# CLONING AND MOLECULAR CHARACTERISATION OF FOUR ALGINATE LYASE GENES FROM *VIBRIO MIDAE* SY9, AN ENTERIC BACTERIUM FROM THE ABALONE *HALIOTIS MIDAE*

**by**

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A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Molecular and Cell Biology, Faculty of Science, University of Cape Town, South Africa.

Cape Town
October 2010

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# ACKNOWLEDGEMENTS

A special thank you to my supervisor, Associate Professor Vernon Coyne, for his support, guidance and advice throughout the duration of this project. Thank you for the financial assistance over the years and for reading my thesis numerous times.

Thank you to the National Research Foundation, The University of Cape Town and The Harry Crossley Foundation for providing me with financial assistance at various times.

A warm thanks to all the members, past and present, of the Marine Biotechnology Unit. It really was a pleasure working with all of you. Thank you for the laughs and fun times we shared and for the advice, support and encouragement along the way.

I am grateful to all the people who make the department of Molecular and Cell Biology tick. Thanks to the departmental assistants, administrative and academic staff for all their mostly behind-the-scenes work. The department would not run as smoothly without you. A special thanks to Di James and Pae-yin of the MCB DNA sequencing unit and MCB DNA service, respectively.

I am especially grateful to Dr Suhail Rafudeen for his invaluable advice on real-time RT PCR and recombinant protein techniques. Thank you for always having an open door and for your encouragement. I would also like to thank Mohammed Jaffer and Dr Brandon Weber at the Electron Microscope Unit for their expert assistance with the immunolocalisation studies, protein purification and ELISA assays. Thank you to Lynthia Paul in the anaerobe unit for her valuable advice and for reading my thesis.

Thank you to all my friends and family for your support throughout my lengthy studies. I know it is not always easy being friends with or being related to a scientist. Thank you to my twin sister Michelle for your constant support and encouragement; you have always been an inspiration to me. Thank you to my parents, Rod and Cindy, for providing me with all the opportunities you have, for believing in me, and for letting me do what makes me happy even when you can't understand it. You have been great parents and role models. Thank you.

To my amazing husband, Justin. Thank you for your love, for believing in me and encouraging me every step of the way. If it wasn't for you I would have given up a long time ago. Thank you for putting up with a sometimes tired and cranky or an unfathomably ecstatic wife and for always knowing what to say. I look forward to making our dreams come true together.

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## ABSTRACT

Alginate is a linear, un-branched polysaccharide of (1-4)-linked β-$_D$-mannuronate (M) and its C-5 epimer, α-$_L$-guluronate acid (G). These uronic acids are arranged in three different block types in the alginate polymer; poly-M, poly-G or poly-MG. Alginate lyases are enzymes that utilize a β-elimination reaction to depolymerise the alginate polymer resulting in cleavage of the (1-4)-*O*-glycosidic linkage between monomers and the formation of an unsaturated uronic acid at the new non-reducing terminus. Alginate lyases have been isolated from a wide range of sources including marine invertebrates and marine bacteria, which often produce more than one alginate lyase enzyme.

*Haliotis midae* is the commercially important abalone species found along the South African coast. Over-fishing and poaching of this species has led to a depletion of the naturally occurring populations and closure of the recreational and commercial fisheries. Abalone farming was initiated and has rapidly increased in response to the increasing demand for this delicacy. However, there are many problems associated with abalone aquaculture, the most significant being disease and the slow growth rates of the animals. The use of probiotics in abalone aquaculture is a potential solution to both of these problems.

Erasmus *et al*. (1997) showed bacteria resident in the gut of *H. midae* were able to degrade complex polysaccharides and suggested that bacterial enzymes play a digestive role in *H. midae* and could positively affect the growth rate of farmed abalone. Macey and Coyne (2005) isolated *Vibrio midae* SY9 from the digestive tract of *H. midae* and demonstrated the bacterium's ability to degrade alginate, gelatin and other proteins, and starch. They showed that when administered as a cocktail of three probiotic strains in a formulated feed, *V. midae* SY9 increased the growth rate of farmed *H. midae* and improved the survival of animals challenged with the pathogen *Vibrio anguillarum*.

Following on from the work of Erasmus and Macey, it would be interesting to determine the role of the *Vibrio midae* SY9 alginate lyase enzyme/s in the probiotic effect of *V. midae* SY9 on farmed *H. midae*. However, in order to investigate this, several other objectives must first be met. It is important to know the characteristics and properties of the *V. midae* SY9 alginate lyase/s and how they function in alginate degradation by *V. midae* SY9 prior to attempting to assign their role in seaweed degradation by *H. midae*. How the alginate lyase/s function to degrade alginate in *V. midae* SY9 might provide clues as to how they might be important in seaweed degradation by *H. midae*. The primary aim of this study was to clone, sequence and characterise the alginate lyase/s of *V. midae* SY9 and to begin to understand the role they play in alginate degradation by this bacterium.

In order to characterise the alginate lyase/s of *V. midae* SY9, three *V. midae* SY9 genomic libraries were screened. The full length sequences of four adjacent alginate lyase genes were identified and named *alyVMI*, *alyVMII*, *alyVMIII* and *alyVMIV*. A putative oligogalacturonate specific porin, a putative pectin degrading protein and a putative deoxygluconokinase were found adjacent to the alginate lyase genes. AlyVMI and AlyVMII were predicted to be ~81 kDa and AlyVMIII and AlyVMIV were predicted to be ~54 kDa and ~57 kDa, respectively. AlyVMIII and AlyVMIV contain the alginate lyase conserved sequence motifs RXELR, QIH and YFKAGXYXQ, where X is any residue, and could therefore be placed into polysaccharide lyase family 7 (PL-7). AlyVMI and AlyVMII do not contain any of the alginate lyase conserved sequence motifs and were placed in family PL-17. It was also determined that *alyVMI* and

*alyVMII* are co-transcribed. This is the first study to report four alginate lyase genes adjacent to each other and to other genes involved in carbohydrate metabolism on a bacterial chromosome.

Alginate lyase genes *alyVMI*, *alyVMII*, *alyVMIII* and *alyVMIV* were cloned into the expression vector pET-29a. Western hybridisation analysis demonstrated that *alyVMI*, *alyVMII* and *alyVMIV* were expressed in the host *E. coli* BL21 (DE3) pLysS and were histidine tagged. AlyVMI and AlyVMII were purified using nickel affinity chromatography, while AlyVMIV could not be purified to homogeneity. Polyclonal antibodies raised against purified recombinant AlyVMI and AlyVMII recognized both AlyVMI and AlyVMII. Pre-absorption of anti-AlyVMI and anti-AlyVMII against *E. coli* BL21 (DE3) pLysS cellular proteins containing alyVMII and AlyVMI, respectively, rendered the antibodies specific for the protein to which they were raised. The antibodies recognized the native and recombinant forms of the proteins and the predicted, recombinant and native molecular weights of AlyVMI and AlyVMII were comparable.

Gene expression studies were conducted to determine whether the *V. midae* SY9 alginate lyase genes are regulated at the transcriptional, translational or post-translational level. *V. midae* SY9 was cultured in alginate media with and without glucose. Cell growth was monitored by absorbance at 600 nm throughout the growth experiment. Real-time reverse transcriptase polymerase chain reaction (RT PCR) was used to monitor the mRNA transcript levels and an indirect ELISA assay was used to monitor the intracellular levels of AlyVMI and AlyVMII over the course of the growth experiment. Alginate lyase activity of the culture supernatants and soluble cell lysates was determined using the thiobarbituric acid assay, while the reducing sugar content of the culture media was determined using the dinitrosalicylic acid assay. The mRNA transcripts of the four genes were found not to be significantly regulated by the presence of glucose in the culture medium, and neither were the intracellular protein levels of the enzymes. However, enzyme activity was only detected when the reducing sugar levels of the culture media were low. Thus, it is suggested that the alginate lyase genes of *V. midae* SY9 are regulated post-translationally under the experimental conditions tested, the mechanism of which remains to be determined.

*Vibrio midae* SY9 was negatively stained using methylamine tungstate and shown to be a rod-shaped bacterium with a single polar flagellum. Transmission electron microscopy of ultra-thin sections of resin embedded or cryo-fixed *V. midae* SY9 immunolabeled with either anti-AlyVMI or anti-AlyVMII antibodies revealed the presence of gold particles in the cytoplasm and periplasm of the bacterium. However, it remains to be determined whether AlyVMI, AlyVMII or both alginate lyases are present in the cytoplasm and periplasm.

Overall, the current study has provided the foundation for further characterisation of the alginate lyases of *Vibrio midae* SY9 and for realising the long term aim of investigating the role of the alginate lyase enzymes of *V. midae* SY9 in seaweed digestion in *H. midae*. The use of effective probiotics will have a positive impact on abalone mariculture in South Africa as a result of improved growth rates and increased resistance to disease of farmed animals fed probiotics.

# ABBREVIATIONS

| | |
|---|---|
| α | alpha |
| β | beta |
| λ | lambda |
| Ψ | psi |
| µ | micro |
| µg | micro gram(s) |
| µl | microliter(s) |
| µm | micrometer(s) |
| µM | micromolar |
| °C | degrees Celsius |
| % | percentage |
| | |
| ABC | ATP binding cassette |
| ADP | adenosine 5' diphosphate |
| AMP | adenosine 5' monophosphate |
| AMPS | ammonium persulphate |
| ASW | artificial sea water |
| ATP | adenosine 5' triphosphate |
| AUBF | adenosine-uridine binding factor |
| | |
| bp | base pair(s) |
| BLAST | basil local alignment search tool |
| BSA | bovine serum albumin |
| | |
| C-terminus | carboxyl terminus |
| cAMP | 3'-5' cyclic adenosine monophosphate |
| CCR | carbon catabolite repression |
| cDNA | complementary deoxyribonucleic acid |
| CF | cystic fibrosis |
| CIP | calf intestine alkaline phosphatase |
| cm | centimeter(s) |
| Cm | chloramphenicol |
| $C_T$ | fluorescent threshold value |
| CTAB | hexadecyltrimethylammonium bromide |
| | |
| Da | Daltons |
| DEPC | diethyl pyrocarbonate |
| $dH_2O$ | distilled water |
| DIG | digoxigenin |
| DNA | deoxyribonucleic acid |
| DNase | deoxyribonuclease |
| dNTPs | deoxy-ribonucleoside triphosphates (dATP, dCTP, dTTP or dGTP) |
| DNS | dinitrosalicylic acid |

| | |
|---|---|
| DRAG | dinitrogenase reductase-activating glycohydrolase |
| DRAT | dinitrogenase reductase ADP-ribosyltransferase |
| EC | enzyme commission number |
| EDTA | ethylenediaminetetra-acetic acid |
| ELISA | enzyme-linked immunosorbent assay |
| EU | European union |
| FT | Fourier transform |
| FSG | fish skin gelatin |
| g | Gram(s) |
| GRAS | generally regarded as safe |
| h | hour(s) |
| ICR | ion cyclotron resonance |
| IPTG | isopropyl-β-D-thiogalactopyranoside |
| k | kilo |
| kb | kilobase(s) |
| kDa | kilodaltons |
| L | liter(s) |
| LA | Luria agar |
| LB | Luria broth |
| LC | liquid chromatography |
| LPS | lipopolysaccharide |
| m | meter(s) |
| M | molar |
| MA | marine agar |
| MALDI | matrix assisted laser desorption/ionisation |
| MB | marine broth |
| mg | milligram(s) |
| min | minutes |
| ml | milliliter(s) |
| mm | millimeter(s) |
| mM | millimolar |
| mol | mole(s) |
| mRNA | messenger RNA |
| MS | mass spectrometry |
| MW | molecular weight |

