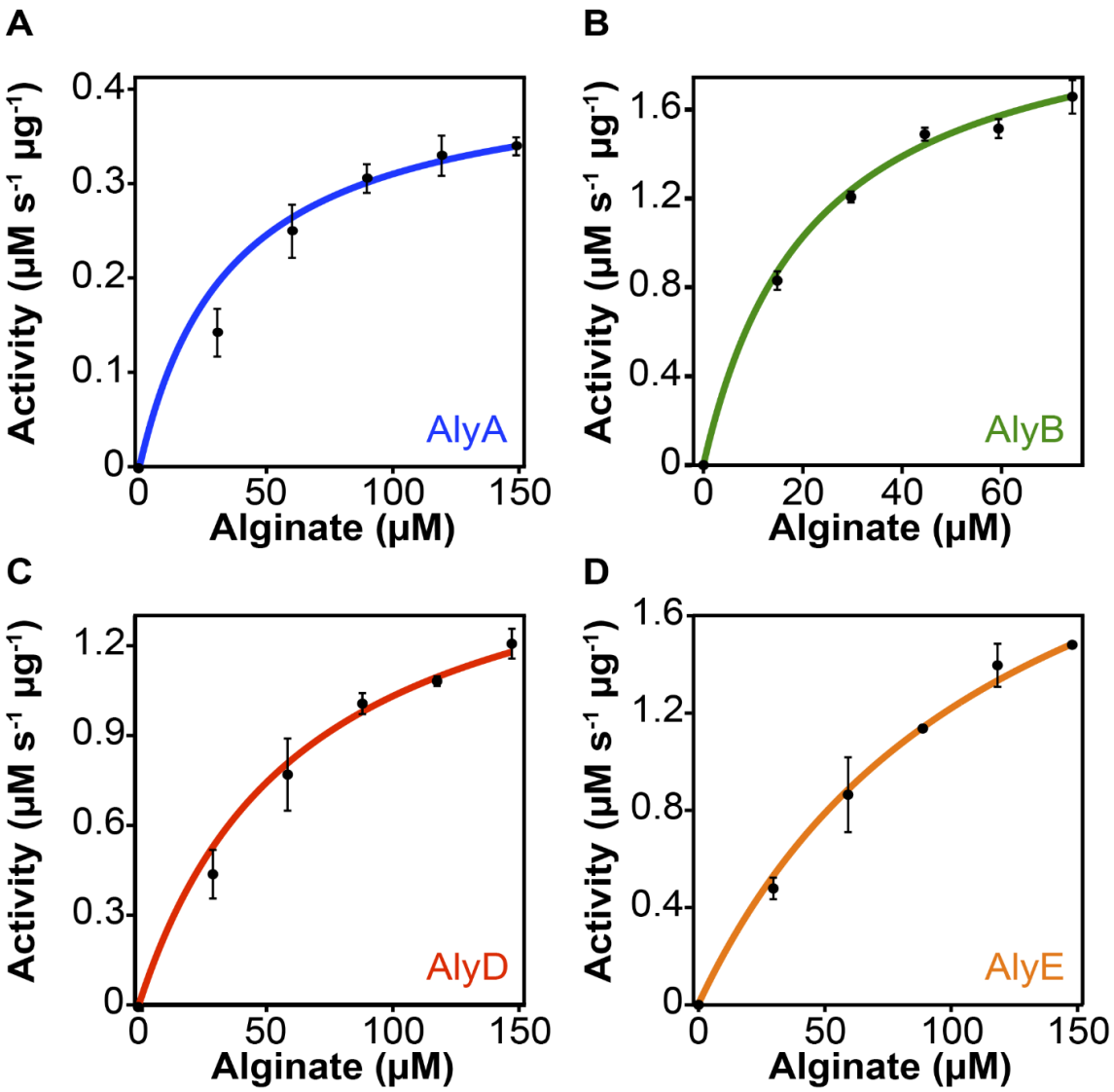

Figure 3.7. Alginate activity curves of AlyA (A), AlyB (B), AlyD (C), and AlyE (D). Experiments were performed at the optimal pH, temperature and NaCl concentration listed in Table 3 with 1 mM $CaCl_2$. The solid curves denote the fit the Michaelis-Menten equation using the parameters given in **Table 3.1**. The mixture was incubated for 20 min.

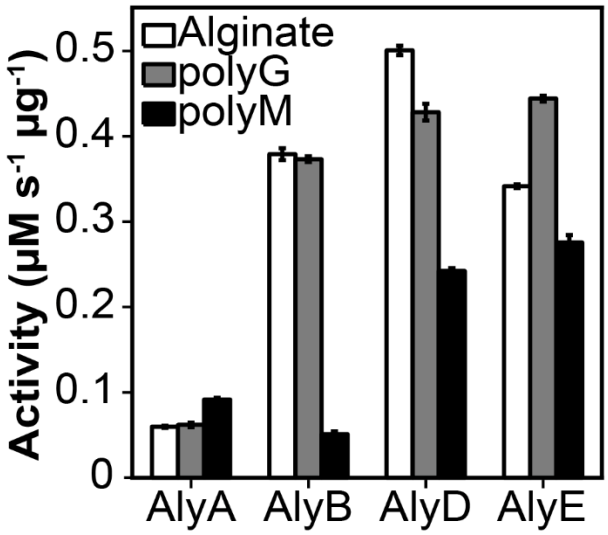

Figure 3.8. Substrate specificity of AlyA, AlyB, AlyD, and AlyE. 0.05% of alginate, polyG-enriched, and polyM-enriched was dissolved in 20 mM APT at pH 7.5 with 250 mM NaCl, incubated for 20 minutes at 20 °C, and the enzymatic activity was determined.

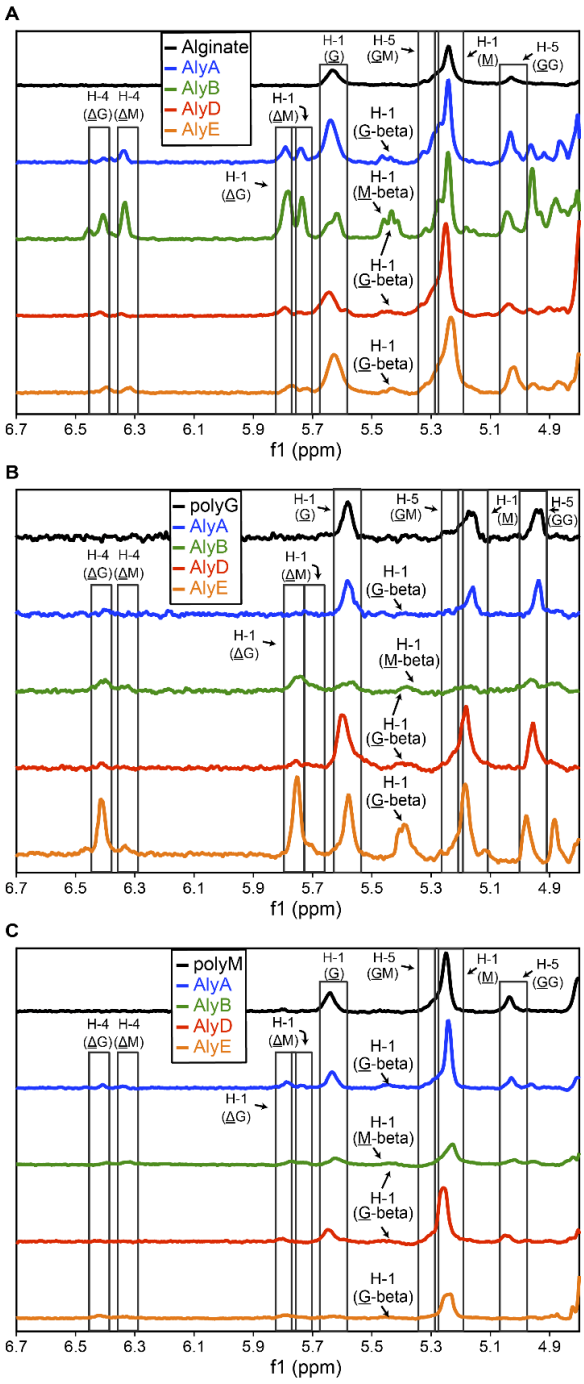

Figure 3.9. ^{1}H NMR (400-MHz) spectra of alginate and alginate derived substrates following degradation with AlyA, AlyB, AlyD, and AlyE. -G, -M denotes signals from internal G or M residues; G-beta, M-beta denotes signals from reducing G or M residues; Δ denotes signals from 4-deoxy-L-erythro-hex-4-enepyranosyluronate non-reducing end residue. Non-underlined residues refer to the neighboring residues to those generating each signal. Protons (H) are numbered to indicate which particular proton causes the signal. a) Alginate b) polyG-enriched alginate c) polyM-enriched alginate.

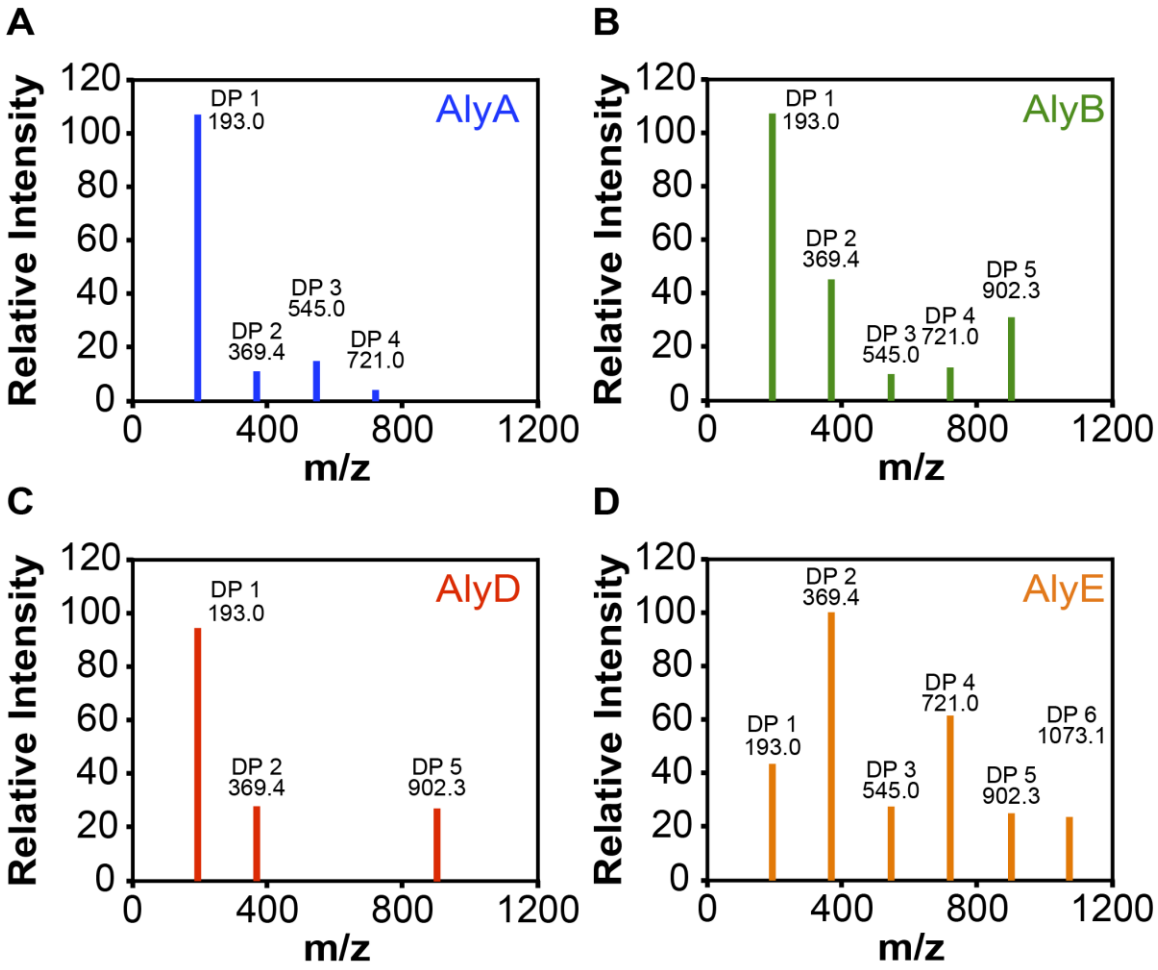

Figure 3.10. Negative-ion electrospray ionization mass spectra of alginate lyase degraded alginate. DP and the respective integers (1-6) refer to the degree of polymerization. *m/z* values are listed below the DP values.

Chapter 4 Degradation of alginate by *Vibrio splendidus* 13B01[1]

4.1 Introduction

Recent works have investigated the alginate lyases from numerous organisms. An alginate lyase from *Vibrio sp*. VKW-34 was found to have a broad tolerance to Na^+ and K^+ ions (103). Alginate lyases from *V. harveyi* AL-128 and *V. alginolyticus* ATCC 17749 were purified and found to have specificity toward polyG and polyM, respectively (98). Two alginate lyases from *Vibrio sp.* O2 were identified via restriction mapping and plate screening and these lyases were found to have polyM specificity (99). An alginate lyase from *Stenotrophomas maltophilia* KJ-2 was purified and found to have optimal activity at pH 8, 30-40 °C, and 1 mM $CaCl_2$. This lyase was found to have polyMG specificity (113). The alginate lyase AlyV5 from *Vibrio sp*. QY105 was isolated and found to have optimal activity at 38 °C, pH 7, 500 mM NaCl, and 1 mM $CaCl_2$. The lyase was found to have polyG specificity (102). Computational modeling was accomplished to understand the binding of the trimer GGG to the alginate lyase AlyVI from *Vibrio sp.* QY105 (101). The study predicted residues within AlyVI that are involved in the stability of the enzyme and the binding of GGG to AlyVI. The L224V/D226G double mutant was predicted to stabilize AlyVI and was then experimentally found to increase k_{cat} more than two-fold.

A recent study has sought to characterize the alginate lyases from *V. splendidus* 12B01 (114). This strain was found to contain four alginate lyases: AlyA, AlyB, AlyD, and AlyE. The enzymes had optimal activity between pH 7.5 and 8.5 and at 20 to 25 °C. Additionally, the lyases were found to act on the G-G or G-M dyad and act endolytically. When comparing growth on alginate to that on glucose as the carbon source, *V. splendidus* 12B01 was found to induce expression of its alginate lyases upon growth on alginate indicating conditional expression of alginate lyases in response to extracellular signals.

[1]NMR and ESI-MS analysis were performed by Geethika Yalamanchili. Enzyme over-expression and *in-vitro* enzyme assays were performed by Matt Plutz. Alginate lyase bioinformatics and secretion experiments were performed by Jan-Hendrik Hehemann.

In this work, we have investigated the marine bacterium *V. splendidus* 13B01. We found that *V. splendidus* 13B01 grows better on alginate than *V. splendidus* 12B01. In order to determine how 13B01 utilizes alginate better than 12B01, we cloned, purified, and characterized a PL6 alginate lyase and five PL7 alginate lyases from *V. splendidus* 13B01. We found that these enzymes are most active between pH 7.5 and 10.0 and at 20-25 °C, consistent with their marine environment (105). The enzymes were found to have enzyme kinetics in agreement with their 12B01 counterparts. We also determined the specificity of each enzyme, and found G-G, M-M, G-M, and M-G dyad specificity. All of the tested lyases were found to be endolytic. Through this work, we identified that *V. splendidus* 13B01 utilizes alginate at faster rates than *V. splendidus* 12B01 due to higher expression and secretion of its alginate lyases and the presence of wider substrate specificity in its alginate lyases.

4.2 Results

4.2.1 *V. splendidus* 12B01 and 13B01 growth rates and secretion

In order to determine how well *V. splendidus* 12B01 and 13B01 utilize alginate, we conducted growth rate experiments of each strain in minimal media supplemented with 0.2% glucose or 1% alginate **(Figure 4.1)**. We found that 12B01 had a growth rate on glucose and alginate of 0.36 ± 0.05 h^{-1} and 0.15 ± 0.01 h^{-1}, respectively, while 13B01 had a growth rate on glucose and alginate of 0.43 ± 0.01 h^{-1} and 0.24 ± 0.02 h^{-1}, respectively. These results show that 13B01 grows 60% faster than 12B01 on alginate as the sole carbon source. There was a more modest (20%) increase in growth rate when 13B01 was grown on glucose in comparison to 12B01.

Further comparison of the secretion of *V. splendidus* 12B01 and 13B01 was made by spotting each bacterium on soft agar medium containing alginate. The secretion of alginate lyases was then inferred by determination of the activity of the secreted alginate lyases. Controlling for cellular amounts, we found that the *V. splendidus* 13B01 secreted lyases had more than seven-fold more activity than the *V. splendidus* 12B01 alginate lyases **(Figure 4.1)**.

4.2.2 *V. splendidus* 13B01 contains six alginate lyases

Via sequence comparison to the recently characterized alginate lyases with *V. splendidus* 12B01 (114), six alginate lyases were identified within the genome of *V. splendidus* 13B01. Four of these lyases correspond to lyases characterized within 12B01: AlyA, AlyB, AlyD, and AlyE. The remaining two lyases are unique to 13B01 and are named AlyF and AlyG. AlyB, AlyD, AlyE, and AlyG contain one polysaccharide lyase family 7 (PL7) alginate lyase domain, while AlyA contains two PL7 domains (30, 31). AlyF contains a polysaccharide lyase family 6 (PL6) alginate lyase domain (31, 115). AlyB and AlyG contain a carbohydrate-binding module family 32 domain (CBM32) in addition to their PL7 domains.

Catalytic sites were identified by sequence comparison to PL6 and PL7 domains **(Figure 4.2)**. PL6 containing alginate lyases employ a Ca^{2+}-dependent mechanism that uses Ca^{2+}, asparagine, and glutamine to neutralize the negative charge on the carboxyl group. Lysine is then used to remove the C-5 proton, while arginine donates a proton to cleave the 1,4-linkage. A double bond is then formed (115). PL7 containing alginate lyases utilize arginine and glutamine to neutralize the carboxyl group negative charge. Histidine then removes the C-5 carbon, while tyrosine donates a proton to the cleave the 1,4-linkage. A double bond is then formed (89, 106, 108).

In order to understand how the 13B01 alginate lyases relate to the corresponding 12B01 lyases, we conducted an alignment **(Figure 4.3)** and then constructed a phylogenic tree of the 12B01 and 13B01 alginate lyases **(Figure 4.2)**. From the phylogenetic tree, the 12B01 and 13B01 alginate lyase counterparts are closely related to one another. The unique to 13B01 alginate lyase, AlyG, was found to be most similar to the AlyB. AlyA contains two alginate lyase domains, and both of these domains were found to be different than AlyB, AlyD, AlyE, and AlyG.

4.2.3 Expression of alginate lyases

In order to determine the effect of alginate on the expression of the six putative alginate lyases, we measured the expression of each lyase using quantitative PCR. To this end, we grew cells in M9 minimal salt medium supplemented with 0.2% glucose, 0.1% (wt/wt) alginate, and 1.0% (wt/wt) alginate. As shown in **Figure 4.4**, growth on alginate strongly induced expression of all six alginate lyase genes in comparison to growth on glucose. Specifically, growth on either alginate concentration induced expression of all lyases more than two-fold with *algG* being induced more than ten-fold. Interestingly, growth on 0.1% and 1.0% alginate yielded no discernable difference in gene expression for any of the six lyases. These results demonstrate that the alginate lyases are not constitutively expressed, but rather are conditionally expressed in response to alginate. Additionally, these results suggest that the lyases may be employed at low alginate concentrations since there was no change in expression between 0.1% and 1.0% alginate.