| | |
|---|---|
| N-terminus | amino terminus |
| NCBI | National Centre for Biotechnology Information |
| ng | nanogram(s) |
| nm | nanometer(s) |
| NTC | no template control |
| OD | optical density |
| o/n | overnight |
| ORF | open reading frame |
| p | plasmid |
| PAGE | polyacrylamide gel electrophoresis |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| PEG | polyethylene glycol |
| PL | polysaccharide lyase |
| pmol | picomole |
| poly(M) | poly-mannuronate |
| poly(G) | poly-guluronate |
| PTS | phospho*enol*pyruvate:sugar phosphotransferase system |
| RBS | ribosome binding site |
| REST | relative expression software tool |
| RNA | ribonucleic acid |
| RNase | ribonuclease |
| rpm | revolutions per minute |
| rRNA | ribosomal RNA |
| RT | reverse transcription |
| s | second(s) |
| SDS | sodium dodecyl sulphate |
| S.E. | standard error |
| sp. | species |
| SSC | sodium chloride tri-sodium citrate buffer |
| T | time |
| TA | tris-acetate buffer |
| TAE | tris-acetate-EDTA buffer |
| TBA | thiobarbituric acid |
| TBS | tris-buffered saline |
| TE | tris-EDTA buffer |
| TEM | transmission electron microscope |
| TEMED | N, N, N', N'-tetramethylethylenediamine |
| TOF | time of flight |
| Tris | tris(hydroxymethyl)aminomethane |

| | |
|---|---|
| U | units |
| UV | ultra violet |
| v | volume |
| VSCFA | volatile short-chain fatty acid(s) |
| X-gal | 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside |

# Chapter 1

## General Introduction

### CONTENTS

## 1.1. Alginate

### 1.1.1. Structure and properties

Alginate is a linear, un-branched polysaccharide of (1-4)-linked β-$_D$-mannuronate (M) and its C-5 epimer, α-$_L$-guluronate (G) (Gacesa, 1988). Although these uronic acids are similar in structure, they adopt different chair conformations to accommodate the bulky carboxyl group to be in the energetically favourable equatorial position (Figure 1.1 a, b). The uronic acid monomers are arranged in three different block types in the alginate polymer (Figure 1.1 c, d, e). The blocks can be homopolymeric, consisting of continuous mannuronate or guluronate residues (poly-M or poly-G), or they can be heteropolymeric and consist of alternating residues (poly-MG). All three block types may be present in a single alginate molecule and are linked by random M and G residues. Four different glycosidic bonds can be present in an alginate molecule, namely M-M, G-M, M-G or G-G (Draget *et al.* 2005; Ertesvåg *et al.* 1998). The geometry of the poly-M, poly-G and poly-MG regions are substantially different because of the conformation of the monomers and their linkages in the polymer (George and Abraham, 2006). Poly-G regions are buckled while poly-M regions have an extended ribbon shape.

Alginate has the ability to form gels in the presence of divalent or multivalent cations, in particular $Ca^{2+}$ (Gacesa, 1988). Monovalent and $Mg^{2+}$ ions do not induce gel formation (Rees and Welsh, 1977). When two poly-G regions are aligned next to each other, a diamond-shaped hole is formed which has dimensions perfect for the binding of calcium ions (George and Abraham, 2006). Divalent cations bind to poly-G regions in a highly co-operative manner and gel networks are formed by the dimerizing of alginate chains with many other chains. The ratio of the two uronic acids (M:G) and their sequence determines the gelling characteristic of the alginate molecule in the presence of calcium ions (Caswell, 1989). Alginates rich in $_L$-guluronate form gels that are strong but brittle with good heat stability, while alginates rich in $_D$-mannuronate form softer but more elastic gels with good freeze-thaw behaviour (Penman and Sanderson, 1972; Rees and Welsh, 1977; Matsumoto *et al.* 1992).

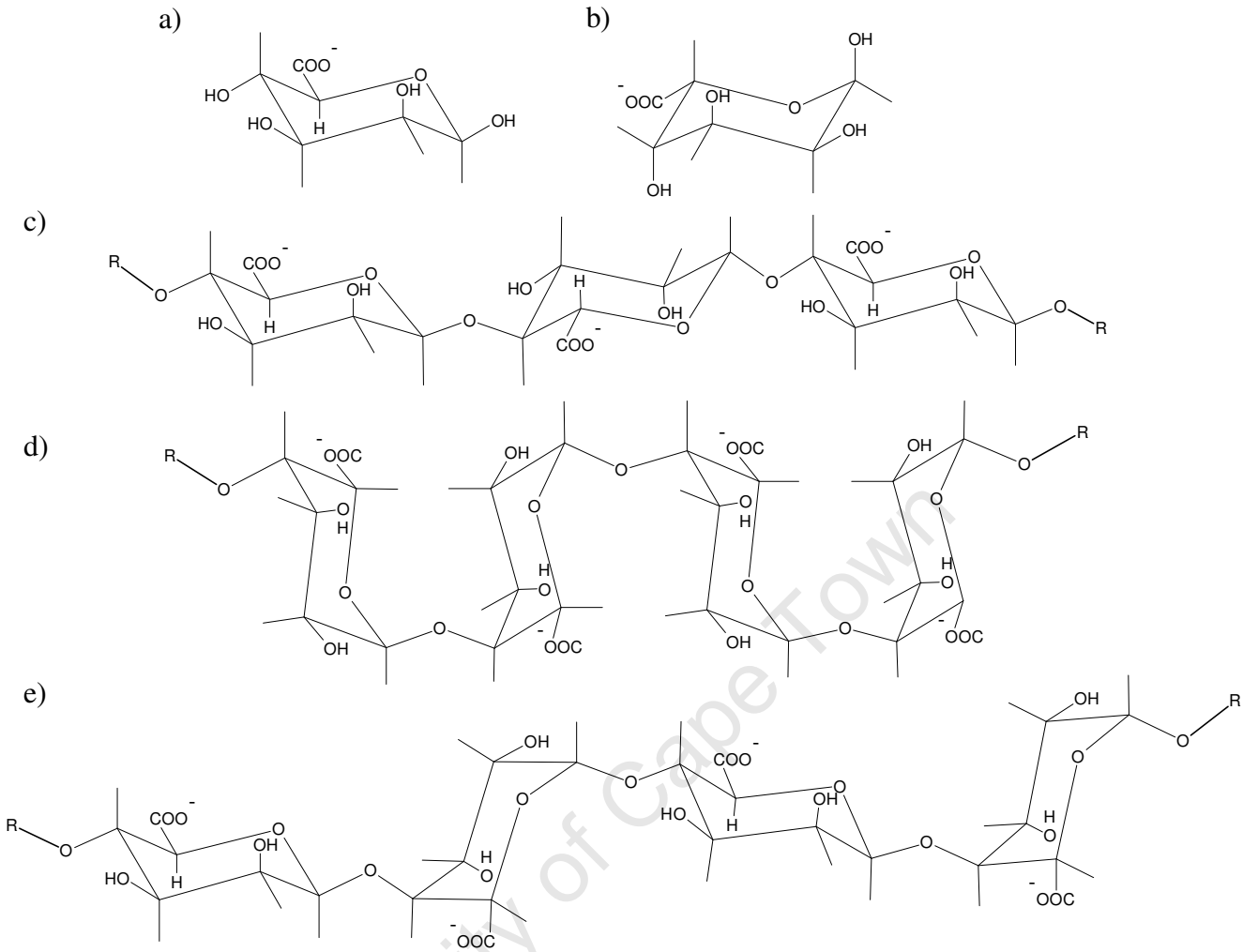


**Figure 1.1.** The structure of alginate. β-$_D$-mannuronate (a), α-$_L$-guluronate (b), polymannuronate (c), poylguluronate (d) and heteropolymeric poly(MG) (e). Adapted from Draget *et al* 2005 and Gacesa, 1988. These figures were drawn using ChemSketch (http://www.acdlabs.com).



### 1.1.2. Occurrence and function

Alginate is found as a gelling polysaccharide in the cell walls and intercellular material of marine brown algae, the *Phaeophyceae*, and comprises between 30% and 40% of their dry weight (Draget *et al*. 2005; Gacesa, 1992). About 60% of the cell wall material of the brown alga *Fucus distichus* is composed of alginate (Doubet and Quatrano, 1982). Alginate in brown algae functions as a structure-forming component (Andresen *et al*. 1977; Draget *et al.* 2005). The

alginate gel matrix present in the intercellular material gives algae mechanical strength and flexibility (Andresen *et al*. 1977).

Some bacteria can also produce alginate. These include *Azotobacter vinelandii* (Gorin and Spencer, 1966), *Azotobacter chroococcum* (Lawson and Stacey, 1954), the opportunistic pathogen *Pseudomonas aeruginosa* (Linker and Jones, 1966) and other Pseudomonads. When facing nutrient deprivation, *Azotobacter* species form metabolically dormant, desiccation-resistant cysts, where the bacteria are surrounded by a protective alginate-containing coat (Kennedy *et al*. 1992; Sadoff, 1975). Alginate produced by mucoid *Pseudomonas aeruginosa* is a key virulence factor in cystic fibrosis (Govan and Deretic, 1996; May *et al*. 1991). The cystic fibrosis lung is colonized by *P. aeruginosa* and consequently a biofilm is formed where the cells are embedded in alginate. Alginate forms a barrier against phagocytic cells and antibiotics, and allows for adhesion and biofilm formation (Govan and Deretic, 1996).

Bacterial alginates differ from algal alginates in that the $\beta$-$_D$-mannuronate residues may be *O*-acetylated at the O-2 or/and the O-3 position, with the O-2 position being preferred (Gacesa, 1988; May *et al.* 1991). Alginate produced by Pseudomonads is synthesized with only $\beta$-$_D$-mannuronate and lack $\alpha$-$_L$-guluronate residues (Govan and Deretic, 1996). *O*-Acetyl modification shields mannuronate residues from epimerization thereby regulating the degree of epimerization (Davidson *et al*. 1977b; May *et al*. 1991). *P. aeruginosa* alginate isolated from cystic fibrosis patients is highly acetylated and may be the reason for the lack of repeating guluronate residues. Acetylation of alginate affects the water-binding properties and ion-binding selectivity of the polymer (Wong *et al*. 2000).

### 1.1.3. Applications

Alginate is a commercially important polysaccharide with many applications in biotechnology and the food industry. Applications for alginate have been found in the food, textile, medical, pharmaceutical, scientific and other industries and are based on its ability to retain water, its gelling, viscosifying and stabilizing properties (Draget *et al*. 2005).

The food industry consumes approximately 50% of the alginate produced (Sabra *et al*. 2001). Alginate has no nutritional value and is generally regarded as safe (GRAS) for use as an additive in foods (Draget *et al* 2006). Alginate is added to certain foods to improve, modify and stabilize the texture. Examples include use as a stabilizer in ice-cream and other processed dairy products; as an emulsifier in salad dressings; and as a thickener and gelling agent in pet food (Chapman and Chapman, 1980; Draget *et al* 2006; Sandford and Baird, 1983). Alginate also has uses in the paint, waterproofing, cosmetic, ceramics, biomedical and pharmaceutical industries. Alginate oligosaccharides have been shown to promote root growth in higher plants such as barley, maize and lettuce (Hu *et al*. 2004; Iwasaki and Matsubara, 2000; Tomoda *et al*. 1994).

An interesting application is the immobilization of living cells in spheres of calcium alginate (Smidsrød and Skjåk-Bræk, 1990). Alginate-immobilized cell systems are used as biocatalysts in many processes; examples include the production of ethanol by yeast cells and the production of monoclonal antibodies from hybridoma cells (Sabra *et al*. 2001). Alginate gel immobilized cells have also been used for cell transplantation. Pancreatic Langerhans islet cells encapsulated in alginate/poly-$_L$-lysine capsules were shown to reverse type I diabetes in large animals (Basta *et al*. 1995; Soon-Shiong *et al*. 1993, 1994). In this application, the alginate capsule acts as a barrier between the transplanted cells and the host immune system. Cell transplantation using alginate micro-encapsulation has also been investigated for the replacement of dopaminergic neurons for the treatment of Parkinson's disease (Yasuhara and Date, 2007). Immunoisolated transplants can also act as active drug delivery systems (George and Abraham, 2006; Leung *et al*. 2010). An example is the potential co-encapsulation of multiple anti-tubercular drugs with a sustained release profile for the treatment of tuberculosis (Ahmed and Khuller, 2008).

## 1.2. Alginate lyases

### 1.2.1. General characteristics

Polysaccharide lyases are carbon-oxygen lyases that cleave polysaccharides containing (1-4)-linked uronic acids by a β-elimination reaction (Anderson, 1998). Alginate lyases utilise a β-elimination reaction to depolymerise the alginate polymer resulting in the cleavage of the (1-4)-*O*-glycosidic linkage between monomers and the formation of an unsaturated uronic acid, 4-deoxy-$_L$-*erythro*-hex-4-ene pyranosyluronate, at the new non-reducing terminus (Gacesa 1988, Gacesa 1992; Wong *et al.* 2000). Some alginate lyases act specifically on the poly-β-$_D$-mannuronate blocks of alginate and some are specific for the poly-α-$_L$-guluronate blocks. Other alginate lyases are non-specific and degrade both blocks and the random MG blocks.

Alginate lyases are single subunit enzymes consisting of a single polypeptide chain and are typically endolytic (Gacesa, 1992). Few exolytic enzymes have been described (Brown *et al.* 1991; Doubet and Quatrano, 1984; Nakada and Sweeny, 1967). An example is HdAlex from *Haliotis discus hannai* that cleaves alginate at the second glycosidic linkage from the reducing terminus producing an unsaturated disaccharide (Suzuki *et al.* 2006). Some enzymes require divalent cations for activity and others do not and in some cases activity is enhanced in the presence of cations (Wong *et al.* 2000).



### 1.2.2. β-Elimination reaction mechanism

β-Elimination reaction mechanisms are thought to be utilised when the products of enzymic digestion are to be further metabolized (Gacesa, 1988). A three step mechanism for the enzymatic depolymerization of alginate has been proposed (Figure 1.2) (Gacesa 1987, 1992). First, the negative charge of the carboxyl group of the substrate must be neutralised. This may occur through the formation of a salt bridge with a positively charged side chain of an amino acid residue, such as lysine, in the active site of the enzyme. An enolate anion intermediate is then formed by the general base-catalyzed abstraction of a proton from C-5 of the uronic acid.

Aspartate, glutamate, histidine, lysine and cysteine residues are able to act as general bases and are candidate amino acids for proton abstraction at C-5. Lastly, a proton is transferred to the oxygen resulting in the formation of a double bond between C-4 and C-5 and the elimination of the 4-*O*-glycosidic bond. It is assumed that a different amino acid residue acts as the proton donor of the leaving group or that a proton is derived directly from the solvent. The unsaturated uronic acid, 4-deoxy-$_L$-*erythro*-hex-4-ene pyranosyluronate, is the resulting oligosaccharide whether the elimination reaction occurs with β-$_D$-mannuronate or α-$_L$-guluronate.

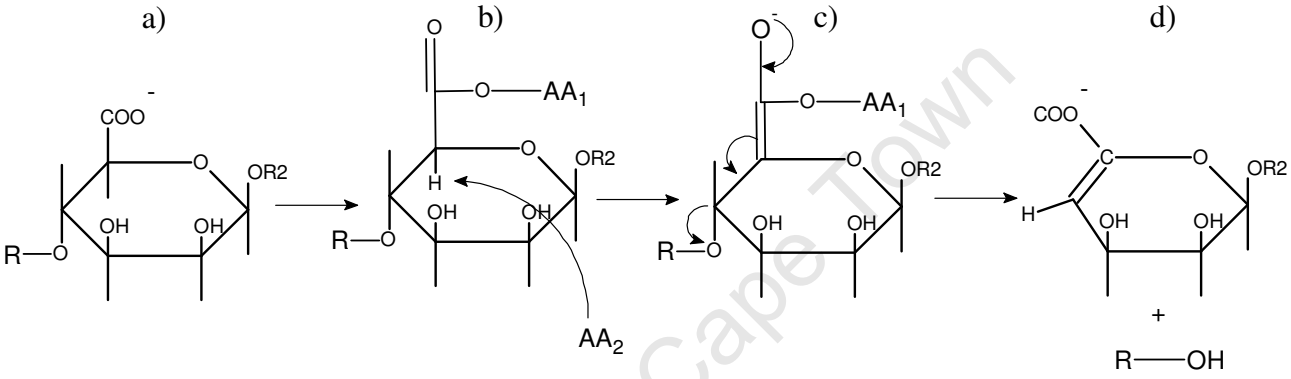


**Figure 1.2.** Three step β-elimination mechanism of alginate lyases. Mannuronate residue in alginate (a), neutralised negative charge of carboxyl group of substrate, general base catalyzed proton abstraction and formation of a resonance stabilized enolate anion (b), transfer of electrons (c) and cleavage of glycosidic bond and double bond formation (d). AA1 and AA2 refer to amino acid residues in the active site of the enzyme. Adapted from Gacesa, 1992 and Svanem *et al*. 2001. Structures were drawn using ChemSketch (http://www.acdlabs.com).



The reaction mechanism of the lyases and mannuronan C5-epimerases are proposed to be the same except in the final step (Gacesa, 1987). In the epimerase reaction the proton at C-5 is replaced stereoselectively instead of the elimination of the 4-*O* substituent group. There is evidence to suggest that the proton abstracted from C-5 is not the same proton that is replaced in the final step of epimerization (Ludoweig *et al*. 1961). This means that the amino acid residue which acts as the general base is not the same residue that acts as the proton donor in the final step.

### 1.2.3. Detection of activity

Methods for detecting alginate lyase activity can be qualitative or quantitative. Qualitative methods are based on the solubility characteristics of alginate and generally incorporate alginate into a solid culture media and are used for identifying native or heterologous (e.g. in *E. coli*) alginate lyases produced by bacteria. Gacesa and Wusteman (1990) described a 'simple plate assay' for the detection of enzyme activity and substrate specificity. Bacteria cultured on alginate-containing media were flooded with solutions of cetylpyridinium chloride or ruthenium red. Regions where alginate had been degraded were detected as clearing zones against an opaque white or dark red background, respectively. The alginate substrate was also substituted with poly(M) or poly(G) rich alginate polymers allowing for the simultaneous detection of activity and substrate specificity. Sawabe *et al*. (1995) modified this assay by incubating the cultures for 5 days before flooding the bacteria with 70% ethanol. Peciña and Paneque (1994) developed a slab-gel electrophoretic method for determining alginate lyase activity after SDS polyacrylamide gel electrophoresis (PAGE). The alginate substrate was included in the acrylamide gel and proteins renatured after electrophoresis. Gels were stained by flooding with cetylpyrdinium chloride.

Quantitative detection methods are based on the formation of unsaturated uronic acids. The formation of cleavage products can be directly followed by an increase in absorbance at 230-240 nm, the UV region specific for the carbon-carbon double bond (-C=C-) (Østgaard, 1993; Preiss and Ashwell, 1962; Michaud *et al*. 2003). Intact alginate and monosaccharides are not detected by this assay (Preiss and Ashwell, 1962). In the thiobarbituric acid (TBA) assay, a chromophore is produced after periodate oxidation of the double bond and subsequent reaction with thiobarbituric acid (Weissbach and Hurwitz, 1959). The chromophore absorbs between 545 and 550 nm. Intact alginate does not react and is not detected by this assay (Preiss and Ashwell, 1962). The assay is sensitive and specific, and has low interference from compounds normally present in crude cell extracts (Gacesa, 1992).

The most sensitive technique, but also most difficult to quantify, is viscometry (Wong, 2000; Gacesa 1992). Another colorimetric method is the orcinol assay which is based on the stability of intact alginate to brief heating in alkali (Preiss and Ashwell, 1962). The intermediate reducing oligosaccharides and monosaccharide end products give negligible reaction with orcinol after alkali treatment.

### 1.2.4. Classification

There are different basis for classifying or grouping alginate lyases. The enzyme commission classification system (IUBMB, 2009, http://www.chem.qmul.ac.uk/iubmb/enzyme/) classifies enzymes according to the chemical reaction they catalyze. The enzyme commission, or EC, number is a four digit code describing the overall reaction the enzyme catalyses (Webb, 1992). The first number (class) describes the basic reaction type, the second and third numbers describe the reaction in additional detail and the final number describes the substrate specificity. Alginate lyases are designated 4.2.2.- since they belong to the lyase class (4), cleave a carbon-oxygen bond (2) and act on polysaccharides (2). The final number differs based on the substrate specificity of each alginate lyase. Alginate lyases are classified as EC 4.2.2.3, $\beta$-$_D$-mannuronate lyase if they are active on poly-M rich alginate, or as EC 4.2.2.11, $\alpha$-$_L$-guluronate lyase if they are active on poly-G rich alginate.

The EC classification system does not consider protein sequence and structure which are important for catalysis (Michaud *et al*. 2003; Nagano, 2005). Polysaccharide lyases have been further classified based on amino acid sequence similarities. The carbohydrate-active enzymes (CAZy) database (http://www.cazy.org) classifies enzymes into families based on protein sequence and structure similarities (Cantarel *et al*. 2009). The classifications correlate with enzyme mechanisms and protein fold more than with enzyme specificity since they are based on amino-acid sequence similarities. Alginate lyases belong to polysaccharide lyase families PL-5, -6, -7, -14, -15, -17 and -18 (http://www.cazy.org). Alginate lyases, EC 4.2.2.3 are found in PL-5, -6, -7, -14, -17 and -18; whereas alginate lyase, EC 4.2.2.11 are only found in PL-7. Enzymes belonging to Family PL-5 have a narrow substrate specificity for poly(M) regions of alginate whereas PL-7 enzymes have diverse substrate specificities (Yamasaki *et al*. 2005).

Alginate lyases can also be grouped into three molecular weight classes: the small lyases ranging from 25-30 kDa, the medium-sized lyases with a molecular weight of ~40 kDa and the large lyases greater than 60 kDa (Osawa *et al*. 2005).

### 1.2.5. Sources and some individual characteristics

Alginate lyases have been isolated from a wide range of sources including brown algae, marine fungi, marine invertebrates, Gram positive soil bacteria, Gram negative bacteria, marine bacteria, viruses and bacteriophages (Gacesa, 1992; Wong *et al*. 2000). The following sections describe alginate lyases purified or cloned from various sources as well as some individual characteristics.

#### 1.2.5.1. Brown algae

Alginate lyases have been identified in crude extracts of *Pelvetia canaliculata* and *Laminaria digitata* (Madgwick *et al*. 1973). Alginate lyases have also been identified in extracts of *Colpomenia sinuosa*, *Eisenia bicyclis*, *Endarachne binghamiae*, *Ishige* spp., *Sargassum sagamianum*, *Spatoglossum pacificum* and *Undaria pinnatifida* (Shiraiwa *et al*. 1975). However, none of the genes encoding for any of the enzymes have been cloned or sequenced.