4.2.4 Purification of alginate lyases

AlyA, AlyB, AlyD, AlyE, AlyF, and AlyG were cloned with a C-terminal 6xHis-tag and expressed in *E. coli* from a T7 promoter induced by IPTG. AlyF was purified under native conditions, while the other lyases were purified under denaturing conditions and re-folded to yield a function enzyme **(Figure 4.5)**. The molecular weights of the native proteins were determined using size exclusion chromatography. Each protein was found to exist as a monomer **(Figure 4.6)**. *alyA*, *alyB*, *alyD*, *alyE*, *alyF*, and *alyG* encode a polypeptide of 580, 522, 345, 345, 538, and 464 amino acids with a predicted molecular mass of 65.1 kDa, 56.8 kDa, 38.2 kDa, 38.2 kDa, 59.2 kDa, and 50.8 kDa, respectively. The denatured molecular masses of AlyA, AlyB, AlyD, AlyE, AlyF, and AlyG were approximately 70 kDa, 60 kDa, 37 kDa, 37 kDa, 60 kDa, and 50 kDa, respectively, as determined by SDS-PAGE **(Figure 4.5)**. We determined the native molecular weights of each protein through size exclusion chromatography on a Bio-Sil SEC-250 column. AlyA, AlyB, AlyF, and AlyG were eluted as a symmetrical peak between ovalbumin and

γ-globulin, while AlyD and AlyE were eluted as symmetrical peak between myoglobin and ovalbumin. AlyA, AlyB, AlyD, AlyE, AlyF, and AlyG were determined to have native molecular masses of 70.7 kDa, 56.9 kDa, 36.0 kDa, 35.6 kDa, 61.9 kDa, and 54.3 kDa, respectively **(Figure 4.6)**. These results indicate that each enzyme migrates as a monomer in gel filtration and are active as a monomer in solution.

4.2.5 Determination of optimal enzymatic conditions

In order to determine the optimal conditions for each lyase, a universal buffer was formulated that allowed for a wide range of pH conditions to be tested. The optimal pH, temperature, and NaCl concentrations for each lyase are listed in **Table 4.1** and the associated data in **Figure 4.7**. The optimal pH for the lyases lies between 7.5 and 10, the optimal temperature was found to be between 20 and 25 °C, and the optimal NaCl concentration lied between 250 and 1000 mM.

In order to determine if divalent cations are necessary for enzymatic function, each lyase was stripped for divalent cations using Chelex 100 resin. Then, divalent cations were individually added to the reaction mixture at 1 mM. $CaCl_2$ was found to increase the enzymatic activity for AlyD and AlyG, while all other cations were found to either decrease or not change activity **(Figure 4.7)**.

4.2.6 Activity of AlyA PL7 domains 1 and 2

In order to determine the enzymatic function of each of the two AlyA PL7 domains, we over-expressed each domain separately with a C-terminal 6xHis-tag and then purified each domain. Then, we determined the enzymatic activity of each domain to degrade alginate. We found that domain 2 of AlyA had 85% activity in comparison to the full AlyA enzyme, while domain 1 of AlyA had no enzymatic activity **(Figure 4.8)**.

4.2.7 Determination of enzyme kinetics

Knowing the optimal pH and NaCl concentrations for each lyase, we determined the kinetic parameters for each lyase by dosing alginate under optimal enzymatic conditions. All six alginate lyases exhibited Michelis-Menten type kinetics **(Figure 4.9)**. The determined kinetic parameters are provided in **Table 4.1**. We found that the K_m parameter varied more than seven-fold, while the maximal velocity (V_{max}) varied almost 30-fold when comparing the six alginate lyases. AlyF was found to have poor activity compared to the other lyases. Comparing AlyA, AlyB, AlyD, AlyE, and AlyG, we found a three-fold range of K_m and a seven-fold range of V_{max}.

4.2.8 Determination of substrate specificity

Substrate specificity was determined using ^{1}H NMR **(Figure 4.10)**. ^{1}H NMR of AlyA degraded alginate produced a doublet corresponding to the anomeric proton of the G-reducing end residue. Hence, AlyA is expected to be acting on GM or GG dyads. The ratio of M units to G units in the degraded alginate was calculated and compared to the ratio of the M units to G units of undegraded alginate. A decrease in the ratio was observed. Therefore, M units were converted to the non-reducing Δ residue. This implies that AlyA is a GM-specific lyase that degrades alginate to a degree of polymerization (DP_n) of approximately 17. AlyB degraded alginate produced a doublet corresponding to the anomeric proton of the G-reducing end residue and a lesser intensity signal corresponding to the M-reducing end residue. Also, a decrease in ratio was observed. Therefore, M units are being converted to the non-reducing Δ residue. This implies AlyB is a GM-specific lyase with mild MM-specificity and degrades alginate to a DP_n of approximately 3. AlyD degraded alginate produced a singlet corresponding to the anomeric proton of the M-reducing end residue and a lesser intensity doublet signal corresponding to the G-reducing end residue. A decrease in the ratio was observed, implying a MM-specific lyase with mild GM-specificity. The DP_n of AlyD degraded alginate is approximately 6. The AlyE degraded alginate produced a doublet corresponding to the anomeric proton of the G-reducing end residues and a very low

intensity signal corresponding to the M-reducing end residues. An increase in the ratio was observed. This suggests AlyE is a GG-dyad specific with very low MG-dyad specificity. The lyase degrades alginate to a DP_n of approximately 6. AlyF degraded alginate produced a doublet corresponding to the anomeric proton of the G-reducing end residues and a decrease in the ratio is observed which implies that it is a GM-dyad specific lyase which degrades alginate to a DP_n of approximately 45. AlyG degraded alginate produced a singlet corresponding to the M-reducing end residue and a lesser intensity doublet signal corresponding to the G-reducing end residue. An increase in the ratio is observed. This suggests that it is a MG-dyad specific lyase with degree of breakdown (DP_n) approximately 8.

Further, action of these enzymes on polyG- **(Figure 4.10)** and polyM-enriched alginate fractions **(Figure 4.10)** was also analyzed. AlyE showed very high degradation activity on polyG-enriched alginate fractions. Degrading activity of AlyD on this fraction also increased considerably. However, the activity of AlyA and AlyB decreased on polyG-enriched substrate. None of the enzymes showed any noticeable activity on polyM-enriched alginate. Therefore, it leads us to conclude that lyase AlyB can be considered to be GM-specific lyase with almost negligible MM-specificity.

4.2.9 ESI-MS analysis

The ESI-MS spectrogram in the negative-ion mode of AlyB, AlyD, AlyE, AlyF and AlyG degraded alginate showed *m/z* 369.4, 545.0, 721.0, 902.3, and 1073.1 [M—H] corresponding to di-, tri-, tetra-, penta-, hexa, and septa-saccharide fragments, respectively (109) **(Figure 4.11)**. AlyA degraded alginate showed peaks at *m/z* 369.4 corresponding to a di-saccharide. Larger oligosaccharides could not be detected due to a weaker signal. Though positive-ion mode was more sensitive, it is much more complex to analyze due to higher ion adduction.

4.2.10 AlyG contributes significantly to the secreted alginate lyase activity

As AlyG is a unique enzyme to 13B01 and it shows high alginate degradation activity, we hypothesized that this enzyme is responsible for the high alginate degradation activity of 13B01 **(Figure 4.1)**. To this end, we built a knockout vector for *alyG*, pAlyG KO, which contains the 1kb regions upstream and downstream *alyG* fused together in the suicide vector pJC4. When conjugated from *E. coli* to *V. splendidus* 13B01, this vector will singly recombine with either region upstream or downstream *alyG*, resulting in the integrated vector **(Figure 4.12)**. Following growth on a medium without chloramphenicol for selection, we selected for cells that have naturally lost the integrated vector through recombination of either duplicate upstream or downstream region. We then found a knockout of *alyG*.

We then compared the secreted alginate lyase activity of 13B01 and Δ*alyG* **(Figure 4.13)**. We found that 13B01 had more than eight-fold greater secreted alginate lyase activity than Δ*alyG*.

4.3 Discussion

Recent work on the alginate metabolism in *V. splendidus* (87, 114) has demonstrated how alginate lyases can be utilized in production of fuels from macroalgae feed stocks (4, 12). In this present study, we characterized the alginate lyases from a strain of *V. splendidus* (13B01) that has faster growth when grown on alginate than the previously investigated 12B01. Additionally, 13B01 was found to have higher secreted alginate lyases activity than 12B01. We characterized six alginate lyases found within *V. splendidus* 13B01: AlyA, AlyB, AlyD, AlyE, AlyF, AlyG. AlyF and AlyG are unique to 13B01.

AlyA, AlyB, AlyD, AlyE, and AlyG contain at least one PL7 domain, while AlyF was found to have a PL6 domain. Through a comparison to other domains, we were able to identify the catalytic residues within each enzyme **(Figure 4.1)**. AlyB and AlyG were also found to contain a CBM32 domain, in addition to their PL7 domains. The CBM32 domain has been shown to bind N-acetylglucosamine and galactose, along with the

disaccharide N-acetyl-D-lactosamine (110). We found that the CBM32 domain essential of enzymatic activity; the PL7 domains of AlyB and AlyG were found to have no activity (results not shown).

The 13B01 lyases that were investigated were found to have an optimal pH for enzymatic activity between 7.5 and 10.0, an optimal temperature between 20 and 25 °C, and an optimal NaCl concentration between 250 and 1000 mM NaCl. These optimal conditions coincide with marine environments (105). These conditions also overlap with the optimal enzymatic conditions of *V. splendidus* 12B01 alginate lyases (114). We also found that Ca^{2+} increased the enzymatic activity of AlyD and AlyG. A similar effect was found with the 12B01 alginate lyases. Ca^{2+} has been previously implicated in stimulating enzymatic activity (38, 112) by weakening ionic interactions between alginate and alginate lyase. We did not find a stimulatory effect for Ca^{2+} on AlyF, but rather an approximately 20% decrease in activity. AlyF contains a PL6 domain which is expected to require Ca^{2+} for activity.

In comparing AlyA, AlyB, AlyD, and AlyE amplified from 12B01 and 13B01, we found general agreement between optimal environmental conditions and kinetic parameters. Interestingly, the 13B01 AlyD is 100% identical to the 12B01 AlyD on the amino acid level, however, we found G-G specificity in the 12B01 version and M-M specificity in the 13B01 version. One possible cause for this discrepancy is due to the location of the 6xHis-tag: the 12B01 AlyD has an N-terminal 6xHis-tag, while the 13B01 AlyD has a C-terminal 6xHis-tag. The dyad specificity of the 13B01 AlyA, AlyD, and AlyE is identical to their 12B01 versions, despite the differing location of their 6xHis-tags.

Previous investigations reported turnover numbers for alginate and oligoalginate lyases that ranged from 0.052 to 164 s^{-1} (32, 87, 101, 103, 104). Most of these values are of similar value to 100-fold larger than the alginate lyases investigated in this study. The most active of the 13B01 lyases - AlyB, AlyD, AlyE, and AlyG - had turnover numbers ranging from 5.2 to 18 s^{-1} compared to a turnover number of 56.9 s^{-1} from Alg17c from *Saccharophagus degredans* 2-40 (32), a turnover number of 11.3 s^{-1} from an unnamed lyase from *Vibrio sp.* TKW-34 (103), and a turnover number ranging from 12.66 to 19.51 s^{-1} from AlyA1 from *Zobellia galactanivorans* (104). In comparing previously reported

K_m values, the *V. splendidus* 13B01 lyases were determined to have K_m values between 40 and 300 µm alginate. Previous investigations have found alginate lyase K_m values between 6.8 µM and 6.2 mM alginate (32, 87, 97, 101, 103, 104). The majority of the reported values lie between 6.8 µM and 240 µM. Previous investigations of alginate lyases have identified enzymes with polyM (87, 98, 99), polyG (97, 98, 100, 102, 104), and polyMG (87, 103) specificity. The enzymes investigated in this study have G-G, M-M, G-M, and M-G specificity, corresponding to polyG, polyM, polyMG, and polyMG, respectively. Most previously investigated alginate lyases were found to have polyM or polyG specificity; *V. splendidus* 13B01 was found to have four alginate lyases with either G-M or M-G specificity.

Since the alginate lyases common to both 12B01 and 13B01 have largely the same optimal conditions and kinetic parameters, we cannot attribute the differences in growth rate under growth on alginate to the actions of AlyA, AlyB, AlyD, AlyE, AlyF, and AlyG alone. We do find a larger variation of substrate specificities in the 13B01 alginate lyases in comparison to the 12B01 lyases: the 12B01 alginate lyases had either G-G or G-M specificity, while the 13B01 lyases had G-M, M-M, G-G, or M-G specificity. The full complement of dyad specificity is not required for alginate metabolism, as evidenced by *V. splendidus* 12B01 growing on alginate despite only having G-G and G-M specific lyases. It is expected that having alginate lyases that degrade all possible G and M interlinkages would allow for more complete degradation of alginate in industrial uses. Additionally, synergy between 13B01 alginate lyases is expected to be greater than the 12B01 lyases due to broader dyad specificity. This, in turn, would lead to fast degradation and incorporation of alginate.

Another possible means by which 13B01 metabolizes alginate better than 12B01 is at the gene expression level. We found that the 13B01 alginate lyases had a larger induction in the presence of alginate than the 12B01 alginate lyases. Additionally, the 13B01 alginate lyases were more sensitive to alginate than the 12B01 alginate lyases. We found maximal alginate lyase expression at 0.1% (wt/wt) alginate in 13B01, while maximal expression was found at 1.0% (wt/wt) alginate in 12B01 (114). These findings allow us to conclude that 13B01 expresses alginate lyases at a higher level than 12B01 in the presence of alginate. 13B01 must possess a metabolic pathway and regulation

network suited toward higher metabolic consumption of alginate, thus providing an attractive target for future genome mining and metabolic engineering. AlyG was found to have the largest induction on alginate growth conditions of the 13B01 alginate lyases. This advantageous regulation pairs well with its fast degradation of alginate at high alginate loadings. When we knocked out *alyG* we did indeed more than 4-fold lower secreted alginate lyase activity than the 13B01 wild-type. This further demonstrates the important role that AlyG plays in the metabolism of alginate by 13B01, and can explain the differences in secretion and growth rates found between 12B01 and 13B01.

Our results demonstrate that the alginate lyases from *V. splendidus* provide an attractive framework for the industrial degradation of macroalgae. Both *V. splendidus* 12B01 and 13B01 contain alginate lyases that allow for the degradation of alginate, however 13B01 contains alginate lyases with wider substrate specificity and a unique alginate lyase, AlyG, with high maximal velocity. Additionally, 13B01 was found to express and secrete its alginate lyase at higher levels than 12B01, so 13B01 serves as an attractive target for future engineering.

Chapter 4 Figures and Tables

Table 4.1. Optimal environmental conditions and enzymatic kinetics of studied alginate lyases. The data and curve fits used to determine the 13B01 lyase kinetic parameters are found in **Figure 4.9**. The 12B01 lyase optimal environmental conditions, kinetic parameters, and specificity were previously determined (114).

		12B01	13B01
AlyA	pH	8.5	8.5
	Temperature (°C)	25	20
	NaCl (mM)	1000	750
	K_m (μM alginate)	36 ± 7	90 ± 30
	V_{max} (μM s^{-1})	0.13 ± 0.01	0.34 ± 0.02
	Turnover (s^{-1})	0.60 ± 0.02	1.9 ± 0.1
	Specificity	G-M	G-M
AlyB	pH	7.5	10.0
	Temperature (°C)	20-25	25
	NaCl (mM)	400	500
	K_m (μM alginate)	22 ± 5	150 ± 30
	V_{max} (μM s^{-1})	0.66 ± 0.06	0.76 ± 0.05
	Turnover (s^{-1})	3.7 ± 0.3	5.2 ± 0.3
	Specificity	G-M	G-M
AlyD	pH	8.0	8.0
	Temperature (°C)	20	20
	NaCl (mM)	400	500
	K_m (μM alginate)	60 ± 2	150 ± 30
	V_{max} (μM s^{-1})	0.52 ± 0.06	1.05 ± 0.04
	Turnover (s^{-1})	4.5 ± 0.5	11.2 ± 0.4
	Specificity	G-G	M-M (mild G-M)
AlyE	pH	7.5	10.0
	Temperature (°C)	25	20
	NaCl (mM)	400	1000
	K_m (μM alginate)	123 ± 6	170 ± 30
	V_{max} (μM s^{-1})	0.83 ± 0.02	1.50 ± 0.07
	Turnover (s^{-1})	7.1 ± 0.2	15.8 ± 0.7
	Specificity	G-G	G-G
AlyF	pH	No Homolog	8.5
	Temperature (°C)		20
	NaCl (mM)		250
	K_m (μM alginate)		40 ± 6
	V_{max} (μM s^{-1})		0.090 ± 0.002
	Turnover (s^{-1})		0.6 ± 0.1
	Specificity		G-M

Table 4.1 (cont.)

AlyG	pH	No Homolog	7.5
	Temperature (°C)		25
	NaCl (mM)		500
	K_m (μM alginate)		300 ± 30
	V_{max} (μM s^{-1})		2.6 ± 0.1
	Turnover (s^{-1})		18.0 ± 0.7
	Specificity		M-G

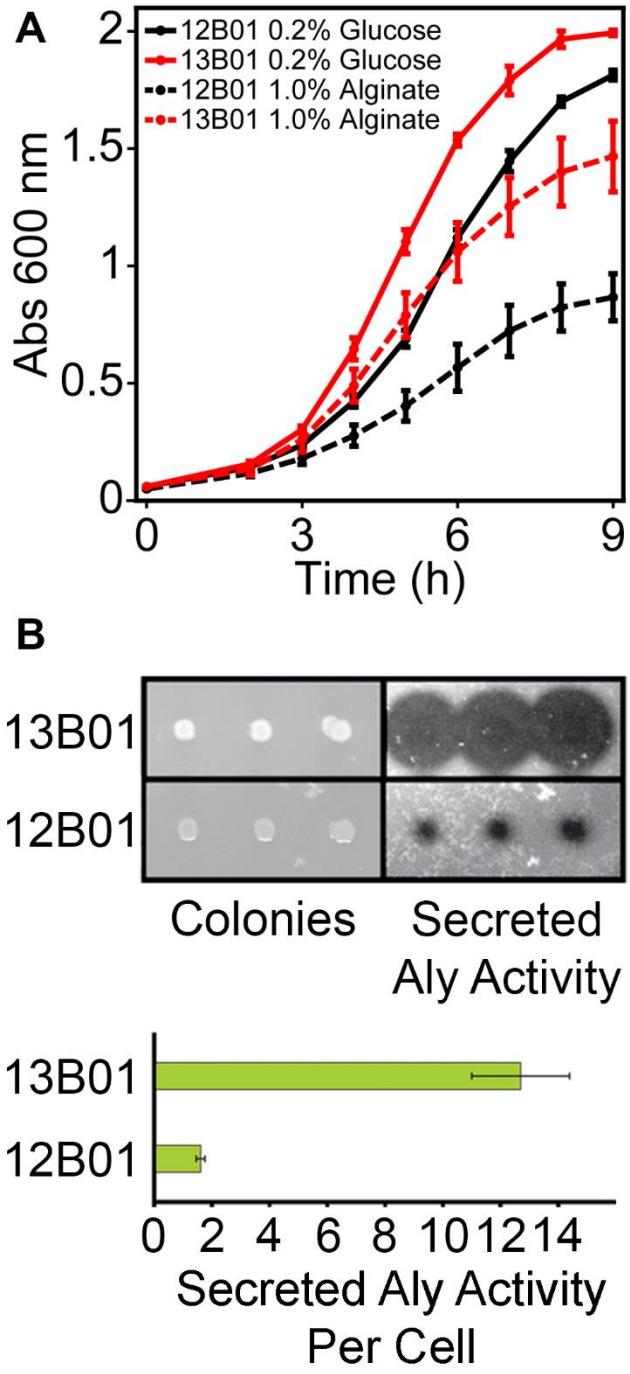

Figure 4.1. A) Time course of *V. splendidus* 12B01 and 13B01 when grown on 0.2% glucose and 1.0% alginate. Each strain was subcultured to 0.05 Abs 600 nm and growth was monitored. Presented data are averages and standard deviations of three replicates. B) Secreted enzymes of *V. splendidus* 12B01 and 13B01. In the upper portion, *V. splendidus* 12B01 and 13B01 were spotted on solid media and their growth was monitored. The activity of secreted alginate lyases (Aly) were then determined. The area of the secreted alginate lyase activity halo was then divided by the colony size to determine the secreted alginate lyase activity per cell.