#### 1.2.5.2. Fungi

Alginate lyase activity has been reported for only a few species of marine fungi that were isolated from decaying seaweed. Schaumann and Weide (1990) screened 72 strains of 19 species of marine fungi for their ability to degrade sodium alginate, calcium alginate or alginate gel. Activity was detected in 18 strains belonging to only three species, namely *Asteromyces cruiciatus*, *Corollospora intermedia* and *Dendryphellia salina*. Wainwright and Sherbrock-Cox (1981) reported alginate lyase activity in *Dendryphellia arenaria* and *D. salina*. Sarrocco *et al*. (2004) determined whether terrestrial *Ascomycetes* were lacking in alginate lyase activity. Of the 1,107 isolates screened for the ability to utilise alginate as a sole carbon source, 27 isolates were found to be positive. These isolates belong to three families, *Pleosporaceae* (13 isolates),

*Valsaceae* (7 isolates) and *Ophiostomataceae* (7 isolates). Production of alginate lyases by fungi seems to be an isolate-specific rather than a species-specific trait (Sarocco *et al*. 2004). There has been little research on alginate lyases from fungi and the genes responsible have not been cloned or sequenced. However, a 35 kDa extracellular poly(M) lyase from *Dendryphellia salina* IFO 32139 has been purified and characterised (Shimokawa *et al*. 1997).

#### 1.2.5.3. Marine invertebrates

Alginate lyases have been detected in many marine invertebrates, most often in crude cell extracts. Some of the enzymes have been purified from crude extracts but few have been cloned and sequenced. Alginate lyase activity was detected in the sea hare *Aplysia depilans* and *A. californica* (Boyen *et al*. 1990b), as well as in a simple homogenate of the crystalline style of the mussels *Choromytilus meridionalis* and *Perna perna* (Seiderer *et al*. 1982) and the surf clam *Spisula solidissima* (Jacober *et al*. 1979).

Two alginate lyases were isolated from the hepatopancreas of the sea hare *Dolabella auricula* and found to be specific for poly(M) regions of the alginate molecule (Nisizawa *et al*. 1968). Alginate lyase VI was purified from the hepatopancreas of *Littorina* sp. (Elyakova and Favorov, 1974). The enzyme is 40 kDa and acts endolytically on poly(M) regions of alginate polymers.

One and three alginate lyases, respectively, were purified from the hepatopancreas of the sea snails *Omphalius rusticus* and *Littorina brevicula* (Hata *et al*. 2009). The *O. rusticus* enzyme, OrAly, is 34 kDa, acts on poly(M) regions of alginate molecules in an endolytic manner and belongs to family PL-14 based on partial amino acid sequence. The *L. brevicula* enzymes, LbAly28, LbAly32 and LbAly35, are 28 kDa, 32 kDa and 35 kDa, respectively. LbAly28, LbAly32 and LbAly35 are also poly(M) specific and act endolytically, but could not be placed in an existing polysaccharide lyase family based on their partial amino acid sequences and may belong to a novel polysaccharide lyase family.

Alginate lyase activity has been detected in hepatopancreas extracts of *Haliotis rufescens* (Richard *et al*. 1971), *H. corrugata* (Nakada and Sweeney, 1967) and *H. midae* (Erasmus *et al*. 1997) but the enzyme/s were not purified or the genes cloned or sequenced. The 34 kDa alginate lyase of *Haliotis tuberculata* was partially purified by cation-exchange chromatography and shown to be specific for poly(M) regions of alginate by NMR spectroscopy and HPLC analysis of reaction end products (Boyen *et al*. 1990b; Heyraud *et al*. 1996a, 1996b). The alginate lyase HiAly was purified from the hepatopancreas of the abalone *Haliotis iris* by ammonium sulphate fractionation and column chromatography (Hata *et al*. 2009). The enzyme is 34 kDa and is specific for poly(M) regions of the alginate polymer. The partial amino acid sequence of HiAly placed it in family PL-14.

Two alginate lyases, HdAly and HdAlex, were purified from the hepatopancreas and digestive fluid, respectively, of *Haliotis discus hannai* and the genes subsequently cloned and sequenced from a cDNA library (Shimizu *et al*. 2003; Suzuki *et al*. 2006). HdAly is 30 kDa and HdAlex is 32 kDa. Both enzymes belong to family PL-14 and are specific for poly(M) regions of alginate. However, HdAly cannot degrade oligomannuronates smaller than a tetrasaccharide whereas HdAlex can degrade trisaccharides. Furthermore, HdAly acts endolytically on alginate polymers whereas HdAlex acts exolytically. HdAly and HdAlex share 67% homology, and 85% and 62% homology, respectively, with SP2 alginate lyase from *Turbo cornutus*.

Two endolytic alginate lyases, SP1 and SP2, were purified from the mid-gut gland of the wreath shell, *Turbo cornutus* (Muramatsu *et al*. 1977). The enzymes were found to be isozymes with the same experimental molecular weight of 32 kDa, the same pH optimum and pH stability as well as thermal inactivation temperature (Muramatsu and Egawa, 1980). The enzymes are both specific for poly(M) regions of alginate. Circular dichroism spectra indicated that SP1 and SP2 were most likely composed of β-sheets and denaturation experiments showed that SP2 has a more rigid conformation than SP1 (Muramatsu *et al*. 1984). The primary structure of SP1 and SP2 was subsequently determined (Muramatsu *et al*. 1996). The amino acid sequence of the two enzymes was identical except for two hydrophobic resides in the C-terminus of SP2 which are thought to be responsible for the greater protein stability of SP2.

#### 1.2.5.4. Bacteria

The greatest research effort has been in isolating bacterial alginate lyases. Indeed, more bacterial alginate lyases have been cloned and sequenced than from any other organism (http://www.cazy.org). In 1996 the primary sequence of only six bacterial enzymes was known (Chavagnat *et al*. 1996) and in 2000, 23 sequences were present in the GenBank database (Wong *et al*. 2000). Currently, more than 200 sequences are known and assigned to a polysaccharide lyase family (http://www.cazy.org). Bacterial alginate lyases can be extracellular, periplasmic or intracellular and it is not unusual for bacteria to have more than one alginate lyase. The following sections describe alginate lyases isolated from various bacteria found in different environments.



##### 1.2.5.4.1. Gram-negative soil bacteria

Kennedy *et al* (1992) partially purified alginate lyases from the periplasm of *Azotobacter chroococcum* and *A. vinelandii* and found them both to be poly(M) specific enzymes. Subsequently, the *algL* genes coding for alginate lyase AlgL from *A. chroococcum* and *A. vinelandii* were cloned, sequenced and expressed in *E. coli* (Ertesvåg *et al*. 1998; Peciña *et al*. 1999). The AlgL alginate lyases of *A. vinelandii* and *A. chroococcum* are 43 kDa and 39 kDa, respectively, contain signal peptides, belong to family PL-5 and have 90% identity to each other as well as 63% and 64% identity, respectively, to AlgL of *Pseudomonas aeruginosa*. Both enzymes are poly(M) specific and, depending on the degree of acetylation, are able to act on acetylated bacterial alginate.

The *algE7* gene which codes for a dual alginate lyase/epimerase was also cloned and sequenced from *Azotobacter vinelandii* (Svanem *et al*. 1999). The deduced and experimental molecular weight is 90 kDa and 105 kDa, respectively (Svanem *et al*. 2001). The enzyme was active on M-rich alginates and cleaved G-GM and G-MM bonds after epimerization of M to G. By substitution analysis, it was shown that the same active site catalyzes the epimerase and alginate lyase activities of the enzyme (Svanem *et al*. 2001).

Alginate lyases from several *Pseudomonas* species including *P. aeruginosa* (Schiller *et al*. 1993; Yamasaki *et al*. 2004), *P. syringae* (Preston *et al*. 2000), *Pseudomonas* sp. OS-ALG-9 (Kraiwattanapong *et al*. 1997; Maki *et al*. 1993), and the marine *Pseudomonas alginovora* (Chavagnat *et al*. 1996) have been cloned and sequenced. *Pseudomonas syringae* is a plant pathogen which produces alginate as an exopolysaccharide (Fett *et al*. 1986). The alginate lyase, AlgL, of *P. syringae* contains a signal peptide, is 42 kDa, occurs in the periplasm and is specific for poly(M) regions of alginate (Preston *et al*. 2000). AlgL has 76%, 63%, 61% and 59% sequence similarity to AlgL of *Halomonas marina*, *P. aeruginosa*, *A. chroococcum* and *A. vinelandii*, respectively.

Two alginate lyases have been cloned from *P. aeruginosa*, namely AlgL and PA1167 (Schiller *et al*. 1993; Yamasaki *et al*. 2004). The two enzymes have different properties: AlgL is 39 kDa, preferably degrades poly(M) rich alginate, resides in the periplasm and belongs to family PL-5; whereas PA1167 is 25 kDa, preferably degrades poly(MG) regions of alginate and belongs to family PL-7. AlgL is predicted to have an $\alpha/\alpha$-barrel structure and PA1167 was found to have a $\beta$-sandwich structure (Yamasaki *et al*. 2004). Two alginate lyases, Aly and AlyII, were also cloned and sequenced from *Pseudomonas* sp. OS-ALG-9 (Kraiwattanapong *et al*. 1997, 1999; Maki *et al*. 1993). Both enzymes are intracellular and specific for poly(M) rich regions of alginate, however, Aly is 46.3 kDa and belongs to family PL-6 whereas AlyII is 79 kDa and belongs to family PL-17.

*Sphingomonas* sp. A1 is a yellow pigmented, Gram-negative alginolytic bacterium which was isolated from soil (Yonemoto *et al*. 1991). This bacterium directly incorporates macromolecules into the cell via a pit-dependent transport system, or super channel, which is specific for the macromolecule (Hisano *et al*. 1995). A mouth-like pit is formed on the surface of the cell through the fluidity and reorganization of pleats on the cell surface (Hisano *et al*. 1995). Alginate is concentrated in the pit and captured by the alginate-binding proteins AlgQ1 and AlgQ2 which deliver the molecule to an ATP-binding cassette (ABC) transporter, consisting of membrane-spanning permeases AlgM1 and AlgM2 and ATP-binding protein AlgS, located in the inner membrane which transports it to the cytoplasm (Mishima 2003; Momma *et al*. 2000). Once in

the cytoplasm, the alginate molecule is cooperatively depolymerized to monosaccharides by the action of three endo- and one exo- alginate lyases (Murata *et al*. 2008).

The four expressed, as well as two cryptic, alginate lyases of *Sphingomonas* sp. A1 have been cloned, sequenced and characterised. A1-I, A1-II, A1-III and A1-IV are expressed in *Sphingomonas* sp. A1 and are located in the cytosol. A1-I is 66 kDa and autocatalyses into A1-II (25 kDa) and A1-III (40 kDa) all of which are endolytic (Murata *et al.* 1993). A1-II is specific for poly(G) regions of alginate, requires pentasaccharides as a minimum substrate, produces tri- and tetrasaccharides and belongs to PL-7 (Yoon *et al*. 2000). A1-III is specific for poly(M) regions of alginate, is able to act on acetylated bacterial alginate and belongs to PL-5. A1-I is able to degrade both poly(M) and poly(G) alginate, requires tetrasaccharides as a minimum substrate, produces di- and trisaccharides and belongs to family PL-5+7. A1-IV is an 86 kDa exolytic lyase which converts the oligosaccharide products of A1-I, A1-II and A1-III to monosaccharides and belongs to PL-15 (Hashimoto *et al*. 2000b).