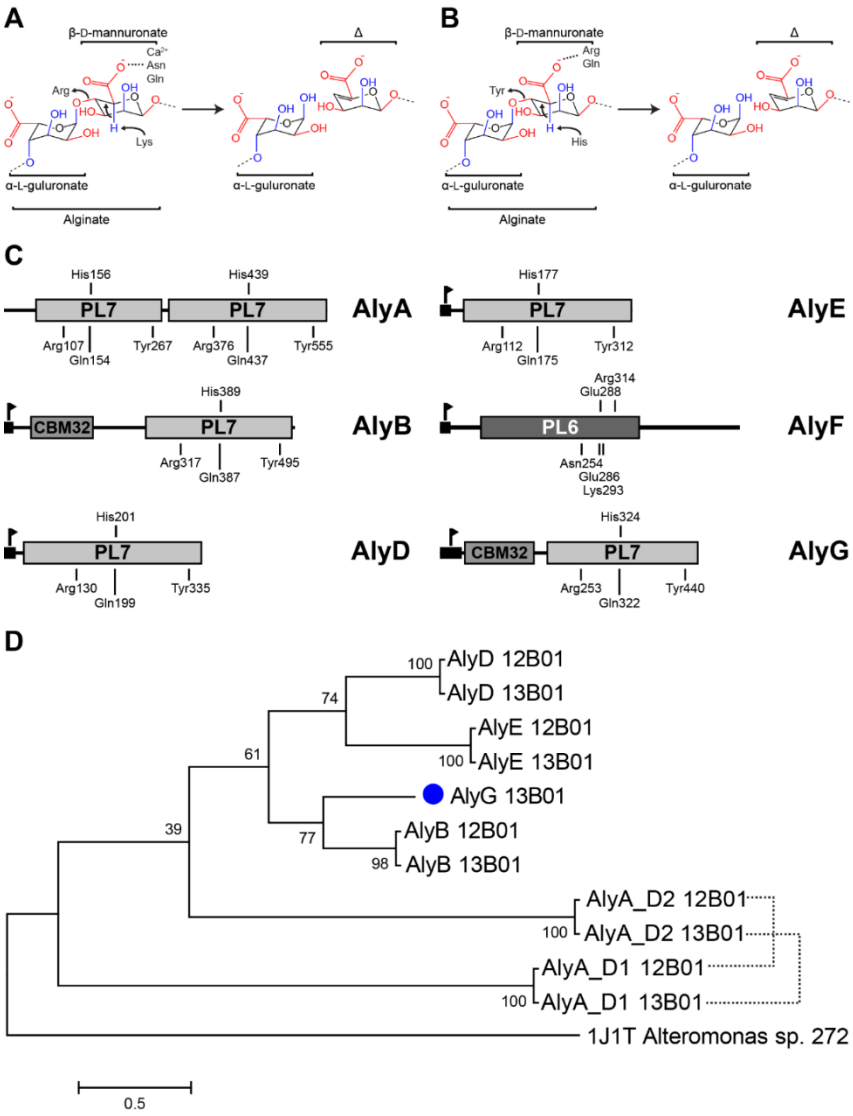

Figure 4.2. A) PL6 mechanism for β-elimination of alginate to form α-L-guluronate and Δβ-D-mannuronate. B) PL7 mechanism for β-elimination of alginate to form α-L-guluronate and Δβ-D-mannuronate. C) Domain structure of alginate lyases AlyA, AlyB, AlyD, AlyE, AlyF, and AlyG, drawn to scale. The indicated amino acid residues are the hypothesized catalytic sites. PL6 is the polysaccharide lyases 6 domain, PL7 is the polysaccharide lyase 7 domain, and CBM32 is the carbohydrate-binding module family 32 domain. Signal peptides are indicated with a flag. D) Phylogenetic tree of relatedness of PL7 alginate lyases. The indicated values at each branch are bootstrap values. The unique 13B01 PL7 alginate lyase is AlyG and is indicated by a blue circle. AlyA_D1 12B01 and AlyA_D2 12B01 indicate the first and second PL7 domains of AlyA from *V. splendidus* 12B01, respectively. AlyA_D1 13B01 and Aly_D2 13B01 indicate the first and second PL7 domains of AlyA from *V. splendidus* 13B01, respectively. The dotted lines connecting AlyA_D1 and AlyA_D2 indicate that each domain belongs to the whole of AlyA.

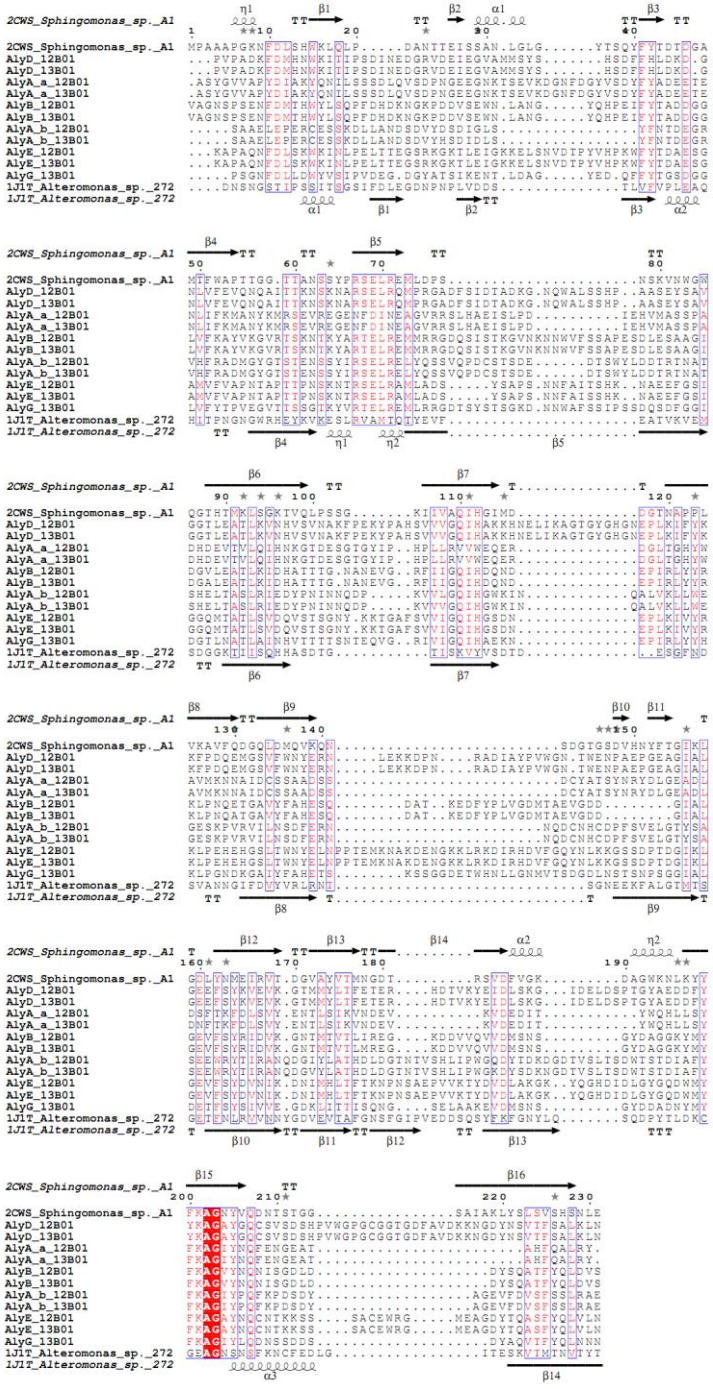

Figure 4.3. Alignment of *V. splendidus* 12B01 and 13B01 PL7 alginate lyases. The 12B01 PL7 lyases AlyA, AlyB, AlyD, and AlyE and the 13B01 PL7 lyases AlyA, AlyB, AlyD, AlyE, and AlyG were aligned to the PL7 alginate lyases from *Sphingomonas sp.* A1 (2CWS) and *Alteromonas sp.* 272 (1J1T).

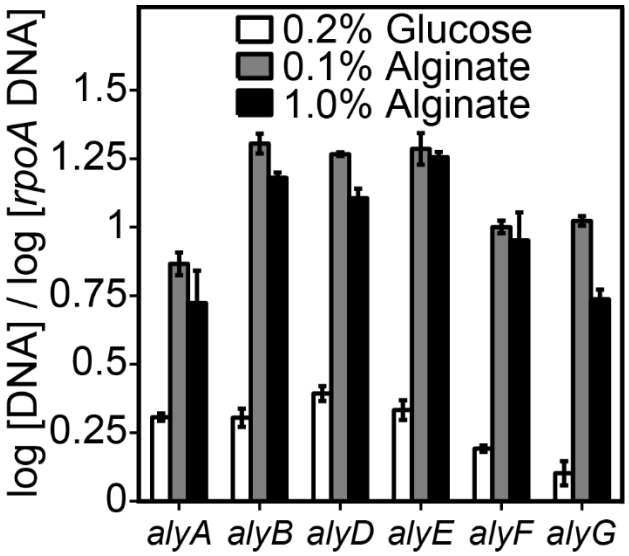

Figure 4.4. Expression of *alyA*, *alyB*, *alyD*, *alyE*, *alyF*, and *alyG* during growth on alginate or glucose. The mRNA levels of *alyA*, *alyB*, *alyD*, *alyE*, *alyF*, and *alyG* in *V. splendidus* 13B01 were determined using RT-PCR. The housekeeping gene *rpoA* was used as an internal standard.

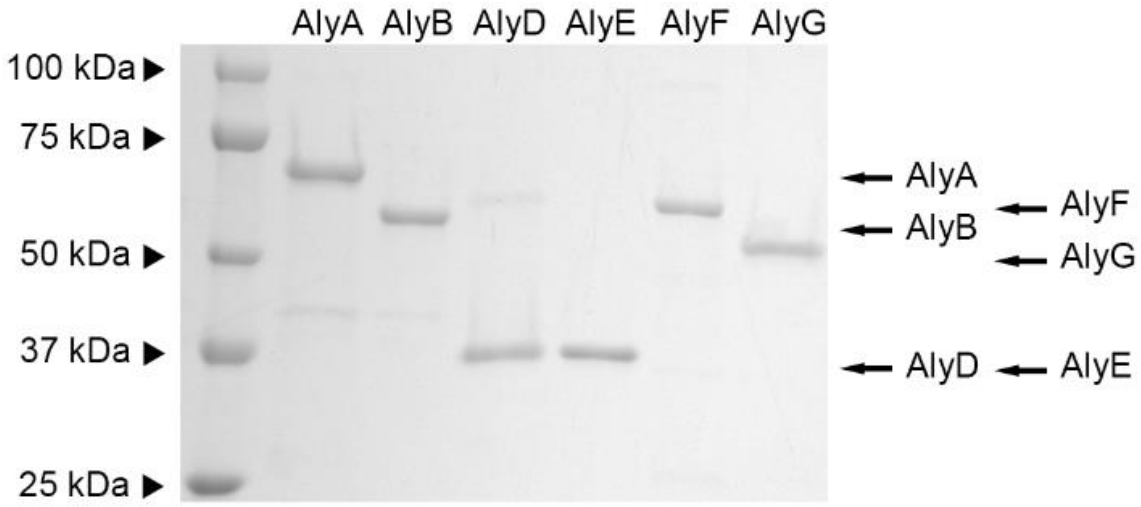

Figure 4.5. SDS-PAGE of purified AlyA, AlyB, AlyD, AlyE, AlyF, and AlyG. The first lane is a molecular marker with indicated molecular weights.

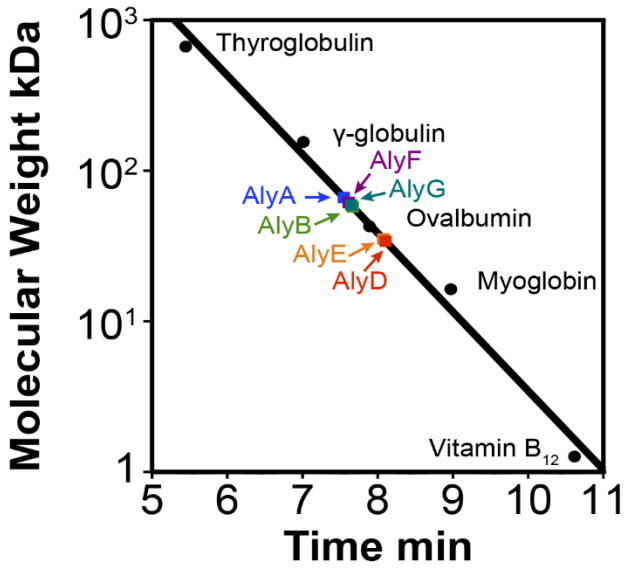

Figure 4.6. Size exclusion chromatography of native alginate lyases. The native size of AlyA, AlyB, AlyD, AlyE, AlyF, and AlyG was determined by comparison to standards thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B_{12} (1.35 kDa). Each protein is indicated by an arrow. AlyD and AlyE overlap due to their similar size. The standards are indicated with a solid circle.

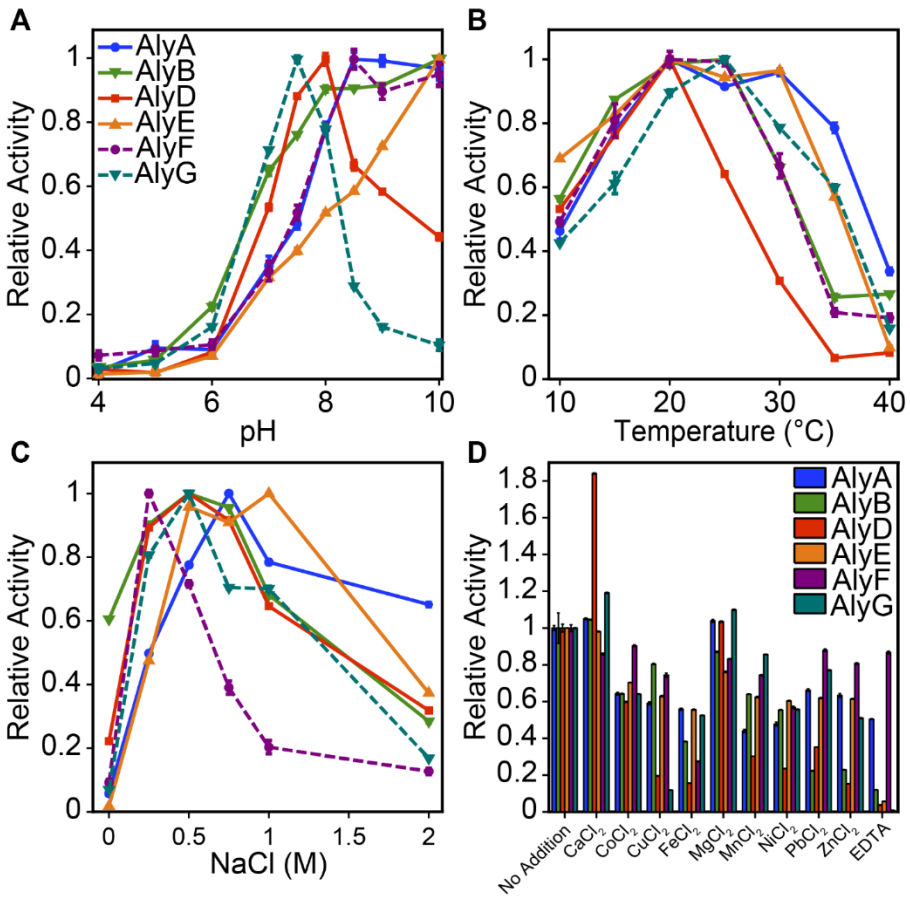

Figure 4.7. Determination of enzymatic conditions. A) The optimal pH for enzymatic activity was determined in 0.2% alginate, 20 mM APT at indicated pH, and 250 mM NaCl incubated at 25 °C for 20 min. Activity was normalized to the maximal pH condition for each enzyme. B) The optimal temperature for enzymatic activity was determined in 0.2% alginate, 20 mM APT at pH 8.5 and 750 mM NaCl, pH 10 and 500 mM NaCl, pH 8.0 and 500 mM NaCl, pH 10.0 and 1000 mM NaCl, pH 8.5 and 250 mM NaCl, and pH 7.5 and 500 mM NaCl for AlyA, AlyB, AlyD, AlyE, AlyF, and AlyG, respectively. Each condition was incubated at indicated temperatures for 20 min. Activity was normalized to the maximal temperature condition for each enzyme. C) The optimal NaCl concentration for enzymatic activity was determined in 0.2% alginate, 20 mM APT at pH 8.5, pH 10.0, pH 8.0, pH 10.0, pH 8.5, and 7.5 for AlyA, AlyB, AlyD, AlyE, AlyF, and AlyG, respectively. Each condition included the indicated NaCl concentration and was incubated at 25 °C for 20 min. Activity was normalized to the maximal NaCl concentration for each enzyme. D) The effect of divalent cations was determined in 20 mM APT at pH 9.0, 750 mM NaCl, and 0.074% alginate; pH 9.0, 750 mM NaCl, and 0.21% alginate; pH 8.0, 500 mM NaCl, and 0.22% alginate; pH 9.0, 750 mM NaCl, and 0.21% alginate; pH 8.5, 250 mM NaCl, and 0.052% alginate; and pH 7.5, 500 mM NaCl, and 0.31% alginate for AlyA, AlyB, AlyD, AlyE, AlyF, and AlyF, respectively. All conditions were incubated at 25 °C for 20 min. Divalent cations and EDTA were added at 1 mM. Activity was normalized to the no addition experiment.

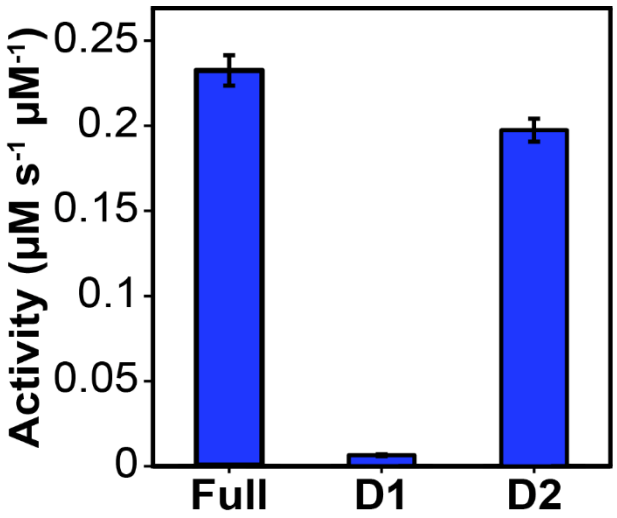

Figure 4.8. Enzymatic activity of full AlyA alginate lyase and AlyA domains 1 (D1) and 2 (D2). The activity is defined as the production of a 2-deoxy-D-glucose equivalent concentration per second per μM protein. The enzymatic activity of full AlyA enzyme and the two alginate lyase domains independently over-expressed was determined in 0.05% of alginate, 20 mM APT at pH 7.5 with 250 mM NaCl and incubated for 20 minutes at 25 °C.

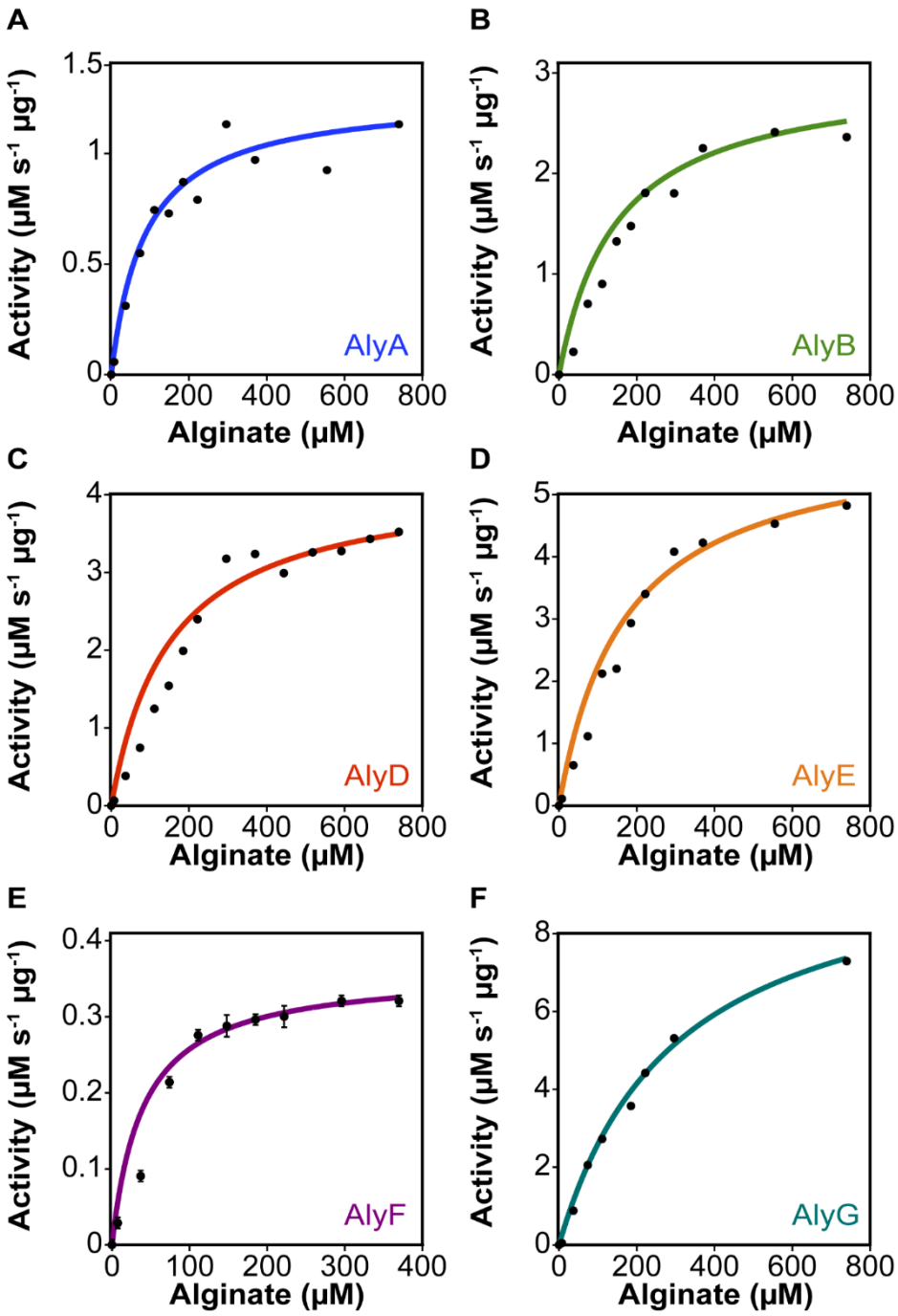

Figure 4.9. Alginate activity curves of AlyA (A), AlyB (B), AlyD (C), AlyE (D), AlyF (E), and AlyG (F). Experiments were performed in 20 mM APT at pH 9.0 and 750 mM NaCl, pH 9.0 and 750 mM NaCl, pH 8.0 and 500 mM NaCl, pH 9.0 and 750 mM NaCl, pH 8.5 and 250 mM NaCl, and pH 7.5 and 500 mM NaCl for AlyA, AlyB, AlyD, AlyE, AlyF, and AlyG, respectively. Alginate was dosed in accordingly. The mixture was incubated for 20 min at 25 °C. The solid curves denote the fit the Michaelis-Menten equation using the parameters given in Table 1.

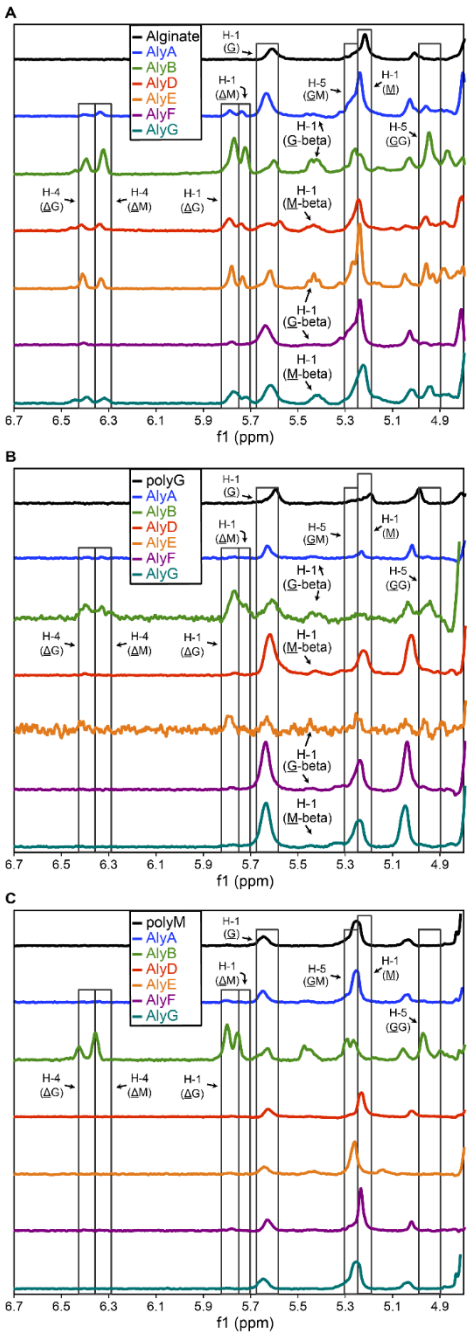

Figure 4.10. ^{1}H NMR (400-MHz) spectra of alginate and alginate derived substrates following degradation with AlyA, AlyB, AlyD, AlyE, AlyF, and AlyG. –G or -M denote signals from internal G or M residues; G-beta or M-beta denote signals from reducing G or M residues; Δ denotes signal from 4-deoxy-L-erythro-hex-4-enepyranosyluronate (DEH) non-reducing end residue. Non-underlined residues refer to the neighboring residues to those generating each signal. Protons (H) are numbered to indicate which particular proton causes the signal. a) Alginate b) polyG-enriched alginate c) polyM-enriched alginate.

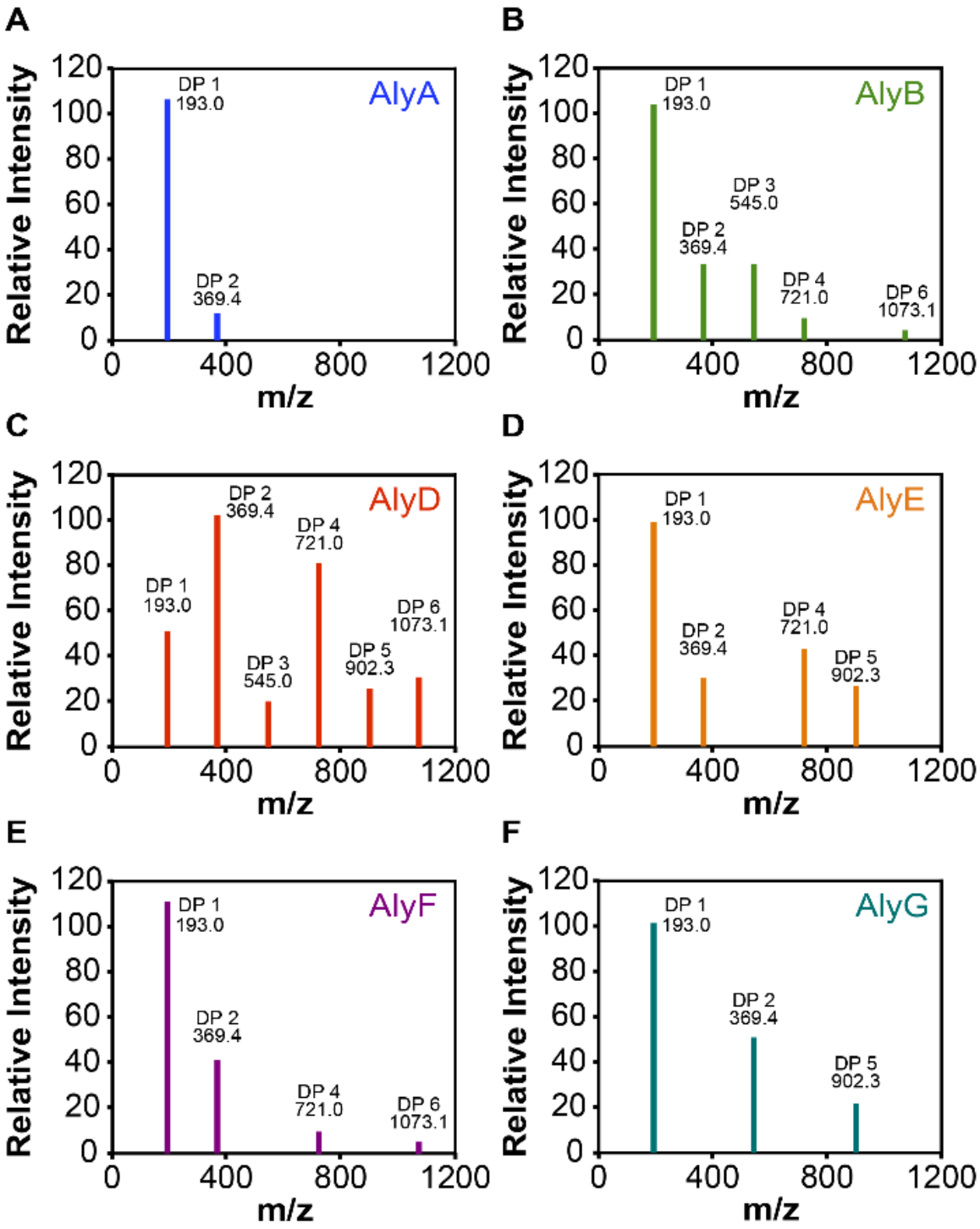

Figure 4.11. Negative-ion electrospray ionization mass spectra of alginate lyase degraded alginate. DP and the respective integers (1-6) refer to the degree of polymerization. *m/z* values are listed below the DP values.

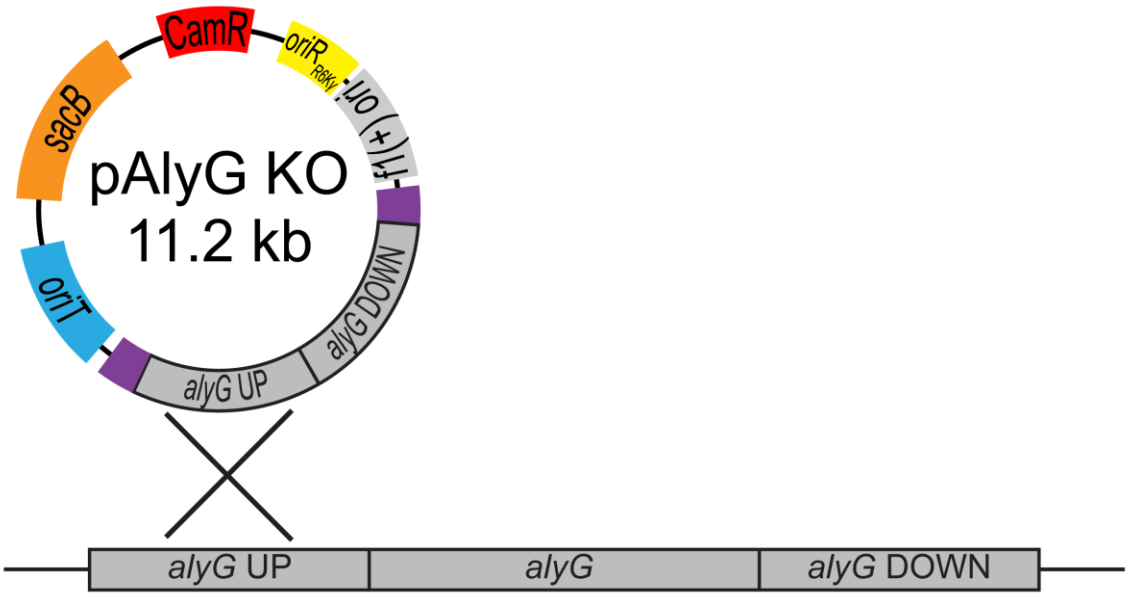

Figure 4.12. Schematic of knockout vector pAlyG KO recombining with the 13B01 genome. Since the vector contains both upstream and downstream regions around *alyG*, recombination can occur at either the UP region, as depicted, or the DOWN region. Either recombination event can lead to successful knockout following the second round of single-recombination.

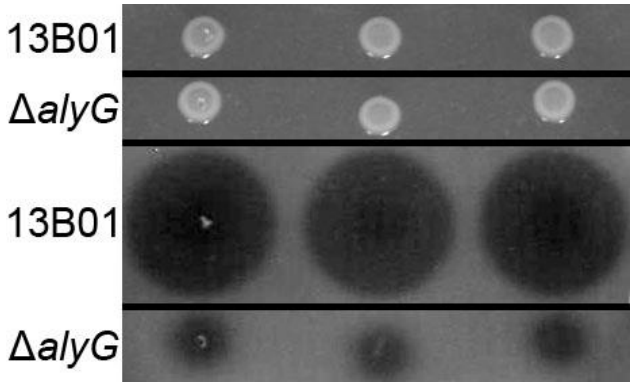

Figure 4.13. Secreted alginate lyase activity. Wild-type 13B01 and Δ*alyG* were spotted on the surface of a marine broth plate supplemented with 0.25% alginate. Following incubation, the alginate lyase degraded alginate was identified by overlaying the plate with cetylpyridinium chloride. The cleared zones are indicated in the lower portion. The upper portion shows the colonies after the initial growth period.

Chapter 5 Characterization of laminarinases in *V. breoganii* 1C10

5.1 Introduction

In this study, we investigated the laminarinases of the marine bacterium *Vibrio breoganii* 1C10. This bacterium contains four putative laminarinases: LamA, LamB, LamC, and LamD. Three of these laminarinases contain a GH16 domain, while LamD contains a GH17 domain. Both GH16 and GH17 employ a retaining mechanism (116). Each of these laminarinases where over-expressed and purified. The optimal environmental condition for each laminarinase was then determined. The enzymes had an optimal pH between 6 and 8.5 and an optimal temperature between 20 and 30 °C. We also determined that each laminarinase is highly induced upon growth on laminarin compared to glucose. Finally, the 1C10 laminarinases were determined to have endolytic activity. This work serves as a comprehensive study of multiple laminarinases contained within a single marine bacterium. Due to the high induction of each of the laminarinase genes upon growth on laminarin and the subsequent degradation of laminarin, *V. breoganii* 1C10 serves as a new platform for the engineering of microorganisms to utilize laminarin for fuels and chemicals.

5.2 Results

5.2.1 *V. breoganii* 1C10 contains four laminarinases

The marine bacterium *V. breoganii* 1C10 contains four putative laminarinases: LamA, LamB, LamC, and LamD. The laminarinases LamA, LamB, and LamC contain one glycoside hydrolase family 16 (GH16) laminarinase domain, while LamD contains one glycoside hydrolase family 17 (GH17) laminarinase domain. LamC also contains two carbohydrate binding module family 4 (CBM4) domains and a CBM11 domain in addition to its GH16 domain. Catalytic sites were identified by sequence comparison to GH16 and GH17 domains (**Figure 5.1**). GH16 and GH17 employ a retaining mechanism to cleave glycosidic linkages (117). This occurs via the action of a glutamic acid and

aspartic acid residue or two glutamic acid residues. In either case, one residue acts as an acid catalyst and the other as a nucleophile. Cleavage occurs in two steps. In the first glycosylation step, the nucleophile attacks the anomeric center to form a glycosol intermediate. Simultaneously, the glycosidic oxygen attacks the proton on the acid catalyst residue, completing the formation of the two residues in complex with the laminarin molecule. The glycosidic linkage is then broken leaving the nucleophile attached to the anomeric center. In the second deglycosylation step, a water molecule is used to attack the anomeric center to cleave the glycosidic linkage, thus completing the cleavage of the original glycosidic bond.

5.2.2 Expression of laminarinases

In order to determine the effect of laminarin on the expression of the putative laminarinases, we measured the mRNA levels of each laminarinase using quantitative PCR. This was accomplished by growing *V. breoganii* 1C10 in NDG minimal medium supplemented with 0.2% glucose, 0.1% (wt/wt) laminarin, and 1.0% (wt/wt) laminarin. As shown in **Figure 5.2**, growth of 1C10 on laminarin strongly induced expression of *lamA, lamB, lamC,* and *lamD* at both tested laminarin loadings. More specifically, *lamA* had a more than 20-fold induction when grown on laminarin compared to growth on glucose. *lamB, lamC,* and *lamD* had a more modest two- to five-fold expression induction on growth on laminarin compared to growth on glucose. None of the laminarinases had any discernable difference in expression when grown on the low or high laminarin loadings. These findings indicate that the 1C10 laminarinases are conditionally expressed and not constitutively expressed. Additionally, our data suggests that the laminarinases are expressed at low laminarin concentrations, since we did not find any difference in expression between 0.1% (wt/wt) and 1.0% (wt/wt) laminarin.

5.2.3 Purification of laminarinases

LamA, LamB, LamC, and LamD were cloned with an N-terminal GST-tag and expressed in *E. coli* from the P_{tac} promoter induced by IPTG. Previous attempts at over-

expression using a 6xHis-tag resulted in no discernable over-expression (results not shown). Following over-expression of the GST-tagged laminarinases, the GST-tags were cleaved and the laminarinases was separated from their tags (**Figure 5.3**).

LamA, LamB, LamC, and LamD without their respective signal peptides encode polypeptides of 494, 417, 899, and 612 amino acids, respectively. Each of these laminarinases then have a calculated molecular mass of 54.6 kDa, 46.9 kDa, 94.4 kDa, and 66.7 kDa for LamA, LamB, LamC, and LamD, respectively. The molecular masses of LamA, LamB, LamC, and LamD were approximately 53 kDa, 41 kDa, 114 kDa, and 66 kDa, as measured by SDS PAGE (**Figure 5.3**). Through gel filtration chromatography, LamA, LamB, LamC, and LamD eluted a symmetric peaks between γ-globulin and ovalbumin and had a molecular weight of 53.9 kDa, 50.3 kDa, 99.3 kDa, and 65.1 kDa, respectively (**Figure 5.4**). These results indicate each laminarinase migrates as a monomer in gel filtration and act as monomers in solution.