A1-II' and A1-IV' are not expressed in *Sphingomonas* sp. A1 and were identified by homology searches of the completed genome (Hashimoto *et al*. 2005; Miyake *et al*. 2004). A1-II' is a 32 kDa endo-acting enzyme which has broad substrate specificity; i.e. acts on poly(M), poly(G) and poly(MG) regions of alginate, and produces di- and trisaccharides (Miyake *et al*. 2004). A1-II' shares 55.1% identity with A1-II and also belongs to PL-7. A1-IV' is 90 kDa, prefers poly(M) regions of alginate, requires tetrasaccharides as a minimum substrate, releases di- and trisaccharides as degradation products and belongs to PL-15 (Hashimoto *et al*. 2005). A1-IV' shares 28.9% identity with A1-IV but is an endolytic enzyme. The crystal structures of A1-II', A1-III and PA1167 have been solved. A1-II' and PA1167 feature an overall glove-like β-sandwich structure (Yamasaki *et al*. 2004, 2005) whereas A1-III has an $\alpha_6/\alpha_5$-barrel fold (Yoon *et al*. 1999, 2001). It is presumed then that A1-I consists of an N-terminal α/α-barrel and a C-terminal β-sandwich (Murata *et al*. 2008).

The genes required for alginate uptake (AlgM1, AlgM2, AlgS, AlgQ1 and AlgQ2) and depolymerization (A1-I, A1-II, A1-III and A1-IV), are arranged in a cluster on the *Sphingomonas* sp. A1 chromosome and appear to operate as an operon (Momma *et al*. 2000). A

homologous cluster of five open reading frames (ORFs) was identified in *Agrobacterium tumefaciens* strain C58 (Ochiai *et al*. 2006). ORFs Atu3021, Atu3022, Atu3023 and Atu3024 are homologous to AlgS, AlgM1, AlgM2, AlgQ1/AlgQ2 and encode a putative sugar ABC transport system while ORF Atu3025 is homologous to A1-IV and encodes an alginate lyase. No gene homologous to *aly* coding for A1-I, A1-II and A1-III in *Sphingomonas* sp. A1 was found in *A. tumefaciens* C58. The alginate lyase gene *atu3025* was cloned and sequenced and the recombinant protein purified (Ochiai *et al*. 2006). Atu3025 is an intracellular 88 kDa exotype alginate lyase that releases monosaccharides as degradation products and belongs to PL-15. Atu3025 shares 55% identity with A1-IV of *Sphingomonas* sp. A1.

#### 1.2.5.4.2. Gram-positive soil bacteria

Alginate lyase activity has also been detected in Gram-positive bacteria such as *Bacillus alginolyticus* (Nakamura, 1987), *B. chondroitinus* (Nakamura, 1987), *B. circulans* (Hansen *et al*. 1984; Hansen and Nakamura, 1985; Larsen *et al*. 1993), *Clostridium alginolyticum* (Kaiser *et al*. 1968) and *Corynebacterium* sp. ALY-1 (Matsubara *et al*. 1998a, 1998b).

The alginate lyase, ALY-1, of *Corynebacterium* sp. ALY-1 was initially purified from culture supernatant by gel filtration (Matsubara *et al*. 1998b) and subsequently cloned, sequenced and expressed in *E. coli* (Matsubara *et al*. 2000). The enzyme is 27 kDa, specific for poly(G) regions of alginate and belongs to PL-7. The crystal structure of ALY-1 has also been solved and the enzyme has a jelly roll β-sandwich fold (Osawa *et al*. 2005).

#### 1.2.5.4.3. Marine bacteria

Alginate lyase activity has been detected in bacteria associated with the brown alga *Fucus distichus* (Doubet and Quatrano, 1982), *F. evanescens* (Ivanova *et al*. 2002), *Laminaria digitata* (Boyen *et al*. 1990a), *Laminaria japonica* (Sawabe *et al*. 1992; Wang *et al*. 2006) and *Sargassum fliutans* (Preston *et al*. 1985) as well as in bacteria isolated from seawater (Kitamikado *et al*. 1990; Kawamoto *et al*. 2006), the gut of abalone (Sawabe *et al*. 1995, 2004a, 2004b, 2007a) and the gut of sea urchins (Sawabe *et al*. 1995). Some enzymes were purified and characterised and

others have been cloned and sequenced. The genes for some marine bacterial alginate lyses have been identified through homology searches of sequenced genes or genomes although no literature regarding further research performed on the enzymes is available. Examples include *Vibrio shilonii* AK1 (sequenced genes GENBANK accession numbers EDL54548 and EDL54549), *Vibrio* sp. MED 222 (sequenced genes GENBANK accession numbers EAQ52860 and EAQ52861), *Vibrio splendidus* 12B01 (sequenced genes GENBANK accession numbers EAP93062, EAP93063, EAP94921, EAP94922, EAP94925 and EAP94396), *Vibrio splendidus* LGP32 (sequenced genome GENBANK accession numbers FM954972 and FM954973) and *Vibrionales* bacterium SWAT3 (sequenced genes GENBANK accession numbers EDK27096 and EDK27097).

#### 1.2.5.4.3.1 Alginate lyases from marine bacteria isolated from seaweed

Marine bacteria A3 and W3 were isolated from *Fucus distichus* (Doubet and Quatrano, 1982). An alginate lyase was partially purified from each strain (Doubet and Quatrano, 1984). The 100 kDa alginate lyase from A3 is intracellular, specific for mannuronate residues and acts exolytically. The extracellular alginate lyase of W3 was found in the culture supernatant, is specific for guluronate residues, acts endolytically and is 35 kDa.

*Pseudomonas alginovora* was isolated from decaying fronds of *Laminaria digitata* (Boyen *et al*. 1990a). The extracellular poly(G) specific alginate lyase was purified by classical techniques and found to be 28 kDa. The intracellular poly(M) specific lyase was subsequently cloned and sequence by Chavagnat *et al*. (1996). The enzyme was histidine-tagged and purified by nickel affinity chromatography and found to be 24 kDa. *Pseudoalteromonas elyakovii* IAM 14594 was isolated from a decaying frond of *Laminaria japonica* and produces extracellular and intracellular alginate lyases (Sawabe *et al*. 1992). The 32 kDa extracellular alginate lyase was purified from culture supernatant and is active on poly(M), poly(G) and poly(MG) regions of alginate (Sawabe *et al*. 1992, 1997). Four intracellular enzymes were identified: one of which was specific for poly(M), two for poly(G) and one for poly(MG) regions of alginate (Sawabe *et al*. 1998a). These four enzymes were specific for oligosaccharides and are unable to degrade intact alginate. Subsequently, the *alyPEEC* gene coding for the 32 kDa extracellular alginate

lyase was cloned and sequenced (Sawabe *et al*. 2001). The enzyme produces tri- to octaoligouronate products and belongs to family PL-18. Expression of *alyPEEC* in *E. coli* was found to be enhanced by calcium compounds (Sawabe *et al*. 2007b).

*Vibrio* sp. QY101 was isolated from a decaying *Laminaria* thallus and produced two extracellular alginate lyases which were purified (Song *et al*. 2003). One of the enzymes has a broad substrate specificity and is 39 kDa, while the other is specific for poly(G) regions of alginate and is 34 kDa. Subsequently, the *alyVI* gene encoding the 34 kDa enzyme was cloned and sequenced (Han *et al*. 2004). The enzyme has 40.5% identity (and 75.1% similarity) to AlyVGII from *Vibrio halioticoli* and belongs to family PL-7. *Vibrio* sp. YWA was isolated from decaying *Laminaria japonica* and an extracellular alginate lyase purified from the culture supernatant by classical techniques (Wang *et al*. 2006). The enzyme was found to be 62.5 kDa and acted on poly(M) regions of alginate but not poly(G) regions.

*Photobacterium* sp. ATCC 433367 was isolated from *Sargassum fluitans* and found to secrete alginate lyases (Preston *et al*. 1985). A poly(M) specific and a poly(G) specific enzyme was partially purified from the culture supernatant (Brown and Preston, 1991). The mannuronate specific enzyme is 29 kDa and acts endolytically whereas the guluronate specific enzyme is 38 kDa and acts endo- and exolytically. The extracellular mannuronate specific enzyme was subsequently cloned (Brown *et al*. 1991). Malissard *et al*. (1993) cloned and sequenced the intracellular poly(M) lyase. This enzyme is 30 kDa and acts on poly(M) regions of alginate as well as acetylated bacterial poly (M) alginate.

*Saccharophagus degradans* 2-40 was isolated from decaying salt marsh cord grass, *Spartina alterniflora*, in Chesapeake Bay, Virginia, United States of America (Andrykovitch and Marx, 1988). This bacterium is able to degrade many complex polysaccharides including agar, alginate, β-glucan, carrageenan, cellulose, chitin, laminarin, pectin, pullulan, starch and xylan (Ensor *et al*. 1999). The chitinase, agarase and cellulase systems of *S. degradans* 2-40 are well characterised (Howard, 2004; Taylor *et al*. 2006; Whitehead *et al*. 2001). Although the alginate lyase system has not been characterised, the genome of *S. degradans* 2-40 has been sequenced (Weiner *et al*.

2008) and six enzymes belong to family PL-6 and PL-7, and one enzyme belongs to family PL-14, PL-17 and PL-18 (www.cazy.org).

#### 1.2.5.4.3.2 Alginate lyases from marine bacteria isolated from seawater and sediments

*Pseudoalteromonas atlantica* AR06 decomposes brown seaweeds and was isolated from seawater in Japan (Uchida and Nakayama, 1993). *P. atlantica* AR06 produces an extracellular alginate lyase and is able to utilise alginate as a sole carbon source. The gene, *alyA*, coding for an extracellular alginate lyase was cloned and sequenced (Matsushima *et al*. 2010). The enzyme has 76% identity with AlyPEEC from *Pseudoalteromonas elyakovii* IAM 14594 and acts on poly(M), poly(G) and poly(MG) regions of alginate to produce di-, tri, and tetramer oligoalginates. One polypeptide is cleaved at different sites in the N-terminus to produce three active enzymes of 43 kDa, 33 kDa and 30.5 kDa which are present at different stages of cultivation of *P. atlantica* AR06. This suggests that the N-terminus is not important for catalysis.

*Vibrio alginolyticus* ATCC 17749, *Vibrio* sp. AL-9 and *V. harveyi* AL-128 were isolated from seawater (Kitamikado, *et al*. 1990). The extracellular enzymes from *V. alginolyticus* ATCC 17749 and *V. harveyi* AL-128 were purified from culture supernatant using classical techniques (Kitamikado *et al*. 1992; Tseng *et al*. 1992a, 1992c). The enzymes are 47 kDa and 57 kDa, and are specific for poly(M) and poly(G) regions of alginate, respectively. Two alginate lyases were purified from the culture supernatant of *Vibrio* sp. AL-9 by classical techniques (Tseng *et al*. 1992b). One enzyme was specific for poly(M) regions of alginate and the other for poly(G) regions. The enzymes are 31 kDa and 25 kDa, respectively.

Two alginate lyases were cloned and sequenced from *Vibrio* sp. O2 which was isolated from seawater from Mihonoseki harbour, Japan (Kawamoto *et al*. 2006). AlyVOA and AlyVOB have a calculated molecular weight of 32 kDa and 31.4 kDa, respectively, and have 92.3% and 32.6% identity to AlxM of *Photobacterium* sp. ATCC 433367. Both enzymes contain a signal peptide and are specific for poly(M) regions of alginate and belong to PL-7.