5.2.4 Determination of optimal enzymatic conditions

A modified version of a universal buffer (114) was employed to allow the testing of a wide range of pH conditions, temperatures, and NaCl concentrations. By varying each of these conditions independently, we could determine the optimal condition for each laminarinase, as listed in **Table 5.1** and **Figure 5.5**. We found LamB, LamC, and LamD to have a broad optimal pH between 6 and 8, while LamA was found to be optimal activity between pH 7.5 and 8.5. All the laminarinases had optimal activity between 20 and 30 °C. LamA, LamB, and LamD also had a broad NaCl tolerance between 50 and 250 mM NaCl. LamC was found to be optimally active between 0 and 1 M NaCl.

In order to determine which divalent cations are necessary for enzymatic activity, we stripped each laminarinase of its divalent cations using Chelex 100 resin. We then added back divalent cations at 1 mM loadings to universal buffer with laminarin. We found that $CoCl_2$ increased activity for LamB and LamC, $FeCl_2$ increased activity for LamA, LamB, and LamD, and $MnCl_2$ increased activity for LamB and LamC (**Figure 5.5**). All other tested additions either decreased activity or had no effect.

5.2.5 Activity of LamC GH16 domain

LamC contains carbohydrate binding domains (CBMs) in addition to its GH16 domain. In order to determine if these domains are necessary for enzymatic activity, we cloned and over-expressed the GH16 domain. Then we determined the activity of the GH16 domain in relation to the full protein. We found that the GH16 domain is active and does not require the CBM domains **(Figure 5.6)**. The GH16 domain was found to have 2.2-fold higher activity than the full LamC enzyme.

5.2.6 Determination of enzyme kinetics

Since we determined the optimal conditions for each laminarinase, we could determine the enzyme kinetic parameters for each laminarinase. This was done by dosing laminarin in the optimal conditioned universal buffer from **Table 5.1**. The laminarinases exhibited Michelis-Menten type kinetics **(Figure 5.7)**. The determined kinetics parameters are found in **Table 5.1**.

5.2.7 ESI-MS analysis

In order to determine whether each laminarinase acted exolytically or endolytically, we determined the ESI-MS spectrogram in the negative-ion mode for LamA, LamB, LamC, and LamD degraded laminarin. Each laminarinase acts endolytically since the spectrogram reported *m/z* values corresponding to di-, tri-, tetra-, and penta-saccharide fragments **(Figure 5.8)**, indicating cleavage products greater than the mono-saccharide.

5.2.8 Laminarinase specificity for β-D-glucan

β-D-Glucan is a glucan containing β-1,3- and β-1,4-linked glucose monomers. In order to determine if LamA, LamB, LamC, and LamD can degrade the β-1,3-linkages within β-D-Glucan, we substituted β-D-Glucan for laminarin in the standard enzymatic

assay. We found that LamB, LamC, and LamD had similar activity towards either laminarin or β-D-Glucan **(Figure 5.9)**. We did find that LamA had approximately 2-fold higher activity on laminarin than β-D-Glucan. This demonstrates that the enzymatic activity of LamA, LamB, LamC, and LamD can be utilized on non-laminarin glucans containing β-1,3-linkages. Further, this suggests that LamB, LamC, and LamD primarily act on β-1,3-linkages.

5.3 Discussion

The breakdown of storage glucans and polysaccharides is a key step in the utilization of macroalgae feed stocks for the production of fuels. Previous work sought to investigate the metabolism of alginate from *Vibrio splendidus* (114). Alginate is a polysaccharide found within macroalgae and is an attractive carbon source for the production of fuels. The enzymes that degrade alginate, called alginate lyases, were cloned and over-expressed and were then characterized. In this work we sought to understand the role laminarinases play in the degradation of laminarin by a microorganism, *V. breoganii* 1C10, that contains four laminarinases: LamA, LamB, LamC, and LamD. These four laminarinases were cloned and over-expressed and were then characterized.

The laminarinases found within *V. breoganii* 1C10 were found to each contain one catalytic domain. LamA, LamB, and LamC were found to contain a GH16 domain, while LamD was found to contain a GH17 domain. These domains have a different 3-D structure, but both catalyze the degradation of β-1,3- and β-1,6-linked glucose. Through homology comparison, we were able to identify the catalytic residues of each domain **(Figure 5.1)**. The laminarinase LamC was found to contain CBM4 and CBM11 domains. The CBM4 (carbohydrate binding module family 4) domain has been implicated in the binding of β-1,3-linked glucans but not to β-1,4-linked glucans, such as cellulose and xylan (118). Similar findings were found for the CBM11 domain (119). We found that the CBM domains for LamC were not necessary for catalytic function.

Previous investigation of differential expression of carbohydrate active enzymes in the marine bacterium *Thermotoga maritima* demonstrated that growth on laminarin

and β-1,3-linked barley induced the expression of a laminarinase and a exoglycosidase (120). A ROK family regulator was also induced upon growth on laminarin, suggesting a transcriptional mechanism for the detection of laminarin in the extracellular environment. We were interested if *V. breoganii* 1C10 had a similar inducement of its laminarinases upon growth on laminarin. We found that each laminarinase was induced upon growth on laminarin in comparison to its monomer glucose. This indicates that *V. breoganii* is also able to detect the presence of laminarin.

In order to characterize the laminarinases LamA, LamB, LamC, and LamD, we cloned and over-expressed each enzyme with an N-terminal GST-tag which was cleaved following purification. We then determined the optimal conditions for each enzyme. We found that each enzyme was robust again the tested environmental pH and NaCl with LamB, LamC, and LamD retaining at least 80% of maximal activity between pH 6 and 8.5 and 50 mM and 600 mM NaCl. LamA retained 80% of maximal activity between pH 7.5 and 8.5 and 50 mM and 400 mM NaCl. All of the laminarinases were most active between 10 and 30 °C. Previous investigations of a laminarinase from *Zobellia galactanivorans* found maximal activity at pH 7.0 (56) in a buffer similar to that used in this study. Labourel and coworkers found higher activity in a Glycine-NaOH buffer but this cannot be directly compared. This enzyme was found to have a K_m value of 5.0 mM laminarin, similar to the enzymes characterized in this study, while the turnover number was 410 s^{-1}, a value more than 60-fold larger than the fastest degrading *V. breoganii* laminarinase, LamD. Additionally, Labourel and coworkers demonstrated much higher enzymatic activity (22-fold higher catalytic efficiency) using laminarin as opposed to β-glucan from barley. We found that LamB, LamC, and LamD had similar activities on both laminarin and β-glucan from barley, while LamA had approximately 2-fold higher activity on laminarin as opposed to β-glucan from barley. These results suggest that the *V. breoganii* 1C10 laminarinases can utilize broader substrates than other laminarinases. Previous investigations have shown laminarinases with preferential activity toward laminarin over lichenan (β-1,3- and β-1,4-linked glucose) (52, 53, 121).

A β-1,3-1,4-glucanase from *Bacillus licheniformis* was found to have optimal activity at pH 7.0 and 55 °C (117), in accordance with the optimal pH range determined in this study. A laminarinase from the larvae *Rhagium inquisitor* has been previously

shown to have at least 75% maximal activity between pH 5 and 7 (121). This laminarinase was shown to have a 50% decrease in activity upon exposure to 9.6 mM Mn^{2+}. We report 20 to 40% increase in activity for LamB and LamC upon exposure to 1 mM Mn^{2+}. The difference of effect of Mn^{2+} upon activity might be due to a larger loading of Mn^{2+} in the *R. inquisitor* laminarinase or due to the differing source of the laminarinase. A laminarinase from the archaeon *Pyrococcus furiosus* has been over-expressed and purified; this enzyme was found to retain 80% maximal activity between pH 4.5 and 7.5, in agreement with LamA, LamB, LamC, and LamD (52). The *P. furiosus* laminarinase was to have a K_m of 0.62 mM laminarin, in comparison to between 3.4 to 6.0 mM laminarin for LamA, LamB, LamC, and LamD. LamA, LamB, LamC, and LamD were found to have specific activities 5- to 30-fold larger than the *P. furiosus* laminarinase, despite the large difference in reaction temperature (25 to 40 °C for *V. breoganii* laminarinases versus 80 °C for *P. furiosus* laminarinase.)

Here we present a characterization of the four laminarinases from the marine bacterium *V. breoganii* 1C10. *V. breoganii* 1C10 differentially expresses its laminarinases and this induction occurs at low laminarin loading. The regulation network allowing for laminarin sensing would be beneficial for metabolic engineering due to the tight regulation and large induction. We also found each laminarinase enzymatically active in a broad range of pH values and NaCl concentrations. We found that laminarinases had similar K_m values to other laminarinases, however, the turnover numbers of LamA, LamB, LamC, and LamD were found to be smaller than a previously investigated laminarinase. Overall, the *V. breoganii* 1C10 laminarin metabolism machinery serves as an attractive platform for the production of fuels and other chemicals, due to the simplicity of implementation in other hosts and the abundance of macroalgae based carbon sources.

Chapter 5 Tables and Figures

Table 5.1. Optimal environmental conditions and enzymatic kinetics of studied laminarinases[1]

	LamA	LamB	LamC	LamD
pH	8.0	7.5	7.0	6.5
Temperature (°C)	30	40	25	30
NaCl (mM)	50	150	150	50
K_m (μM laminarin)	3.4 ± 0.4	6.0 ± 0.6	4.5 ± 0.6	5.2 ± 0.8
V_{max} (μM s^{-1})	0.42 ± 0.02	0.15 ± 0.01	0.24 ± 0.02	0.92 ± 0.09
Turnover (s^{-1})	2.3 ± 0.1	0.69 ± 0.03	2.3 ± 0.2	6.1 ± 0.6

[1]The data and curve fits used to determine the kinetic parameters are found in **Figure 5.7**.

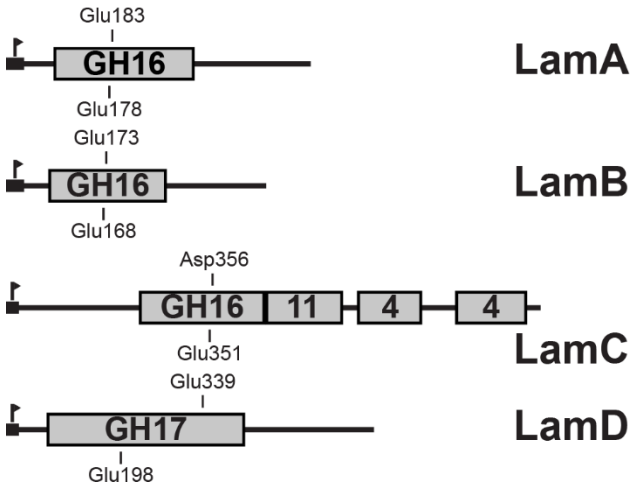

Figure 5.1. Domain structure of laminarinases LamA, LamB, LamC, and LamD, drawn to scale. The indicated amino acid residues are hypothesized catalytic sites. GH16 is the glycoside hydrolase family 16 domain and GH17 is the glycoside hydrolase family 17 domain. The domain indicated by 11 indicates the carbohydrate-binding module family 11 domain, while 4 indicates the carbohydrate-binding module family 4 domain. Each signal peptide is indicated with a flag.

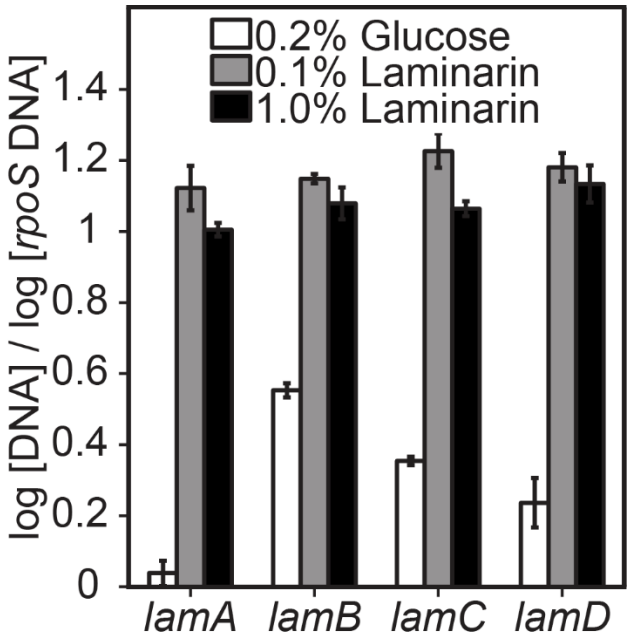

Figure 5.2. Expression of *lamA*, *lamB*, *lamC*, and *lamD* during growth on laminarin or glucose. The mRNA levels of *lamA*, *lamB*, *lamC*, and *lamD* in *V. breoganii* 1C10 were measured using RT-PCR. The housekeeping gene *rpoS* was used as an internal standard.

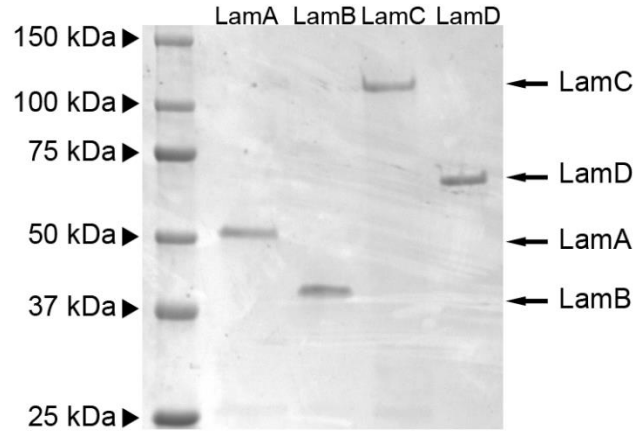

Figure 5.3. SDS-PAGE of purified LamA, LamB, LamC, and LamD. The first lane is a molecular marker with indicated molecular weights.

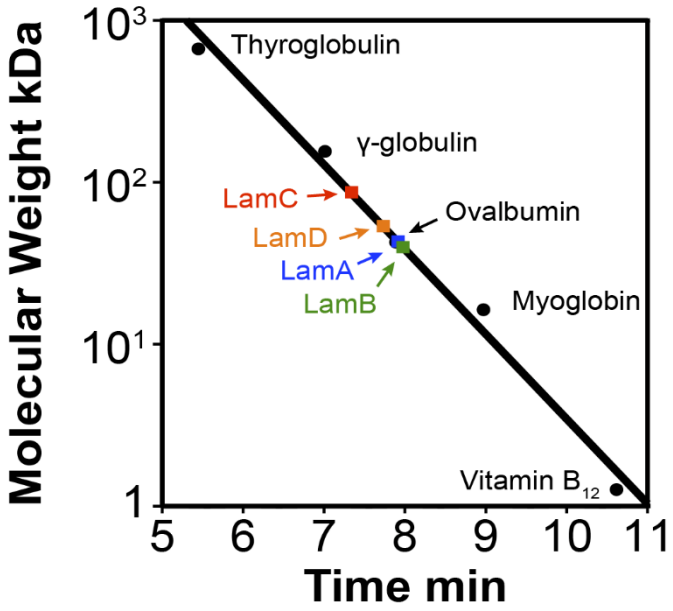

Figure 5.4. Size exclusion chromatography of laminarinases. The native sizes of LamA, LamB, LamC, and LamD were determined through comparison to standards thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B_{12} (1.35 kDa). Each protein is indicated by an arrow. LamA, LamB, and ovalbumin overlap due to their similar sizes. Standards are indicated with a solid circle. The solid line is the interpolation of the standards.

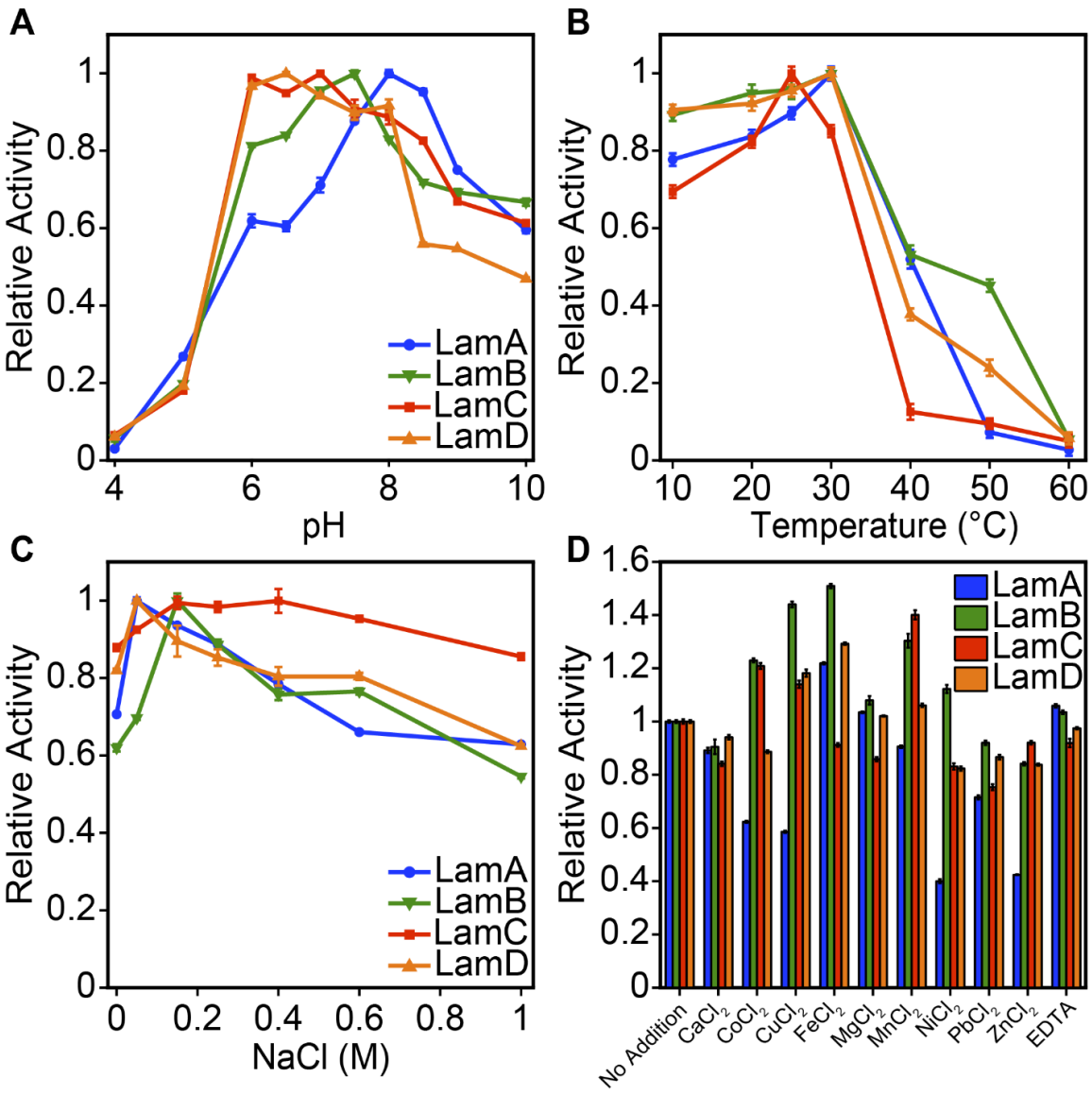

Figure 5.5. Determination of optimal enzymatic conditions. A) The optimal pH for laminarinase activity was determined in 0.05% laminarin, 20 mM AMT at indicated pH, and 250 mM NaCl incubated at 20 °C for 2 hours. Activity was normalized to the maximal pH condition for each enzyme. B) The optimal temperature for laminarinase activity was determined in 0.05% laminarin, 20 mM AMT at pH 7.5, and 250 mM NaCl. Each condition was incubated at indicated temperatures for 2 hours. Activity was normalized to the maximal temperature condition for each enzyme. C) The optimal NaCl concentration for enzymatic activity was determined in 0.05% laminarin, 20 mM AMT at pH 7.5. Each condition included the indicated NaCl concentration and was incubated at 20 °C for 2 hours. Activity was normalized to the maximal NaCl concentration for each enzyme. D) The effect of divalent cations was determined in 0.05% laminarin, 20 mM AMT at pH 7.5, and 250 mM NaCl incubated at 20 °C for 2 hours. Divalent cations and EDTA were added at 1 mM. Activity was normalized to the no addition experiment.

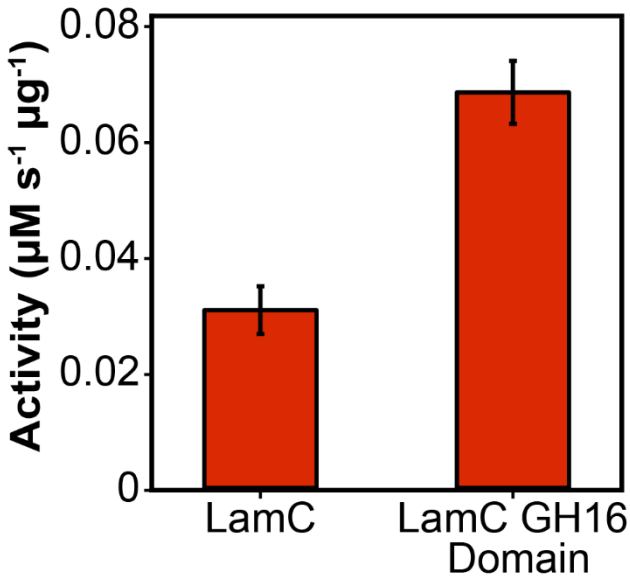

Figure 5.6. Enzymatic activity of full LamC laminarinase and LamC GH domain. The enzymatic activities of full LamC enzyme and the GH domain were independently over-expressed and then determined in 0.05% of laminarin, 20 mM AMT at pH 7.5 with 250 mM NaCl and incubated for 2 hours at 20 °C.

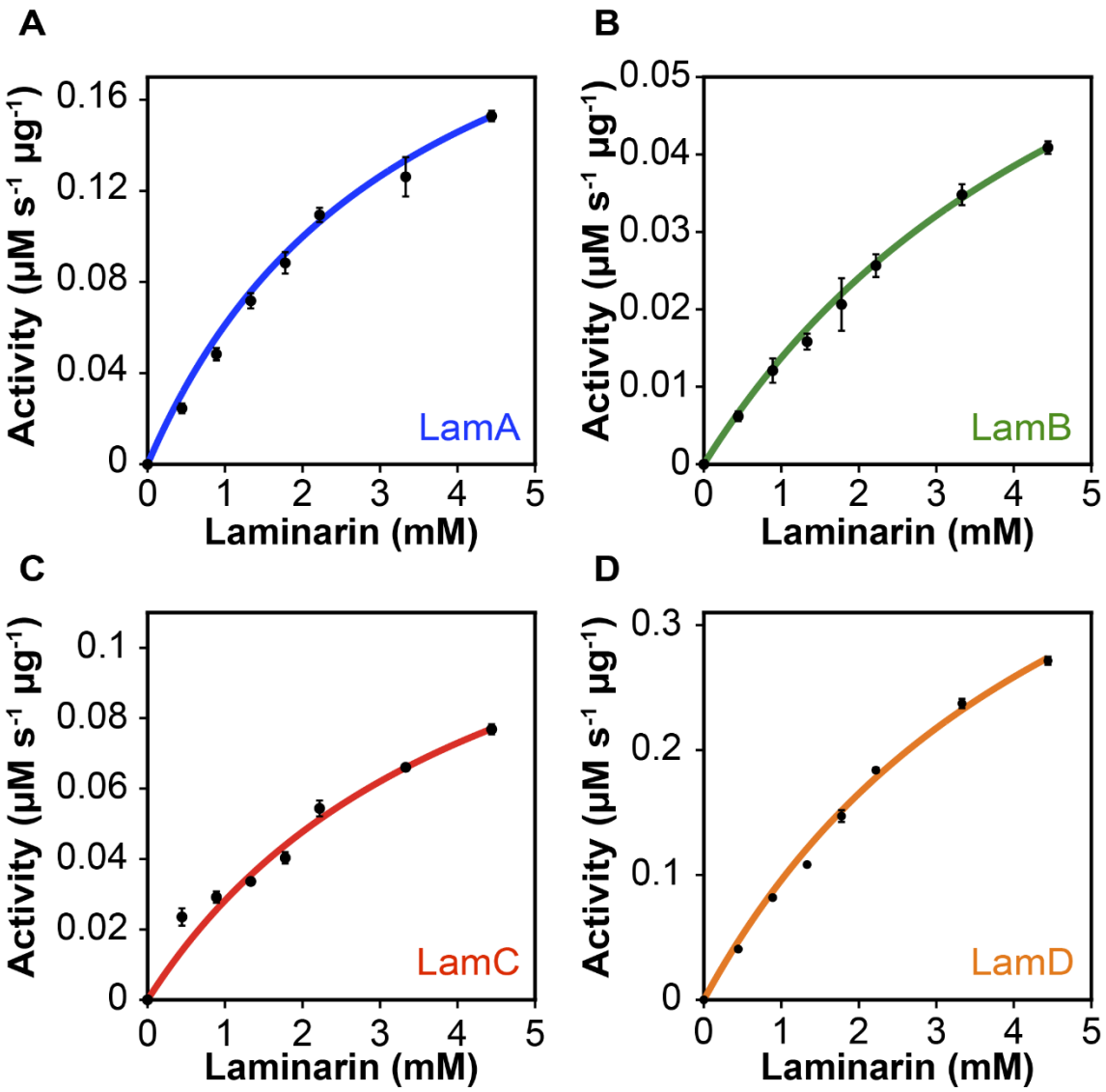

Figure 5.7. Laminarin curves of LamA (A), LamB (B), LamC (C), and LamD (D). Experiments were performed at the optimal pH, temperature and NaCl concentration listed in Table 1 with 1 mM $FeCl_2$ for LamA, LamB, and LamD and 1 mM $MnCl_2$ for LamC. The mixture was incubated for 2 hours. The solid curves denote the fit the Michaelis-Menten equation using the parameters given in Table 1.

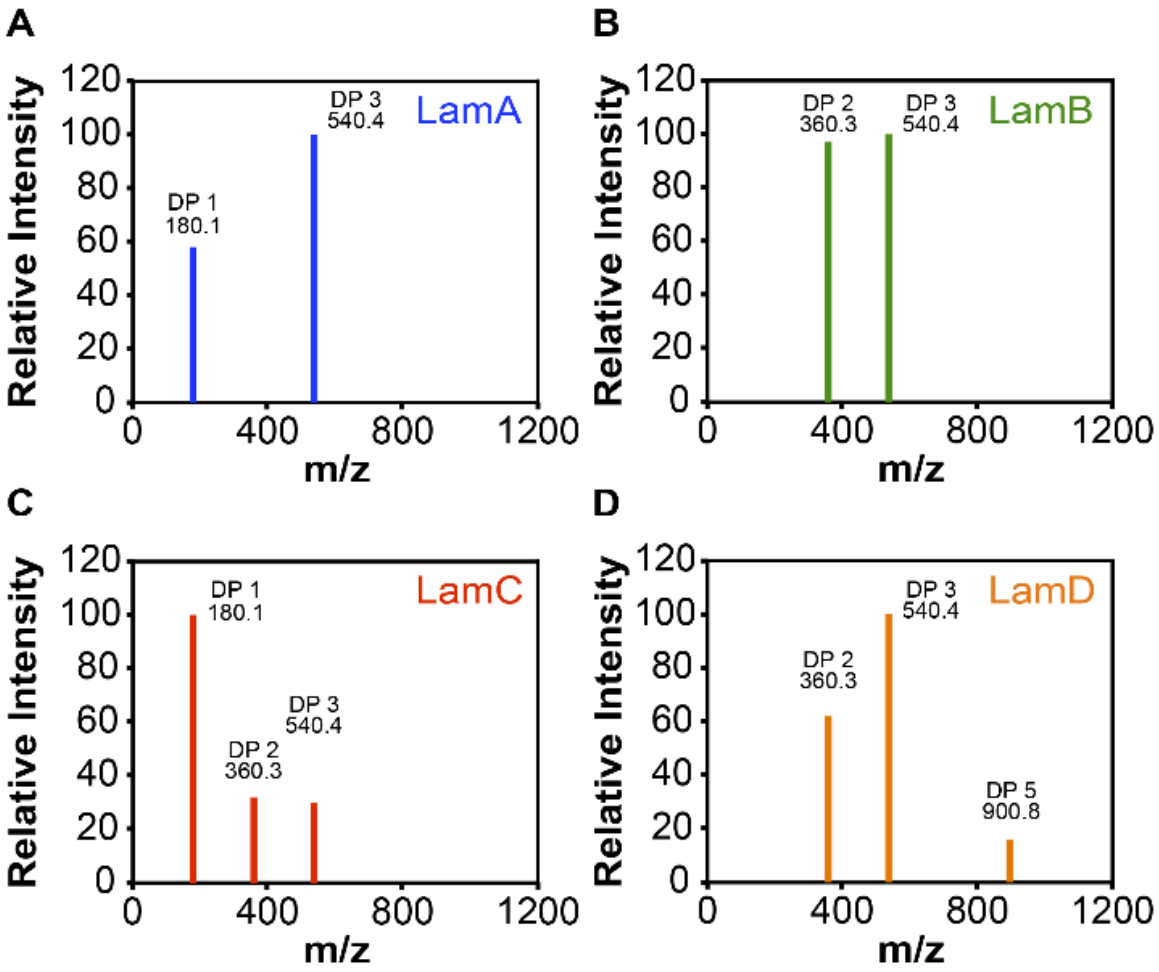

Figure 5.8. Negative-ion electrospray ionization mass spectra of laminarinase degraded laminarin. DP and the respective integers (1-5) refer to the degree of polymerization. *m/z* values are listed below the DP values.

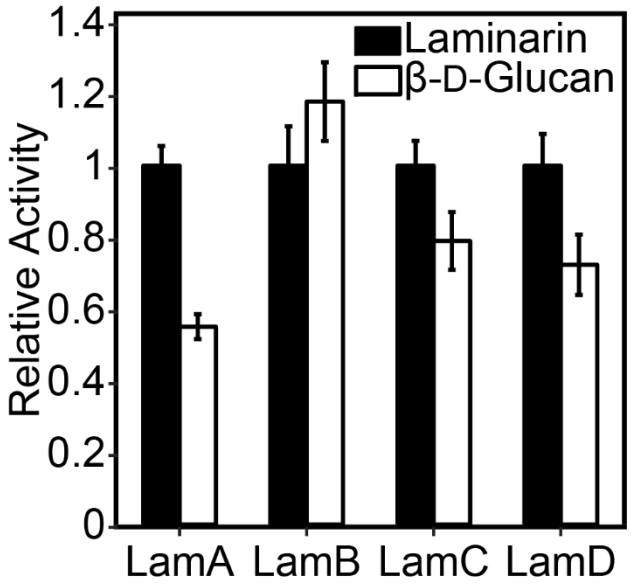

Figure 5.9. Enzymatic activity of LamA, LamB, LamC, and LamD when grown on laminarin and β-D-Glucan. The activity was determined in 0.05% of either laminarin or β-D-Glucan, 20 mM AMT at pH 7.5 with 250 mM NaCl and incubated for 2 hours at 20 °C.

Chapter 6 Characterization of alginate lyases of *V. breoganii* 1C10 and analysis of the PL7 family[1]

6.1 Introduction

Previous work (114) (see also **Chapter 3**) has investigated the alginate lyases from the marine bacterium *Vibrio splendidus* 12B01. This work over-expressed and purified four alginate lyases: AlyA, AlyB, AlyD, and AlyE. This work found that the lyases had optimal activity between pH 7.5 and 8.5 and between 20 °C and 25 °C. We also found that the lyases were either polyG or polyMG specific and act via an endolytic mechanism. Through analysis of the expression of each lyase under differing carbon sources, we demonstrated that each lyases was not constitutively expressed, but rather expressed when *V. splendidus* 12B01 is grown under alginate as the sole carbon source. Additionally, one alginate lyase, AlyB, was found to be expressed at higher levels under low alginate conditions than AlyA, AlyD, and AlyE.

The marine bacterium *Vibrio splendidus* 13B01 is a close relative to *V. splendidus* 12B01, and, as such, possess homologs to AlyA, AlyB, AlyD, and AlyE with large amino acid similarity (more than 98%). *V. splendidus* 13B01 also possesses two unique alginate lyases: AlyF and AlyG. An initial characterization of the growth rates and secretion of alginate lyases found that *V. splendidus* 13B01 has faster growth rates and a seven-fold larger secreted alginate lyase activity than *V. splendidus* 12B01. In order to determine the cause of the higher growth rates and greater secreted alginate lyase activity, the six alginate lyases of *V. splendidus* 13B01 were over-expressed and characterized (see **Chapter 4**). The 13B01 alginate lyases were found to have activities similar to their 12B01 counterparts, so the higher secreted alginate lyase activity is due to higher levels of secretion of alginate lyases in 13B01. To understand why 13B01 secretes alginate lyases at higher levels than 12B01, we measured the mRNA levels of the six alginate lyases in 13B01. In contrast to 12B01, the 13B01 alginate lyases were maximally expressed at both low and high alginate loadings, indicating that 13B01 is better able to metabolize alginate over a wide range of alginate. Additionally, the induction levels (the ratio of expression at either low or high alginate to glucose) of AlyA, AlyB, AlyD, and

[1]NMR analysis was performed by Geethika Yalamanchili.

AlyE in 13B01 were higher than 12B01 indicating that these alginate lyases are expressed at higher amounts than 12B01. AlyG was found to have a large enzymatic activity and high induction level in comparison to the other 12B01 and 13B01 alginate lyases. Additionally, we knockout out *alyG* and found four-fold smaller secreted alginate lyase activity, indicating the important role AlyG plays in alginate degredation. This unique enzyme provides 13B01 an advantage in alginate degradation and subsequent growth rates. These effects point to 13B01 expressing its alginate lyases at higher level than 12B01, which leads to higher alginate lyase secretion and greater alginate degradation than 12B01. These effects are hypothesized as leading to the faster growth rates of 13B01 in comparison to 12B01. All in all, 13B01 serves as a better source of alginate lyases and the corresponding secretion and regulation machinery than 12B01.

Further investigation of marine bacteria capable of degrading and metabolizing alginate has identified an organism that degrades alginate and secretes alginate lyases at similar levels as 13B01. This organism is *Vibrio breoganii* 1C10. A preliminary sequence search of its genome has identified 12 putative alginate lyases. Alignment to the 12B01 and 13B01 alginate lyases identified several homologs to previously characterized enzymes. However, the majority of the 1C10 enzymes were more distantly related. The 1C10 lyases were over-expressed and purified. Like the 12B01 and 13B01 lyases, the 1C10 lyases were found to be optimally active between pH 7 and 10 and 20 °C and 30 °C. We also found that these enzymes had similar activities to the 12B01 and 13B01 enzymes, but we found a wider dyad specificity in the 1C10 enzymes. This work serves to expand the space of alginate degradation by characterizing enzymes further divergent than the 12B01 and 13B01 enzymes.

6.2 Results

6.2.1 *V. breoganii* 1C10 secretes alginate lyases at high levels

Initial screening demonstrated that *V. breoganii* 1C10 is an organism capable of degrading and metabolizing alginate (data not shown). To confirm this finding, we spotted *V. splendidus* 12B01 and 13B01 along with 1C10 on a soft agar medium containing 0.25% alginate. The secretion of alginate lyases was then determined by staining the plate with cetylpyridinium chloride to show areas cleared of alginate.

Controlling for colony size, we found *V. breoganii* 1C10 to have more than 4-fold higher secreted alginate lyase activity than *V. splendidus* 12B01. 1C10 had 70% of the *V. splendidus* 13B01 secreted alginate lyase activity **(Figure 6.1)**.

6.2.2 Identification of twelve alginate lyases within *V. breoganii* 1C10

Through a genome search within the *V. breoganii* 1C10 genome, we identified twelve alginate lyases within its genome. These lyases were analyzed for their particular domains. Through comparison to other alginate lyase domains, we identified ten alginate lyases containing at least one PL7 domain, while the remaining two lyases contain a PL6 domain. Through sequence alignment to other PL6 or PL7 domains, we were able to identify the catalytic sites within the twelve alginate lyases **(Figure 6.2)**.

Through comparison to the 12B01 and 13B01 alginate lyases previously studied **(see Chapter 3** and **Chapter 4)**, we built a phylogenetic tree depicting the relatedness of the PL7-containing alginate lyases from *V. splendidus* 12B01, and *V. splendidus* 13B01, and *V. breoganii* 1C10 **(Figure 6.3)**. From the amino acid alignment, we found that the 12B01 AlyA and 1C10 1C10 had a 64% sequence identity, while 12B01 AlyB and 1C10 AlyB had an 89% sequence identity. The 12B01 AlyD and 1C10 AlyD were found to have 88% sequence identity, while the 12B01 AlyE and 1C10 AlyE were found to have 59% sequence identity.

We, similarly, performed a sequence alignment and phylogenic analysis on the two 1C10 PL6-containing alginate lyases and the PL6-containing alginate lyase AlyF from *V. splendidus* 13B01 **(Figure 6.4)**. AlyN from 1C10 was found to have 72% sequence identity with AlyF from 13B01.

6.2.3 Over-expression and purification of alginate lyases

Each of the twelve alginate lyases were cloned with an N-terminal 6XHis-tag and expressed in *E. coli* BL21(DE3) from the pET28A T7 promoter. Each lyase was then purified under denaturing conditions and refolded. In preliminary characterization, AlyK was found to have no enzymatic activity (data no shown). A native purification was also

attempted for AlyK, and this protein was also found to have no enzymatic activity (data not shown).

6.2.4 Determination of optimal enzyme conditions

In a process identical to the previous alginate lyase characterization, the optimal conditions for each lyase was determined by varying the temperature, pH, and NaCl concentration in a 20 mM APT reaction condition. The optimal environmental conditions for each lyase are listed in **Table 6.1**. The 1C10 lyases were found to have optimal enzymatic activity between pH 7 and 10, consistent with the 12B01 and 13B01 alginate lyases. The optimal temperature was found to be 20 and 30 °C for the 1C10 lyases, again consistent with the 12B01 and 13B01 lyases. The optimal salt condition for the 1C10 lyases was found to be between 50 and 400 mM NaCl. This range is smaller than the optimal NaCl concentration for the 12B01 lyases (400 to 1000 mM NaCl).

As alginate lyases have previously been shown to be positively affected by divalent cations **(Chapter 3** and **Chapter 4)**, we sought to investigate the role divalent cations play in the 1C10 alginate lyases. To this end, we stripped each alginate lyase of divalent cations and reintroduced divalent cations individually at 1 mM loadings. We found that the 1C10 lyases were positively affected by Ca^{2+}. The same effect was found in the 12B01 and 13B01 alginate lyases.