*Agarivorans* sp. JAM-A1m and *Vibrio* sp. JAM-A9m were isolated from deep-sea sediments collected in Japan (Kobayashi *et al*. 2009; Uchimura *et al*. 2009). Alginate lyase A1m was purified from culture supernatant by classical techniques (Kobayashi *et al*. 2009). The enzyme is 31 kDa, acts on poly(G) and poly(MG) regions of alginate and is active under high alkaline conditions (pH 10). The gene encoding the enzyme was subsequently shotgun cloned from genomic DNA and sequenced. A1m shares 54% identity with AlyA of *Klebsiella pneumonia* subsp. *aerogenes*. Alginate lyase A1mU was also shotgun cloned from *Agarivorans* sp. JAM-A1m and sequenced (Uchimura *et al*. 2009). A1mU has a calculated molecular weight of 30 kDa and shares 76% identity with AlyVOA of *Vibrio* sp. O2. Both A1m and A1mU contain a signal peptide and belong to family PL-7.

Three alginate lyase genes were cloned from *Vibrio* sp. JAM-A9m by PCR of genomic DNA and sequenced (Uchimura *et al*. 2009). A9mC and A9mL have a calculated molecular weight of 25 kDa and 54 kDa, respectively, and share 77% and 87% identity with an alginate lyase from *Vibrio splendidus* sp. 12B01. A9mT has a calculated molecular weight of 29 kDa and is 57% homologous to AlxM of *Photobacterium* sp. ATCC 433367. A9mT and A9mL contain a signal peptide whereas A9mC does not. Recombinant A9mT was purified by classical techniques. The recombinant enzyme has an experimental molecular weight of 28 kDa, is activated in the presence of salt and is specific for poly(M) regions of alginate.

#### 1.2.5.4.3.3 Alginate lyases from marine bacteria isolated from marine animals

Sawabe and co-workers have isolated many alginolytic strains from the gut of abalone. *Vibrio halioticoli* was isolated from the gut of the Japanese abalone *Haliotis discus hannai* (Sawabe *et al* 1995, 1998b); *Vibrio superstes* was isolated from the gut of the Australian abalone *Haliotis laevigata* and *H. rubra* (Hayashi *et al*. 2003); *Vibrio gallicus* was isolated from the gut of the French abalone *Haliotis tuberculata* (Sawabe *et al*. 2004a); *Vibrio neonatus* and *V. ezurae* were isolated from the gut of the Japanese abalone *Haliotis discus discus*, *H. diversicolor diversicolor* and *H. diversicolor aquatilis* (Sawabe *et al*. 2004b); and *Vibrio comitans*, *V. rarus* and *V. inusitatus* were isolated from the gut of the Japanese abalone *Haliotis discus discus*,

*H. gigantea* and *H. madaka*, as well as from the Californian abalone *H. rufescens* (Sawabe *et al*. 2007a).

Although many alginolytic *Vibrio* strains have been isolated from abalone, the literature only cites the cloning and sequencing of alginate lyase genes from *Vibrio halioticoli* (Sugimura *et al*. 2000). *V. halioticoli* produces six alginate lyases of which four are guluronate specific and two are mannuronate specific. The genes for three of the poly(G) specific lyases, AlyVG1, AlyVG2 and AlyVG3, were cloned and sequenced (Sugimura *et al*. 2000). AlyVG1 has a theoretical molecular weight of 39 kDa and shares 36.2% identity with AlyA of *Klebsiella pneumoniae*. AlyVG2 and AlyVG3 are calculated to be 36 kDa and 27 kDa, respectively, and share 23.9% and 18.7% identity with AlxM of *Photobacterium* sp. ATCC 433367. The three genes were shown to be unlinked on the *V. halioticoli* chromosome by Southern hybridisation analysis.

Alginate lyase activity has been detected in bacteria isolated from other marine invertebrates other than abalone. An example is *Vibrio* sp. YKW-34 which was isolated from the gut of the turban shell, *Turbinidae batillus cornutus* (Fu *et al*. 2007) and *Pseudoalteromonas citrea* KMM 3297 which was isolated from the coelomic fluid of the holothurian *Apostichopus japonicus* (Bakunina *et al*. 2002). The extracellular alginate lyase from *Vibrio* sp. YKW-34 was purified from culture supernatant by classical techniques (Fu *et al*. 2007). The enzyme is 60 kDa, acts on both poly(M) and poly(G) regions of alginate and requires $Na^+$ or $K^+$ ions for activity. Three intracellular alginate lyases, A1I, A1II and A1III, were purified from *Pseudoalteromonas citrea* KMM 3297 by classical techniques (Alekseeva *et al*. 2004). The enzymes are 25 kDa, 79 kDa and 61 kDa, respectively, and act on both poly(M) and Poly(G) regions of alginate. A1I produces tri-, tetra-, and penta- oligouronates, whereas A1II and A1III produce longer fragments. The enzymes are activated by the presence of NaCl, $MgCl_2$ and $MgSO_4$.

#### 1.2.5.5. Bacteriophages and viruses

Alginate lyases have been found to be associated with phages that infect alginate-producing bacteria (Bartell *et al*. 1966; Eklund and Wyss, 1962) and function in facilitating penetration of the phage through the polysaccharide capsule surrounding the host cell (Leiman *et al*. 2007). These enzymes form part of the phage tail-spike assembly (Glonti *et al*. 2010) and are either freely diffusible (Eklund and Wyss, 1962) or bound to the phage particle (Adams and Park, 1956).

Plaques of *Azotobacter* phages are characterised by a distinctive halo surrounding the central area of lysed cells which consists of cells which have had their alginate capsules degraded (Eklund and Wyss, 1962). Davidson *et al*. (1977a) characterised a poly(M) specific enzyme from an *Azotobacter vinelandii* phage. The phage enzyme was shown to be distinct from the host enzyme by its molecular weight, pH optimum, Michaelis constant and stability. The phage enzyme was reported to be ~30-35 kDa and the host enzyme greater than 50 kDa. Pike and Wyss (1977) reported the alginate lyase of Azoto-phage A31 to be ~35-42 kDa.

Bacteriophages associated with mucoid *Pseudomonas aeruginosa* also exhibit the characteristic translucent halo surrounding plaques which do not contain phage (Bartell *et al*. 1966). Only two alginate lyases from *P. aeruginosa* phages have been described (Bartell *et al*. 1966; Glonti *et al.* 2010). The alginate lyase from phage PT-6 was shown to be a freely diffusible 37 kDa enzyme (Glonti *et al.* 2010). Bartell *et al* (1966, 1968) described a *P. aeruginosa* phage alginate lyase that was estimated to be 180 kDa by gel filtration and is likely to be a component of the phage tail spike (Sutherland, 1977).

Virus encoded alginate lyases have only been reported from *Chlorella* viruses (www.cazy.org). CL2 from *Chlorella* virus CVN1 is 39 kDa and has weak homology to the mannuronate lyase SP2 from *Turbo cornutus* (Suda *et al*. 1999). The enzyme vAL1 from *Chlorella* virus CVK2 shares 93% identity with CL2, but was shown not to be an alginate lyase, but rather a novel polysaccharide lyase which cleaves chains of β- or α-1,4-linked glucoronic acids producing 4,5-unsaturated glucuronic acid-containing oligosaccharides (Sugimoto *et al*. 2000, 2004).

### 1.2.6. Protein structure

#### 1.2.6.1. Primary structure

Alginate lyases typically do not share a high level of amino acid sequence homology (Wong *et al*. 2000). Sequence analysis revealed that most residues are hydrophilic, there is a central hydrophilic region, a hydrophobic sequence in the C-terminus and a slightly charged C-terminal end (Wong *et al*. 2000). Although alginate lyases are not highly homologous, some sequence motifs are highly conserved. INNHSYW and WLEPYCALY are motifs present in the central region and C-terminus of 40 kDa alginate lyases (Wong *et al*. 2000). The motif INNHSYW is not found in G-specific or 30 kDa lyases but is conserved as the slightly altered motif, E/FNNVSY, in mannuronate C5-epimerases. It has been suggested that both of these sequence motifs may be alginate or mannuronan binding motifs. RXELR, QIH and YFKAGXYXQ, where X is a variable residue, are conserved sequence motifs found in the N-terminus, central region and C-terminus, respectively, of family PL-7 lyases.

#### 1.2.6.2. Secondary and tertiary structure

The crystal structures of some alginate lyases from family PL-5 and PL-7 have been solved which has provided insight into the residues involved in catalysis as well as the catalytic mechanism of action. Alginate lyases seem to be predominantly composed of either α-helices or β-strands with a final topology based on the dominant secondary structure. Murata *et al.* (2008) found that polysaccharide lyases can be grouped into six folds: parallel β-helix, β-sheet abundant flattened oval disk, α/α-barrel, α/α-barrel + anti-parallel β-sheet, β-sandwich and β-propeller.

Aly-1 from *Corynebacterium* sp., A1-II' from *Sphingomonas* sp. A1 and PA1167 from *Pseudomonas aeruginosa* belong to family PL-7 and have similar overall structures. Aly-1 consists of two α-helices and 14 β-strands arranged in two curved anti-parallel β-sheets which form a jelly-roll β-sandwich structure (Osawa *et al*. 2005). A1-II' consists of four short α-helices and nine β-strands arranged in two large β-sheets which forms an overall glove-like β-sandwich structure (Yamasaki *et al*. 2005). PA1167 consists of three α-helices and 15 β-strands arranged

into two sheets producing an overall glove-like β-sandwich fold (Yamasaki *et al*. 2004). All three proteins feature a rigid active cleft with two short flexible loops which form a lid-like structure over the active cleft. A1-II of *Sphingomonas* sp. A1 resembles A1-II' and PA1167 in primary structure and Murata *et al*. (2008) suggested that A1-II would probably also adopt a β-sandwich structure as a basic frame. A1-III of *Sphingomonas* sp. A1 of family PL-5 was found to consist of 12 α-helices arranged in an $\alpha_6/\alpha_5$-barrel fold (Yoon *et al*. 1999, 2001). By homology modeling using the coordinates for A1-III, Yamasaki *et al*. 2004 found that AlgL of *Pseudoalteromonas aeruginosa* also has an α/α-barrel structure. A1-I of *Sphingomonas* sp. A1 autocatalyses into A1-II and A1-III (Murata *et al*. 1993). It is presumed then that A1-I consists of an N-terminal α/α-barrel and a C-terminal β-sandwich (Murata *et al*. 2008).

#### 1.2.6.3. Structure-function relationships

Family PL-5 enzymes, A1-III of *Sphingomonas* sp. A1 and AlgL of *Pseudoalteromonas aeruginosa*, share common structural features such as an α/α-barrel fold, a lid loop for recognition of substrates or catalytic reaction, aromatic and positively-charged amino acid residues for the binding of uronic acids, and a catalytic tyrosine residue (Murata *et al*. 2008). The lid loop of A1-III covers a tunnel-like active cleft and is flexible in order to capture the substrate (Yoon *et al*. 1999, 2001). The substrate is bound in the active site by hydrogen bonds and van der Waals interactions formed with aromatic and positively-charged amino acid residues present in the active cleft. Tyr246 functions as the proton abstracter and donator in the catalytic reaction through activation by formation of a Tyr68-Tyr246 pair. By mutational analysis, Mikami *et al*. (2002) showed that the movement of the lid loop is essential for the enzymatic reaction through the formation of the Tyr68-Tyr246 pair and thus the activation of Tyr246, the binding of substrate and the release of products. A1-III contains the conserved alginate lyase motif NNHSYW but the catalytic Tyr246 is not the tyrosine residue in NNHSYW (Yoon *et al*. 1999).