6.2.5 Determination of enzyme kinetics

Knowing the optimal environmental conditions for each lyase, we can determine the enzymatic kinetic parameters for each lyase. This was accomplished by dosing alginate under the optimal environmental conditions listed in **Table 6.1** and 1 mM $CaCl_2$. We found that each lyase exhibits Michaelis-Menten type kinetics. The determined enzymatic parameters are listed **Table 6.1**.

6.2.6 Substrate specificity of alginate lyases

An important characteristic of an alginate lyase is the dyad specificity by which it cleavages alginate. This specificity was determined by degrading alginate by each alginate lyase. The degraded alginate were then analyzed by NMR to determine which peaks on the spectra are enhanced or decreased. As an alginate lyase degrades alginate, the newly liberated DEH and the corresponding dyad partner can be determined, which allows for determination of the dyad specificity. This analysis was undertaken for the 1C10 alginate lyases and the determined dyad specificity is found in **Table 6.1**. The specificities of each lyase are updated on the phylogenic trees prepared for each PL family.

6.2.7 Localization of alginate lyases within their hosts

Within a gram-negative bacterial host, proteins can be predicted to exist within one of the following general locations: cytoplasm, inner membrane, periplasm, outer membrane, and extracellular. Each of these locations has particular motifs found in a signal peptide or other signaling sequence within an mRNA fragment, which allows the cell to direct the folded or unfolded protein to its designated location. Using the prediction toolboxes PSORTb and CELLO, we were able to predict the cellular location of each alginate lyase from *V. splendidus* 12B01 *V. breoganii* 1C10 **(Table 6.2)**. We found that the 12B01 and 1C10 AlyA lyases were predicted to exist within the outer membrane and 1C10 AlyL was predicted to exist within the periplasm. All other lyases were predicted to be secreted outside their hosts to degraded alginate.

6.3 Discussion

In the analysis of the 1C10 lyases, we sought a broader understanding of the role alginate lyases play in the degradation of alginate by marine bacteria. Previously, we have shown that the alginate lyases from 12B01 and 13B01 have all had optimal activity

under similar conditions. From the characterization of the 1C10 lyases, we saw the same range of optimal activity conditions for each lyase. This demonstrates that novelty and multiplicity in alginate lyases cannot be attributed to the optimal environmental conditions for each lyase.

6.3.1 The *V. splendidus* 12B01 lyases provide a framework for understanding alginate metabolism

Therefore one must consider the other enzyme characteristics to determine how the set of alginate lyases within an organism influences metabolism of alginate. At the lowest level, the expression of each alginate lyase mRNA is a means by which its host organism can tailor its metabolism of alginate. As evidenced by the high induction upon growth on alginate, a*lyB*, *alyD*, and *alyE* from 12B01 are expected to be expressed at high levels upon growth on alginate. Additionally, *alyB* was found to be near maximally expressed at low alginate loadings, indicating that *alyB* is utilized at low alginate loadings in the marine ecosystem. This hypothesis is further evidenced by the low K_m toward alginate that AlyB demonstrated. A model can be hypothesized wherein the expression of each alginate lyase was evolved to be differentially expressed upon growth on alginate. Since AlyA and AlyB have different dyad specifies than AlyD and AlyE, these pairs can be independently regulated to provide finer control of alginate metabolism than would be afforded by a single alginate lyase with either G-G or G-M dyad specificity.

The metabolism model within *V. splendidus* 12B01 can be further expanded by considering the degree of polymerization of the degraded alginate generated by each lyase **(Figure 6.5)**. Our work shows that AlyA and AlyB generated degraded products shorter than those of AlyD and AlyE. This indicates that AlyA and AlyB attack alginate on the ends of the molecule with an average degree of polymerization of the degraded products between 4 and 11. AlyD and AlyE attack further inside the polymer with an average degree of polymerization of the degraded products of between 16 and 21. A consequence of this model is that AlyA and AlyB are predicted to act on smaller fragments of alginate, which is further confirmed by these enzymes having smaller K_m values towards alginate. As degradation of alginate proceeds, alginate lyases will

degrade alginate into smaller fragments, so AlyA and AlyB will be utilized on those smaller fragments more effectively than AlyD and AlyE, due to their higher affinities toward the decreasing supply of alginate.

The above picture of the degradation of alginate by *V. splendidus* 12B01 does not take into account the action of AlyA versus AlyB. This pair of enzymes have been shown to have similar attributes to one another, so what is the role of AlyA in comparison to AlyB? A partial answer to this question lies in the predicted localization of AlyA. This lyase is predicted to localize to the outer membrane of *V. splendidus* 12B01, thus forming a spatial separation between these enzymes. While AlyA was shown to have a lesser enzymatic activity than AlyB, the close proximity to its host will allow AlyA activity to be more easily utilized up by 12B01 than a secreted enzyme that must diffuse its products back to the host. Additionally, since AlyA is predicted to be attached to its host, it can be expressed at lesser amounts than secreted enzymes since secreted enzymes are lost once secreted. Thus, the action of the AlyA and AlyB pair can be described thusly: AlyA and AlyB are G-M lyases that act on smaller fragments of alginate with tighter affinity than AlyD and AlyE. Since AlyA is predicted to be localized of the outer membrane of 12B01, it is expressed at lower amounts than AlyB. AlyB is predicted to be secreted by 12B01 and is expressed at higher levels than AlyA, so in order to be a cost effective lyase, it has higher activity than AlyA.

The role of AlyD and AlyE in alginate metabolism by *V. splendidus* 12B01 cannot be discerned from the localization analysis outlined above. Both of these enzymes are predicted to be secreted into the cellular environment, so they do not serve spatially different roles. These enzymes have similar affinities toward alginate and cleave alginate exolytically on the G-G dyad. Also, both of these enzymes have similar gene expression patterns and enzymatic activity, so one cannot differentiate these enzymes by these characteristics. When considering their location on the phylogenetic tree, these enzymes are not closely related in comparison to the other 12B01, 13B01, and 1C10 PL7-containing enzymes, thus AlyD and AlyE might have originated from different sources in the evolution of 12B01. A consequence of this is that AlyD and AlyE serve the same role within 12B01, and no evolutionary pressure exists to select for one of these enzymes.

6.3.2 *V. splendidus* 13B01 degrades alginate better than *V. splendidus* 12B01

V. splendidus 13B01 possesses the alginate lyases AlyA, AlyB, AlyD, and AlyE, so similar conclusions can be made for the roles of AlyA, AlyB, AlyD, and AlyE as were made for *V. splendidus* 12B01. Therefore, any differences in the metabolism of alginate by 13B01 compared to 12B01, can be attributed to intrinsic differences in metabolic regulation and the action of additional alginate lyases in 13B01.

When comparing the gene expression of 12B01 and 13B01, two conclusions can be made on the regulatory network of these organisms. Firstly, the induction of the 13B01 lyases (expression under alginate compared to that of glucose) was greater than the 12B01 lyases. Secondly, 13B01 was more sensitive to alginate than 13B01, since the 13B01 lyases were maximally expressed at the low alginate loading, while the 12B01 lyases were moderately expressed. These findings show that 13B01 possesses a regulatory mechanism better suited than 12B01 for fast degradation of alginate.

V. splendidus 13B01 also possesses two additional alginate lyases than 12B01: AlyF and AlyG. AlyF was shown to have small enzymatic activity compared to the other 12B01 and 13B01 alginate lyases, so its effect on 13B01 alginate metabolism is expected to be modest. AlyG was shown to have a large enzymatic activity, and through knockout of *alyG*, we showed a large decrease in secreted alginate lyase activity. This demonstrates that AlyG contributes significantly to the secreted alginate lyase activity of 13B01, contributing more than 50% of the difference between 12B01 and 13B01 secreted alginate lyase activity. The regulatory differences between these strains is expected to make up the remaining difference in secreted lyase activity. This demonstrates how a single high activity lyase and changes to the regulation in response to alginate can dramatically affect the metabolism of alginate in two closely related organisms.

6.3.3 Alginate metabolism in *V. breoganii* 1C10

V. breoganii 1C10 is a marine bacterium containing twelve putative alginate lyases in its genome. In this work, we have shown that it contains eleven functional alginate lyases. Through over-expression and purification, we were able to show that

these enzymes have optimal activity under marine seawater conditions, as has been shown with the alginate lyases from *V. splendidus* 12B01 and 13B01. Thus, differences between these enzymes must lie with another factor.

In characterizing these enzymes, we determined the enzyme kinetics for each enzyme. We found that the K_m parameter varies over an 8.5-fold range, while the turnover number varies over a 7.9-fold range. This demonstrates a wide range under which the 1C10 lyases are active during growth on alginate. A similar finding is found with the 13B01 lyases; a 7.5-fold range in K_m and 30-fold range in turnover number were found. In *V. splendidus* 12B01, a similar range in kinetic parameters were also found with a 5.6-fold range in K_m and 11.8-fold range in turnover number. A difference between these organisms can be found in how well they secrete alginate lyases to degrade alginate. 12B01 has been shown to secrete alginate lyases at much lower levels than 13B01, indicating less degradation of alginate in culture conditions. 1C10 was shown to have a secreted alginate lyases activity similar to 13B01. This demonstrates that 1C10 is able to degraded alginate with similar efficiency as 13B01.

In comparing the alginate lyase enzymatic activity of 13B01 and 1C10, a marked difference in turnover number can be seen between these strains. The most active 1C10 lyase (AlyJ) has a turnover number of $7 \pm 1\ s^{-1}$, while AlyG, AlyE, and AlyD from 13B01 has a turnover numbers of $18 \pm 0.7\ s^{-1}$, $18.8 \pm 0.7\ s^{-1}$, and $11.2 \pm 0.4\ s^{-1}$, respectively. The other lyases in both strains have turnover numbers less than $5\ s^{-1}$. Comparing the 13B01 lyases to the 1C10 lyases, one would expect much lower secreted enzymatic activity than the experimentally determined difference. This demonstrates that the *in-vitro* enzymatic activity of each lyase is not sufficient to predict how a host organism degrades alginate.

An explanation for the enhanced secreted alginate lyase activity found in 1C10 might lie in its lyase dyad specificity. 13B01 alginate lyases were demonstrated to have a more diverse dyad specificity than 12B01. The addition of AlyG to 13B01 brought M-G dyad specificity to this organism. The action of AlyG on 13B01 was demonstrated to reduce secreted alginate lyase activity more than 4-fold. Therefore, the increased dyad specificity in 13B01 is expected to influence the large secreted alginate lyase activity in 13B01. In *V. breoganii* 1C10, we have shown lyases with M-M, G-M, G-G, and M-G

dyad specificity. Additionally, several enzymes were shown to have more promiscuous specificity with two dyad preferences. Like 13B01, we expect 1C10 to degrade alginate efficiently due to the wide variety of dyad specificities found in its alginate lyases.

A model can be now be formulated describing the influence alginate lyases have on their host organism. *V. splendidus* 12B01 has been demonstrated to have poor secreted alginate lyase activity which influences its slower alginate metabolism. *V. splendidus* 13B01 has evolved to degrade alginate at high levels by expressing its alginate lyases at higher levels than 12B01 and incorporation of a novel alginate lyase, AlyG. AlyG was shown to significantly increase the secreted alginate lyase activity of 13B01, most probably due to high activity and a novel dyad specificity. *V. breoganii* 1C10 has also evolved to degrade alginate efficiently through secretion, but considering the enzymatic activity of its alginate lyases, one would not expect the large alginate degradation that we demonstrated. Rather, *V. breoganii* 1C10 expresses many alginate lyases that have modest activity to efficiently degrade alginate. 1C10 and 13B01 both serve as solutions to the problem of degrading alginate within a competitive environment. 13B01 evolved its alginate degradation by the incorporation of a single enzyme to significantly improve alginate degradation. 1C10 evolved its alginate degradation by utilizing many enzymes to degrade alginate. Each of those enzymes had enzymatic activity lesser than the 13B01 alginate lyases.

Chapter 6 Figures and Tables

Table 6.1. Optimal environmental conditions and enzyme kinetics of alginate lyases.

	AlyA	AlyB	AlyD	AlyE
pH	8.5	9	8	N/D
Temperature (°C)	25	20	25-30	N/D
NaCl (mM)	400	50	400	N/D
K_m (μM alginate)	150 ± 20	36 ± 7	21 ± 5	N/D
V_{max} (μM s^{-1})	0.08 ± 0.01	0.36 ± 0.02	0.24 ± 0.02	N/D
Turnover (s^{-1})	0.4 ± 0.05	2.0 ± 0.1	2.0 ± 0.2	N/D
Specificity	M-M	G-M	G-G	G-M/G-G
	AlyH	AlyI	AlyJ	AlyL
pH	10	7	7.5	N/D
Temperature (°C)	25	25	25	N/D
NaCl (mM)	400	100	100	N/D
K_m (μM alginate)	60 ± 20	68 ± 8	180 ± 60	N/D
V_{max} (μM s^{-1})	0.34 ± 0.05	0.47 ± 0.02	1.4 ± 0.2	N/D
Turnover (s^{-1})	3.2 ± 0.5	4.5 ± 0.2	7 ± 1	N/D
Specificity	M-M	G-G	G-G	G-M/M-M
	AlyM	AlyN	AlyO	
pH	7.5	9.5	8	
Temperature (°C)	25	25	25	
NaCl (mM)	250	150	400	
K_m (μM alginate)	20 ± 3	50 ± 10	130 ± 30	
V_{max} (μM s^{-1})	0.02 ± 0.01	0.16 ± 0.01	0.09 ± 0.01	
Turnover (s^{-1})	0.06 ± 0.03	0.89 ± 0.06	0.7 ± 0.08	
Specificity	G-M	G-G/M-G	M-G/M-M	

Table 6.2. Predicted locations of alginate lyases within its host.

	Cellular Location
12B01 AlyA	Outer Membrane
12B01 AlyB	Extracellular
12B01 AlyD	Extracellular
12B01 AlyE	Extracellular
1C10 AlyA	Outer Membrane
1C10 AlyB	Extracellular
1C10 AlyD	Extracellular
1C10 AlyE	Extracellular
1C10 AlyH	Extracellular
1C10 AlyI	Extracellular
1C10 AlyJ	Extracellular
1C10 AlyK	Extracellular
1C10 AlyL	Periplasm
1C10 AlyM	Extracellular
1C10 AlyN	Extracellular
1C10 AlyO	Extracellular

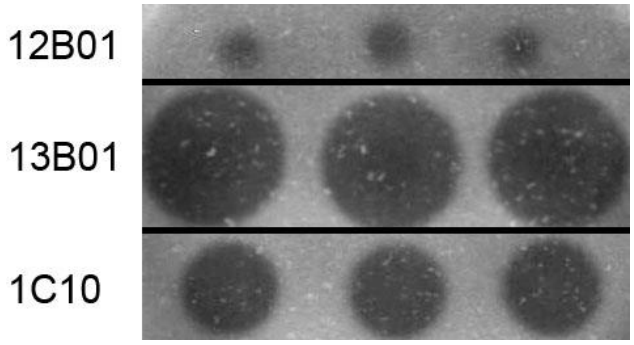

Figure 6.1. Secreted alginate lyase activity. *V. splendidus* 12B01 and 13B01 and *V. breoganii* 1C10 were spotted on the surface of a marine broth plate supplemented with 0.25% alginate. Following incubation, the alginate lyase degraded alginate was identified by overlaying the plate with cetylpyridinium chloride.

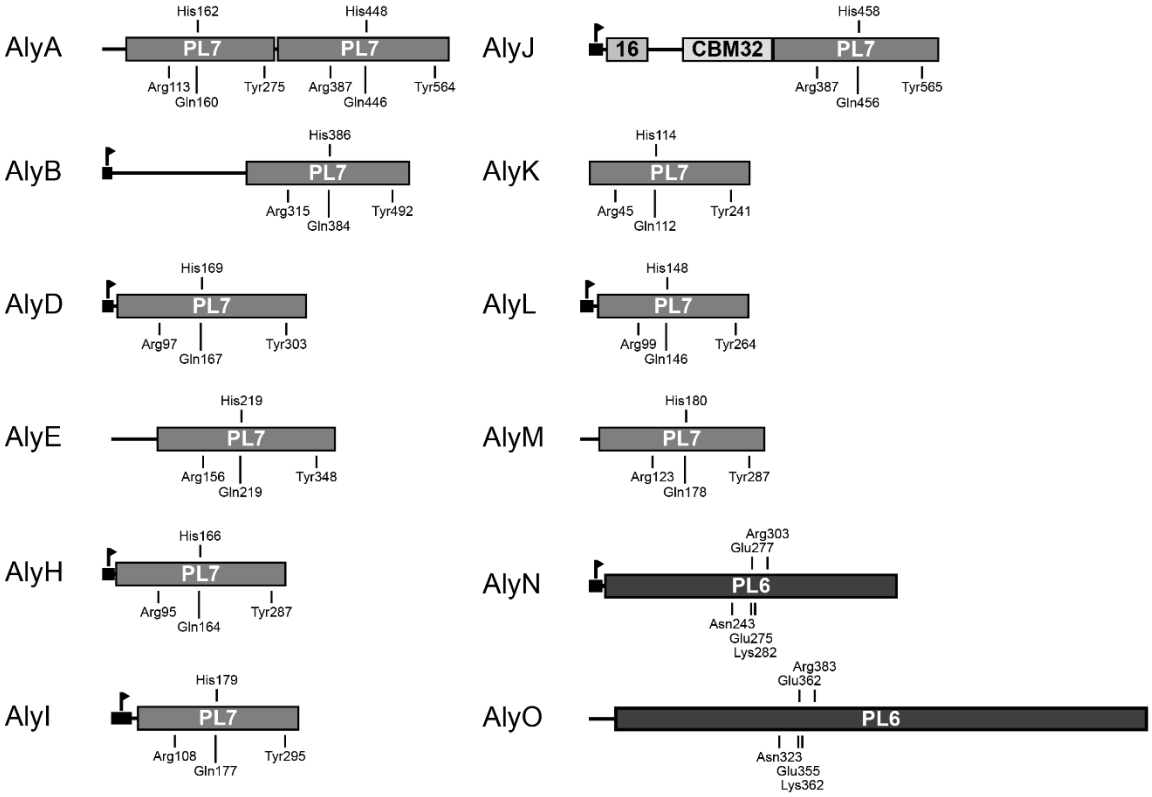

Figure 6.2. Domain structure of alginate lyases AlyA, AlyB, AlyD, AlyE, AlyH, AlyI, AlyJ, AlyK, AlyL, AlyM, AlyN, and AlyO from *V. breoganii* 1C10. The indicated amino acid residues are hypothesized catalytic sites. PL7 is the polysaccharide lyase family 7 domain, while PL6 is the polysaccharide lyases family 6 domain. The domain indicated by 16 is the carbohydrate-binding module family 16 domain, while CBM32 is the carbohydrate-binding module family 32 domain. Each signal peptide is indicated with a flag.

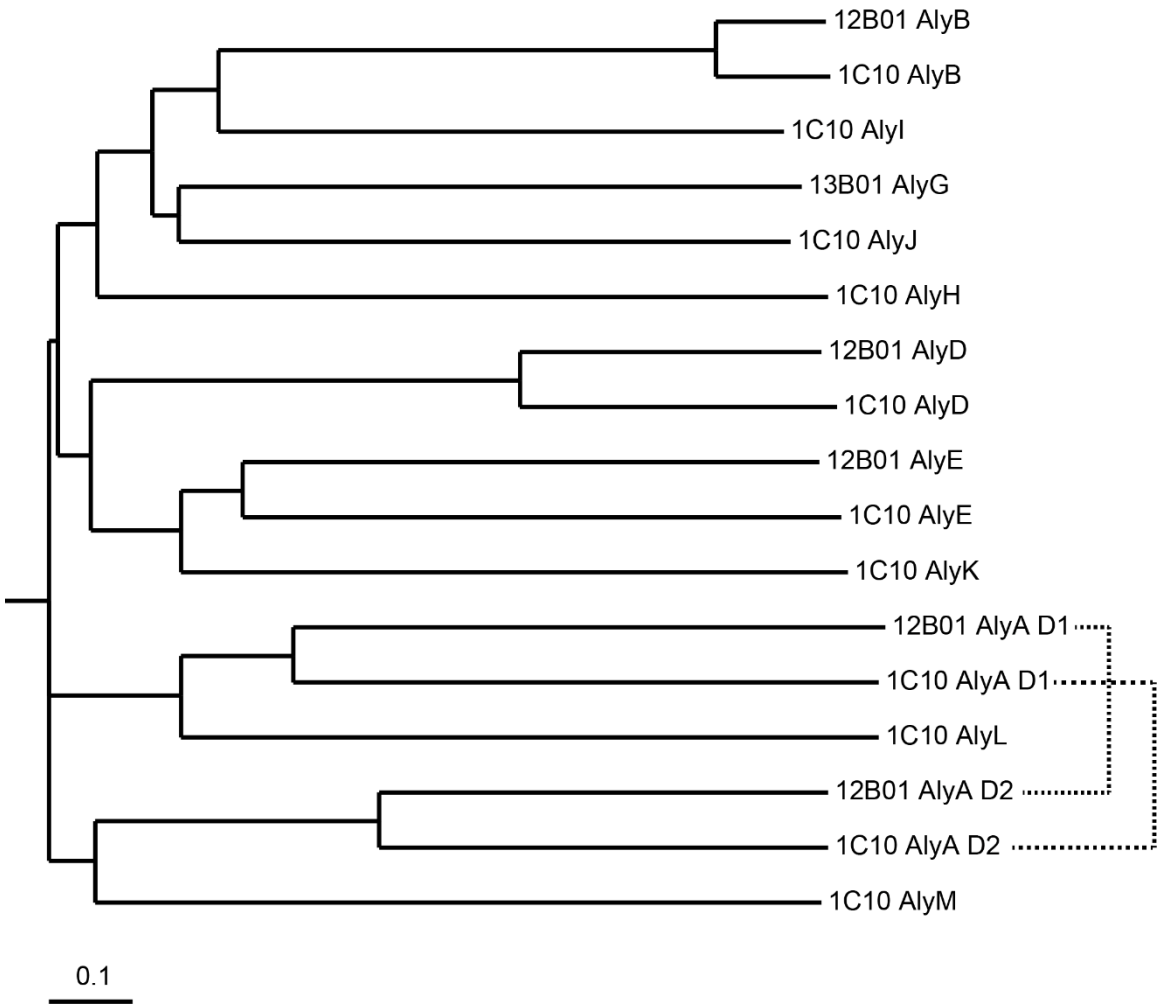

Figure 6.3. Phylogenetic tree of *V. breoganii* 1C10 alginate lyases containing PL7 domains. The *V. splendidus* 12B01 alginate lyases AlyA, AlyB, AlyD, and AlyE were included for comparison and have been analyzed previously **(Chapter 3)**. The *V. splendidus* 13B01 alginate lyase AlyG was also included for comparison as has been previously analyzed **(Chapter 4)**. The distance between enzymes indicates the similarity of each amino acid sequence. The dotted line connects the two domains of each AlyA.

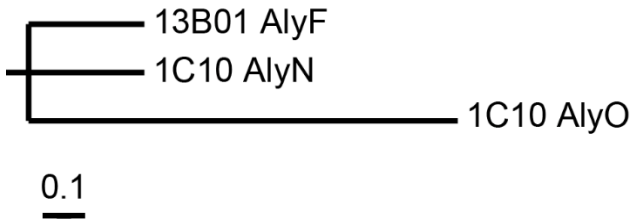

Figure 6.4. Phylogenetic tree of *V. breoganii* 1C10 alginate lyases containing PL6 domains. The *V. splendidus* 13B01 alginate lyase AlyF was also included for comparison as has been previously analyzed **(Chapter 4)**. The distance between enzymes indicates the similarity of each amino acid sequence.

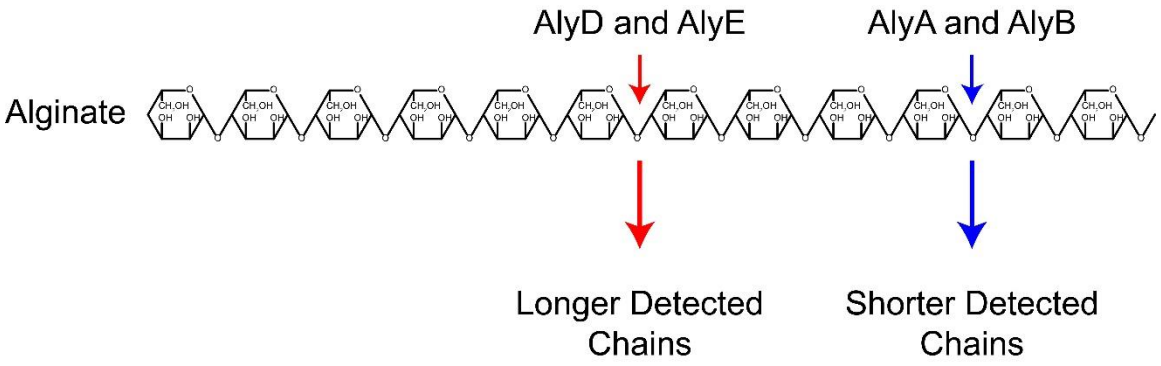

Figure 6.5. Model of the action of AlyA, AlyB, AlyD, and AlyE in *V. splendidus* 12B01. AlyA and AlyB are hypothesized to attack closer to the ends of alginate than AlyD and AlyE due to the smaller sizes of degraded alginate.

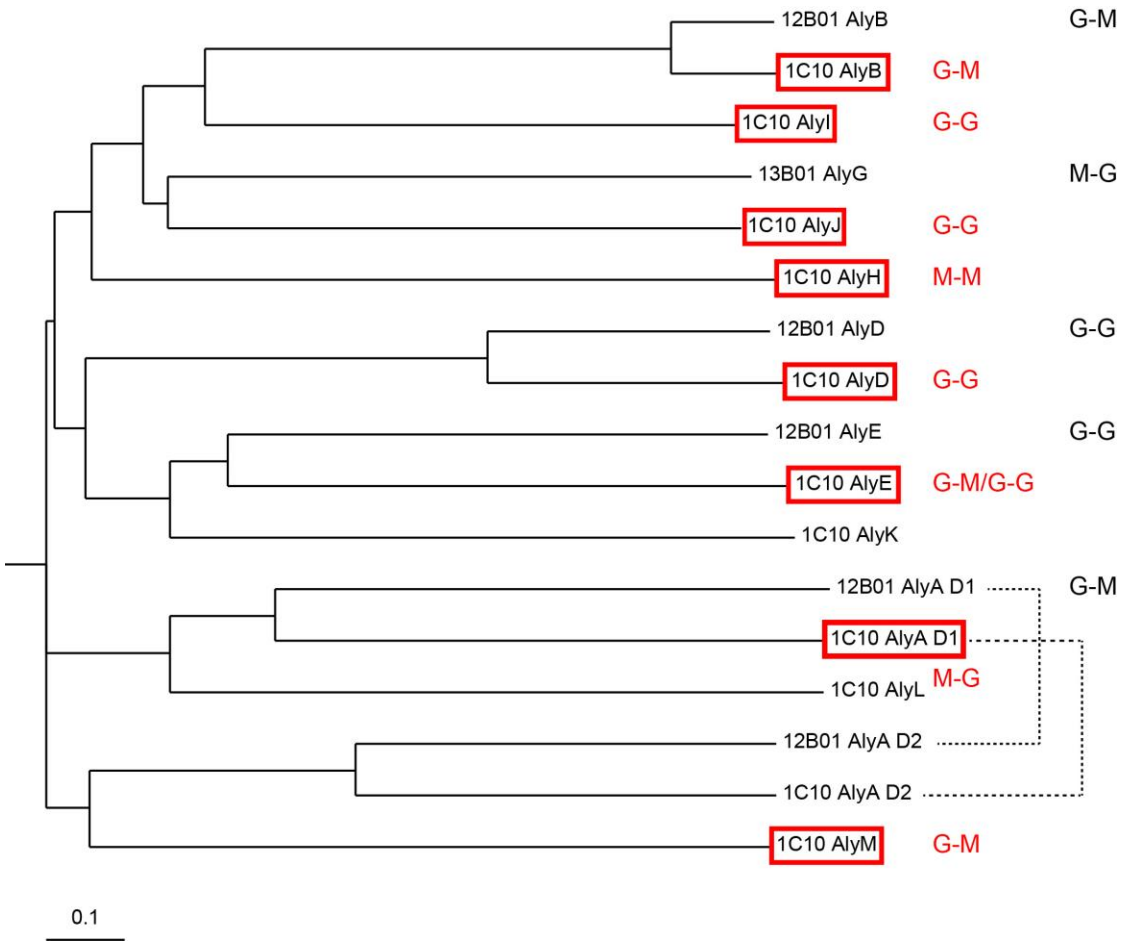

Figure 6.6. Phylogenetic tree of *V. breoganii* 1C10 alginate lyases containing PL7 domains. The *V. splendidus* 12B01 alginate lyases AlyA, AlyB, AlyD, and AlyE were included for comparison and have been analyzed previously **(Chapter 3)**. The *V. splendidus* 13B01 alginate lyase AlyG was also included for comparison as has been previously analyzed **(Chapter 4)**. The distance between enzymes indicates the similarity of each amino acid sequence. The dotted line connects the two domains of each AlyA. To the right of each enzyme, the dyad specificity is listed. Boxed enzymes are the 1C10 lyases.

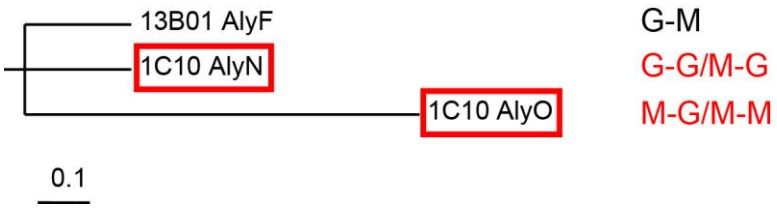

Figure 6.7. Phylogenetic tree of *V. breoganii* 1C10 alginate lyases containing PL6 domains. The *V. splendidus* 13B01 alginate lyase AlyF was also included for comparison as has been previously analyzed **(Chapter 4)**. The distance between enzymes indicates the similarity of each amino acid sequence. To the right of each enzyme, the dyad specificity is listed. Boxed enzymes are the 1C10 lyases.

Chapter 7 Conclusions and future work

7.1 Conclusions and Contributions

In this work, we have studied the metabolism of alginate and laminarin by the marine bacteria *V. splendidus* 12B01, *V. splendidus* 13B01, and *V. breoganii* 1C10. 12B01 and 13B01 were shown to degrade and metabolize alginate, which 1C10 was able to degraded and metabolize alginate and laminarin. Each of these organisms expresses and secretes enzymes which facilitate the metabolism of alginate or laminarin by degrading these polymers into smaller fragments. These fragments are then broken up into their constitutive monomers and metabolized by metabolic pathways within the organisms. In order to utilize these pathways in metabolic engineering to produce biofuels from alginate and laminarin, we must understand how the host organism degrades these carbon sources. The enzymes that accomplish this were investigated in this work. To this end, we developed a pipeline for the characterization of degrading enzymes by over-expressing and purifying these enzymes. Then, we could determine under what conditions these enzymes were active and their activity. This information would then allow a comparison of these enzymes to inform metabolic engineering of alginate and laminarin degrading microorganisms.

We found that the laminarinases from *V. breoganii* 1C10 are expressed in the presence of laminarin and we could over-express and purify these enzymes **(Chapter 5)**. We demonstrated that these enzymes readily degrade laminarin, which liberates glucose monomers, thus laminarinases would easily be implemented in a metabolic engineering strategy. No additional metabolic pathways are necessary for the metabolism of laminarin; rather, only expression and secretion of the 1C10 laminarinases is required in an engineered host. We also demonstrated that these enzymes are optimally active at typical culture conditions, so these enzymes are expected to be active in the extracellular environment.

We also investigated the alginate lyases from *V. splendidus* 12B01 and 13B01 and *V. breoganii* 1C10. These enzymes could also be over-expressed and purified **(Chapter 3, Chapter 4, and Chapter 6)**. We have shown that these enzymes are also

enzymatically active under conditions amenable for industrial fermentation. These enzymes can be expressed and purified for *in-vitro* alginate degradation or expressed in recombinant organisms for fermentation. Since the constituent monomer of alginate is not natively fermented by industrial organisms (4, 12), additional metabolic engineering is needed to produce a strain capable of consuming alginate as the sole carbon source. However, we do expect that the analyzed enzymes will be relevant to industrial fermentation since they are active at typical culture conditions. These enzymes were shown to have a high salt tolerance, so these alginate lyases will be robust across culture and degradation conditions.

7.2 Future Direction: Laminarinases from *V. breoganii* 1C10

We found the laminarinases from *V. breoganii* 1C10 were conditionally expressed in the presence of laminarin, when compared to growth on glucose, indicating that 1C10 can precisely sense its surroundings. We found that LamA, LamB, LamC, and LamD degraded laminarin with a turnover number between 0.69 and 6.1 s^{-1}. Therefore, these enzymes can be incorporated in a metabolic strain for the production of ethanol. Genetic manipulation of *V. breoganii* 1C10 is complicated by the high salt requirements for this organism, so typical electroporation techniques have so far been unsuccessful. Genetic manipulations of its chromosome are feasible, as is recombinant gene expression, however, these techniques are laborious. Additionally, this strain has not been modified for fermentation, since it has only recently been isolated. Therefore, future work on laminarin utilization for the production of ethanol should target expression of laminarinases in a typical metabolic engineered host.

The bacterium *E. coli* is a member of the class γ-proteobacteria, along with *V. breoganii*. Further, the families Vibrionaceae and Enterobacteriaceae are closely related, simplifying transfer of enzymes between family members. Thus, LamA, LamB, LamC, and LamD are expected to be easily transferred to the host *E. coli* for expression and secretion in an engineered strain. Enzymes from the *Vibrio* genus have previously been expressed in *E. coli* (12), so we expect these laminarinases to allow the degradation of laminarin by *E. coli*. We demonstrated that LamA, LamB, LamC, and LamD have

enzymatic activity have greater than other characterized laminarinases (52), however the 1C10 laminarinases were found to have lower activity than another studied laminarinase (56). However, we present a picture of all the laminarinases from an organism. Additionally, we demonstrated that the 1C10 laminarinases degraded a non-laminarin storage glucan, indicating their versatility in other 1,3-linked glucans.

We do not yet understand the role each laminarinase plays in the degradation of laminarin. Since the laminarinases were shown to have similar enzymatic rates and each degrades laminarin individually, we expect that the laminarinases exhibit a synergistic influence on one another. Cellulose degrading enzymes have been shown to exhibit synergy in activity (122), so this serves as a model for to understanding if the 1C10 laminarinases influence another during laminarin degradation, and then, quantifying their influence on another.

In this work, we have only sought to characterize the set of laminarinases from a single marine organism. Since we target metabolic engineering of a strain to degrade laminarin and metabolic convert the feedstock into ethanol, we must get a wider understanding of laminarases from marine organisms. To this end, more laminarinases from marine organisms must be identified, and these can be fed into the enzyme characterizing pipeline outlined in this work. As more enzymes are characterized, a broader understanding of the role laminarinases play in laminarin degradation can be developed, which will further broaden the search space for metabolic engineering. Additionally, a rational protein engineering strategy could be simultaneously implemented to improve the enzymatic activity of the characterized laminarinases. Domain exchange between cellulases has been demonstrated to improve their temperature stability and enzymatic activity (123, 124). This technique could be utilized to improve the stability and enzymatic activity of laminarinases to produce enzymes for *in-vitro* laminarin degradation at high temperatures.

7.3 Future Direction: Alginate lyases from *V. splendidus* 12B01 and 13B01 and *V. breoganii* 1C10

Through RT-PCR analysis of gene expression levels we found that both *V. splendidus* 12B01 and 13B01 conditionally regulate the expression of their alginate lyases in response to alginate in their surroundings. We found that 13B01 is more sensitive to alginate since its alginate lyases are expressed at higher levels at lower alginate loadings. 13B01 serves as a more attractive host for further metabolic engineering, due to the more sensitive regulatory system for alginate lyase expression. If these elements were transferred to a new host, we would expect more efficient alginate lyase expression and secretion than what was found in previous 12B01 metabolic engineering (12). Alternatively, future metabolic engineering can utilize know secretion signals in *E. coli* to efficiently secrete these enzymes, independent of enhanced regulatory signals. As 13B01 contains an additional high activity lyase, AlyG, 13B01 metabolic engineering is expected to result in larger alginate degradation than 12B01 engineering with either strategy.

In our work on *V. breoganii* 1C10 lyases, we identified additional alginate lyases which showed high activity. These lyases were not as active as the 13B01 lyases, however, 1C10 was demonstrated as having high secreted alginate lyase activity. Thus, the 1C10 lyases are expected to cooperate with one another to efficiently degraded alginate. 1C10 contains lyases which alone do not majorly contribute to the significant secreted alginate lyase activity, but rather we hypothesize that synergistic action between lyases with broad dyad specificity result in the significant secreted lyase activity. This hypothesis can be confirmed by conducting pairwise incubation of alginate lyases with alginate and then determining the activity. This will allow a determination of the effect of synergy on the 1C10 lyases. From this, we expect to determine which lyases act in concert to degrade alginate that allow 1C10 to degrade alginate effectively. Along with this effort, determination of synergy between alginate lyase should also include the lyases of *V. splendidus* 12B01 and 13B01. An interesting course this experiments could take is to determine synergy between an enzyme with high activity and one with low activity.

Singularly, many enzymes appear to have little contribution to the secreted alginate lyase activity, however, these enzymes might simply show weak activity when incubated alone. A corollary to this hypothesis lies in the matching of enzymes with differing dyad specificity. Could pairing weak and strong enzymes that differ in dyad specificity allow for enzymatic activity greater than the sum of its parts?

Our analysis of the PL7-containing enzymes from *V. splendidus* 12B01, *V. splendidus* 13B01, and *V. breoganii* 1C10 allow us to begin to enumerate enzymes which we predict could be developed in an enzyme mixture to degrade alginate *in-vitro*. Cellulase cocktails have been developed (125) which mix together enzymes from disparate sources to develop strains capable of fermenting cellulose into ethanol. Similar engineering can be done with the alginate lyases discussed in this work. The combination of the alginate lyases AlyB from 12B01; AlyD, AlyE, and AlyG from 13B01; and AlyI and AlyJ from 1C10 are expected to provide a full dyad specificity coverage. Additionally, these enzymes were determined to exhibit fast alginate degradation (turnover numbers ranging from 4 to 18 s^{-1}).

Since we have established enzymes which exhibit fast alginate degradation and broad substrate specificity, can we further engineer these enzymes to degrade alginate even more efficiently and with higher temperature tolerance? In a similar procedure to that proposed to engineer the 1C10 laminarinases, we can exchange the alginate lyase domains of AlyB, AlyE, AlyE, AlyG, AlyI, and AlyJ to seek these improvements. Overall this work demonstrates that alginate lyases are a diverse class of enzyme that can be utilized for industrial degradation of marine feedstocks for the production of ethanol and other biofuels. We have only scratched the surface of the potential of these enzymes and we hope that work continues to engineer hosts to degrade alginate and laminarin for the production of fuels and value added chemicals.

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