The localization of the conserved residues to three adjacent β-strands, A3, A4 and A5, in the center of a large cleft led to the identification of the active cleft of family PL-7 enzymes (Yamasaki *et al*. 2005). YXSELREM was located to strand A3, QIH was located to strand A5 and YFKAGXYXQ was located to strand A4. The side-chains of the three adjacent β-strands in

the active cleft of PL-7 enzymes are extremely well ordered and rigid when compared to side chains on the surface of the protein which suggested that they are important in substrate binding and catalysis (Yamasaki *et al*. 2005). The three step catalytic β-elimination mechanism is described in Section 1.2.2. Ogura *et al*. (2008) proposed that a glutamine, histidine and tyrosine residue are the catalytic residues of *Sphingomonas* sp. A1 alginate lyase A1-II'. Gln189 (Q of QIH) is proposed to neutralise the negative charge on the C6 carboxylate anion, His191 (H of QIH) acts a general base and abstracts a proton from C5 and Tyr284 (**Y** of YFKAGX**Y**XQ) acts as a general acid and donates a proton to the glycosidic bond to be cleaved. Similarly, His104 (H of QIH), Tyr193 (**Y** of **Y**FKAGXYXQ) and Tyr199 (**Y** of YFKAGX**Y**XQ) are proposed to be the catalytic residues of *Pseudomonas aeruginosa* alginate lyase PA1167 (Yamasaki *et al*. 2004). In contrast, Tyr246 of PL-5 *Sphingomonas* sp. A1 alginate lyase A1-III acts as both the proton acceptor and the proton donor (Yoon *et al*. 2001). This is believed to be a novel β-elimination mechanism where one residue acts as the general acid and base.

Family PL-7 enzymes have two short flexible loops which form a lid-like structure over the active cleft through formation of a hydrogen bond between two asparagine residues at the edges of the loops (Ogura *et al*. 2008). Ogura *et al*. (2008) demonstrated by mutation analysis that the flexibility of the loops is essential for substrate binding and accommodation of substrate in the active site of A1-II'. The presence of positively charged residues in the active site allows A1-II' to bind to acidic alginate molecules.

Family PL-7 alginate lyases have diverse substrate specificities. Some act specifically on poly(M), poly(G) or poly(MG) blocks of the alginate molecule. Osawa *et al*. (2005) postulated that the overall structure of the enzyme was not directly associated with substrate specificity, but rather, the local position of substrate binding residues determined substrate specificity. Ogura *et al*. (2008) showed that this was the case in A1-II' which has broad substrate specificity and is able to hydrolyse poly(M), poly(G) as well as poly(MG) blocks of alginate. The binding of M or G in the active site is achieved through recognition of carboxyl groups by specific residues in the active site and appropriate interactions with hydroxyl groups of the substrate. PL-18 alginate lyase AALyase from *Pseudoalteromonas* sp. strain 272 also has broad substrate specificity (Iwamoto *et al*. 2002) and adopts a β-sandwich fold. Residues important for substrate binding in

A1-II' were well conserved in AALyase (Ogura *et al*. 2008). Furthermore, the arrangement of these residues was almost identical in A1-II' and AALyase. Therefore, the structural characteristics of the active site are important for broad substrate specificity in alginate lyases.

### 1.2.7. Biological function

Bacteria which do not produce the polysaccharide alginate but produce alginate lyases are usually able to utilise alginate as a sole carbon source (Wong *et al*. 2000). This indicates that the biological role of alginate lyase enzymes in non-alginate producing organisms is to assist assimilation of alginate molecules for use in general metabolism of the cell.

Molluscan-produced alginate lyases are involved in the degradation of alginate present in the algae they feed on (Inoue *et al*. 2010). The enzymes assist in the assimilation of intracellular algal nutrients and alginate degradation products are used by the mollusc as a carbon source (Hata *et al*. 2009; Rahman *et al*. 2010).

Alginate lyases have a different function in alginate producing organisms which do not use alginate as a carbon source. In *Pseudomonas aeruginosa*, the alginate lyase AlgL is required for alginate production (Albrecht and Schiller, 2005). Jain and Ohman (2005) demonstrated that AlgL forms part of a multiprotein scaffold which directs alginate polymers through the periplasm for secretion across the outer membrane. AlgL has a structural role in the scaffold and degrades free alginate polymers in the periplasm which would otherwise accumulate in the periplasmic space and cause the cell wall to lyse. AlgL was also suggested to have a role in biofilm formation (Boyd and Chakrabarty, 1994). High-molecular weight alginate is required for attachment of cells to surfaces. Increased alginate lyase activity led to an increase in bacterial detachment from surfaces. *Azotobacter vinelandii* forms metabolically dormant cysts which are surrounded by a coat of alginate (Kennedy *et al*. 1992; Sadoff, 1975). The alginate lyase, AlgL, of *A. vinelandii* resides in the periplasm and has been proposed to prime the biosynthesis of alginate molecules, determine the length of the polymers and degrade the cyst coat during germination (Ertesvåg *et al*. 1998).

### 1.2.8. Applications

Alginate lyases have been used to examine the fine structure of algal and bacterial alginates (Heyraud *et al*. 1998; Østgaard, 1993). Alginate lyases have also been used in combination with other enzymes to remove the cell walls of brown seaweeds in the production of protoplasts (Gacesa, 1988; Wong *et al*. 2000). The production of protoplasts is important for the genetic manipulation of seaweed. Species of brown algae from which protoplasts have been isolated successfully include zygotes of *Fucus distichus* (Kloareg and Quatrano, 1987), *Laminaria* species (Boyen *et al*. 1990a), *Laminaria digitata* (Butler *et al*. 1989), *Laminaria saccharina* (Butler *et al*. 1989), and *Macrocystis pyrifera* (Kloareg *et al*. 1989). Recently, protoplasts have been isolated from *Laminaria japonica* using recombinant alginate lyase and cellulase from abalone (Inoue *et al*. 2010). The advantage of using recombinant enzymes for protoplast isolation is that they are pure and free from other contaminating enzymes that may affect protoplast viability and that enzyme activity is consistent (Reddy *et al*. 2008).

There is interest in alginate lyases as a treatment of *Pseudomonas aeruginosa* infections in cystic fibrosis (CF) patients. *P. aeruginosa* biofilms in the CF lung are characterised by a layer of alginate which promotes bacterial attachment to tracheal epithelium as well as tolerance to antibiotics and host defences. Hatch *et al*. (1998) showed that *P. aeruginosa* alginate blocked the diffusion of aminoglycoside antibiotics. Alginate lyase treatment of *P. aeruginosa* biofilms would degrade the exopolysaccharide leaving the cells more vulnerable to host immune defences and antibiotic treatment. Alkawash *et al*. (2006) found that co-administration of alginate lyase from *Bacillus circulans* ATCC15518 with antibiotics increased the efficacy of antibiotic treatment against *P. aeruginosa* biofilms. Bacterial products, leukocytes and other debris, such as DNA, contribute to bronchial thickness (May *et al*. 1991). The combined use of alginate lyase and deoxyribonucleases (DNAse) has also been proposed (Schiller *et al*. 1993). Not all alginate lyases are active against bacterial *O*-acetylated alginate and therefore would not be suitable for therapy purposes. Only a few lyases effective against acetylated alginate have been isolated. These include AlgL from *P. aeruginosa* (Schiller *et al*. 1993), AlgL from *Pseudomonas* sp. QD03 (Xiao *et al*. 2006), AlgL from *Azotobacter vinelandii* (Ertesvåg *et al*. 1998), $AlxM_B$ from *Photobacterium* sp. ATCC 433367 (Malissard *et al*. 1993), A1-I and A1-III

of *Sphingomonas* sp. A1 (Yonemoto *et al*. 1992; Hisano *et al*. 1993) and recently, an enzyme from *P. aeruginosa* phage PT-6 (Glonti *et al*. 2010).

An alternative to antibiotic therapy is the use of bacteriophages (Donlan, 2009). *Pseudomonas aeruginosa* phages produce alginate lyases that are able to degrade the alginate capsule and reduce its viscosity. It has been shown that these enzymes positively influence the adsorption of the phage to the bacterial cell surface and that phages are able to diffuse through the alginate exopolysaccharide matrix. Some of the drawbacks of biofilm control by antibiotic treatment, such as antibiotic resistance and presence of exopolysaccharide matrix, may be overcome by the use of phages (Donlan, 2009; Sutherland *et al*. 2004).

### 1.3. Probiotics in aquaculture

World aquaculture has grown from a production of 1 million tonnes in the 1950s to a production of 51.7 million tonnes with a value of US$ 78.8 billion in 2006 (FAO, 2009). Aquaculture is growing more rapidly than other areas of animal food production. In 2006, aquaculture accounted for 47% of the world's fish food supply and 90% of fish food production in China originates from aquaculture. The production of molluscs was 27% of total global aquaculture production at 14.1 million tonnes and a value of US$ 11.9 billion. Aquaculture accounts for 65% of global mollusc production when capture fisheries are excluded. The importance of aquaculture products will increase as over-harvesting of natural populations continues and as world demand for seafood increases (Kasercodi-Watson *et al*. 2008).



However, there are many difficulties associated with aquaculture. High stocking densities as well as other farming practices lead to increased physical and biological stress experienced by animals which affects their health and growth rate. The high stocking densities in aquaculture, as with any other intensive farming practice, allows for the easy spread of disease and complicates the management of disease outbreaks. The high incidence of disease has led to an increase in the use of antimicrobial drugs which have not only been used for therapy, but also for the prevention of disease (prophylactic use) as well as continuously fed as a growth enhancer (van den Bogaard

and Stobberingh, 2000). The overuse of antibiotics has led to the emergence of resistant bacterial strains due to an increase in selective pressure (Verschuere *et al*. 2000).

Antibiotic resistant strains have originated from shrimp, fish and eel farms (Alcaide *et al*. 2005; Chelossi *et al*. 2003; Karunasagar *et al*. 1994). The concern is that the resistance will be transferred to human pathogens which would pose a human health risk. Indeed, there have been reports of antibiotic resistance genes of farm origin that are now present in human-associated bacteria (Schwarz *et al*. 2001; van den Bogaard and Stobberingh, 2000; Witte, 2000). However, there is a debate about whether this is likely to occur or not, the argument being there is not enough data to support the transferal of resistance genes (Acar *et al*. 2000; Phillips *et al*. 2004).

Despite there being no clear answer, the use of several antimicrobial drugs has been banned by the European Union (EU). The growth promoter, avoparcin, was banned in 1997 and in 2005, the use of all non-therapeutic antimicrobials was banned (Delsol *et al*. 2005; Turnridge, 2004). Since this event, alternatives such as improved husbandry, the use of vaccines and stimulants of the non-specific immune system, as well as the use of probiotics, have yielded positive results, one example being the Norwegian salmon industry (Maroni, 2000; Verschuere *et al*. 2000). The study of probiotics for farmed aquatic animals has increased with the increasing demand for environment-friendly aquaculture (Gatesoupe, 1999).



### 1.3.1. Definition

The term probiotic is derived from the Greek ‘pro bios’ which means ‘pro life’ (Gismondo *et al*. 1999). Lilly and Stillwell (1965) coined the name to describe the substance produce by one microorganism which stimulated the growth of another. This definition was modified by Parker (1974) to “organisms and substances which contribute to intestinal microbial balance”. Fuller (1989) introduced the concept that probiotics are beneficial to the health of the host animal with the definition “a live microbial feed supplement which beneficially affects the host animal by improving its microbial balance”. Salminen *et al*. (1999) broadened the definition to not limit the delivery of probiotics to food and to include non-viable bacteria with the definition “probiotics

are microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well-being of the host".

Traditionally, interest in probiotics has centered on terrestrial organisms and considering the intimate relationship aquatic organisms share with their environment, the definition of a probiotic requires revision. The aquatic environment is different from the terrestrial environment in that the immediate ambient environment has a larger influence on the health status of the animal (Verschuere *et al*. 2000). Hosts and microorganisms share the same ecosystem and bacteria are ingested during osmoregulation and feeding. Pathogens are also supported in the surrounding environment and are able to multiply independently of the host animal. Aquatic microorganisms have the choice of living in association with the host, either in the intestinal tract, gills or skin, or not. Gram *et al*. (1999) recognized this and proposed the definition: "a live microbial supplement which beneficially affects the host animal by improving its microbial balance". Moriarty (1999) observed that microorganisms can modify the bacterial composition of water and sediments and suggested that the definition include the addition of naturally occurring bacteria to aquaculture tanks and ponds. Verschuere *et al*. (2000) expanded on these two definitions and proposed: "a live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment". Thus the concept of probiotics as biological control agents is included in these three definitions. A prebiotic on the other hand, is defined as a non-digestible food ingredient, mainly oligosaccharides, that selectively stimulate the growth and/or the activity of specific health promoting bacteria (probiotics) in the gastrointestinal tract thereby having a positive influence on host health (Gibson and Roberfroid, 1995).

There are many different understandings of the term probiotic. Balcázar *et al*. (2006) summarized probiotics as a term that is "generally used to denote bacteria that promote the health of other organisms". Irianto and Austin (2002) also highlighted the importance of the health benefits to the host whether by the entire organism or a component of it. All aquatic definitions also include the statement that there it is no longer a requirement for the probiotic to be acting in the gastrointestinal tract (Kesarcodi-Watson *et al*. 2008).

### 1.3.2. Possible modes of probiotic action and selection criteria

Although many studies on probiotics have been published, the mode of probiotic action is not always clear (Verschuere *et al*. 2000). This is due in part to the methodological and ethical limitations associated with animal studies (Balcázar *et al*. 2006). However, the knowledge gained from human and agricultural probiotics has assisted aquaculture probiotic studies (Verschuere *et al*. 2000).

The possible modes of action of probiotics include: competition for adhesion sites, competition for chemicals or available energy, enhancement of the immune response, enzymatic contribution to digestion, improvement of water quality, interaction with phytoplankton, production of inhibitory compounds, source of macro- or micronutrients, alteration of enzymatic activity of pathogens, inhibition of virulence gene expression and disruption of quorum sensing (Balcázar *et al*. 2006; Farzanfar, 2006; Kasercodi-Watson *et al*. 2008; Merrifield *et al*. 2010; Tinh *et al*. 2008; Verschuere *et al*. 2000). These modes of action are not mutually exclusive and a probiotic can possess more than one.

There are certain characteristics or properties that a bacterial strain must possess to be considered as a probiotic. The essential criteria are that a bacterium must be non-pathogenic to the host, other aquatic organisms and human consumers; should be free of antibiotic resistance markers; it should reach the desired location and remain there long enough to achieve an effect; and it should benefit the host in some way (Farzanfar, 2006; Gómez and Balcázar, 2008; Merrifield *et al*. 2010; Verschuere, 2000). Other desirable properties include being amenable to large scale production; remaining viable during storage; being indigenous to the host or rearing environment; and the ability to adhere to and colonize the gut (Farzanfar, 2006; Gómez and Balcázar, 2008; Merrifield *et al*. 2010). It is not likely that one strain will fulfill all the criteria and the simultaneous use of more than one probiotic strain as well as the use of probiotics together with prebiotics (synbiotics) should be considered and may be a way to achieve greater benefits (Merrifield *et al*. 2010).

## 1.4. Abalone aquaculture

### 1.4.1. Classification and occurrence of the South African abalone, *Haliotis midae*

Abalone are marine molluscs that belong to the phylum Mollusca, Class Gastropoda, family Haliotidae and genus *Haliotis* (Bevelander, 1988). Haliotis means 'sea ear' and refers to the shape of the shell. Abalone are found in tropical, temperate and cold waters ranging from the low tide line to more than 30 meters deep. Seaweed is the major food source for adult abalone in the wild; *Plocamium*, *Laminaria* and *Ecklonia* being the preferred species (Bevelander, 1988; Erasmus, 1996).

There are approximately 100 species of abalone worldwide, of which only 15 are commercially exploited (Bevelander, 1988; Britz, 1990). There are five endemic species of abalone in South Africa: *Haliotis midae*, *H. parvum, H. spadicea*, *H. queketti* and *H. speciosa* (Branch *et al*. 2005). *H. midae* occurs from St Helena Bay on the West coast to just north of Port St Johns on the East coast of Southern Africa and is the only species of commercial importance in South Africa (Tarr, 1992).

Abalone is a highly prized food particularly in the Far East. An increasing demand for abalone has led to the depletion of the natural stocks of *H. midae* along the South African coastline due to over-fishing and poaching to such an extent that the recreational fishery was closed in 2004 (Troell *et al*. 2006) and the commercial fishery in 2007. The only feasible answer to meet the demand and replenish natural stocks is the farming or aquaculture of abalone (Britz, 1990). Currently, 22 abalone farms exist in South Africa and a further 5 are scheduled for development (Troell *et al*. 2006). However, there are many problems associated with abalone aquaculture, the most significant being disease and the slow growth rates of the animals. Disease is a problem in any intensive animal rearing practice and generally 8-12 years is required for abalone to reach a legal harvestable size. The use of probiotics in abalone aquaculture is a potential solution to both of these problems.

### 1.4.2. Probiotics in abalone aquaculture

There has been some research on probiotics for molluscs including the Pacific oyster *Crassostrea gigas*, the scallop *Pecten maximus*, the Chilean scallop *Argopecten pupuratus* and the Manila clam *Ruditapes philippinarum* (Kesarcodi-Watson *et al*. 2008); as well as the abalone *Haliotis midae* (Macey and Coyne, 2005, 2006; ten Doeschate and Coyne, 2008) and *H. gigantea* (Iehata *et al*. 2009, 2010). This section will focus on the published probiotic research of abalone.

Erasmus *et al*. (1997) showed bacteria resident in the gut of *Haliotis midae* were able to degrade complex polysaccharides such as alginate, agarose, carrageenan, carboxymethylcellulose and laminarin. It was suggested that these bacteria play a digestive role in *H. midae* and may affect the growth rate of *H. midae*. *Pseudoalteromonas* sp. strain C4 was isolated by Erasmus *et al*. (1997). Subsequently, one bacterium, *Vibrio midae* SY9, and two yeasts strains, *Cryptococcus* sp. SS1 and *Debaromyces hansenii* AY1, were isolated from the gastrointestinal tract of *H. midae* (Macey and Coyne, 2005). *Pseudoalteromonas* sp. strain C4 is able to degrade alginate, cellulose and gelatin and *V. midae* SY9 is able to degrade alginate, gelatin and starch. Both strains produce extracellular proteases.

Macey and Coyne (2005) showed that when administered together as a cocktail in a formulated feed, *Cryptococcus* sp. SS1, *D. hansenii* AY1 and *V. midae* SY9 improved the growth and survival rate of *Haliotis midae*. An improved growth rate of up to 33% in larger sized animals was attributed to an increase in intestinal protease activity which correlated with an increase in protein digestion and absorption in the intestine. Furthermore, the number and phagocytic activity of circulating haemocytes was higher in probiotic fed abalone and 62% survived seven days after challenge with the pathogen *Vibrio anguillarum* compared to 25% of non-treated animals. Macey and Coyne (2006) subsequently showed that these three strains have the ability to colonize the gastrointestinal tract of *H. midae* and were detected 15 days after the cessation of feeding. Thus, these strains have great potential as probiotics for farm reared abalone.

ten Doeschate and Coyne (2008) showed that *H. midae* fed a kelp-based diet supplemented with *Pseudoalteromonas* strain C4 had an increased growth rate compared to animals fed the unsupplemented kelp diet. There was increased alginate lyase activity in the digestive tract of probiotic treated animals suggesting that strain C4 contributes enzymes for the digestion of complex algal polysaccharides. It was also shown that strain C4 acts as a source of protein for *H. midae*, supplementing the poor protein content of kelp.

*Vibrio halioticoli* was isolated from the gut of the Japanese abalone *Haliotis discus hannai* (Sawabe *et al*. 1995, 1998b). *V. halioticoli* produces six alginate lyases, four poly(G) specific and two poly(M) specific, and it is suggested that the bacterium plays an integral role in alginate degradation in the abalone gut (Sugimura *et al*. 2000). Since the abalone alginate lyases HdAly and HdAlex isolated from *H. discus hannai* are specific for poly(M) regions of alginate, and the alginate lyases of *V. halioticoli* are predominantly specific for poly(G) regions of alginate, it was suggested that *H. discus hannai* and *V. halioticoli* degrade alginate co-operatively (Sawabe *et al*. 1995; Sawabe, 2006). Furthermore, Sawabe *et al*. (2003) showed that *V. halioticoli* produces the volatile short-chain fatty acids (VSCFA) acetate and formate. In ruminants and termites, gut microbes produce acetate which is absorbed by the host and used as a precursor for the synthesis of proteins, sugars and lipids (Breznak, 1982; Hungate, 1966). It was suggested that *V. halioticoli* contributes to the nutrition and energy metabolism of abalone by the production of VSCFAs (Sawabe *et al*. 2003).

Iehata *et al*. (2009) reported the colonization of lactic acid bacteria in the digestive tract of the abalone *Haliotis gigantea*. *Lactobacillus* sp. strain a3 and *Enterococcus* sp. strain s6 were isolated from terrestrial environments, are resistant to bile salts and inhibited the growth of three abalone pathogens. Animals were fed a supplemented commercial diet for 3 weeks and the strains detected 3 to 5 days after cessation of feeding. There was an increase in protease activity as well as in the concentration of the VSCFAs acetate and formate in the digestive tract of animals receiving the probiotic supplemented feed compared to animals receiving unsupplemented feed.

Iehata *et al*. (2010) subsequently demonstrated colonization of the *Haliotis gigantea* gut by host-derived *Pediococcus* sp. Ab1. Abalone fed a commercial diet supplemented with *Pediococcus* sp. Ab1 for 6 weeks had increased alginate lyase activity and formate production in the gut when compared to abalone fed an unsupplemented diet. *Pediococcus* sp. Ab1 also altered the gut microbial population and was present in the digestive tract 12 days after the cessation of feeding. Although not demonstrated, it was suggested that through these qualities, strain Ab1 could increase the growth rate of abalone and thus act as an effective potential probiotic. Taken together with the results of Macey and Coyne (2006), it was suggested that host-derived probionts had a greater potential for colonizing the gut than those isolated from other environments.

### 1.5. Aims of this study

As outlined above, Macey and Coyne (2005) demonstrated an increase in the growth rate of farmed *Haliotis midae* following feeding with a probiotic supplemented formulated diet for eight months. ten Doeschate and Coyne (2008) suggested that the improved growth rate achieved with probiotic-supplemented feed was a consequence of increased availability of nutrients for absorption in the gut, an increased pool of digestive enzyme present in the gastrointestinal tract as well as the probiotics providing the abalone with an additional nutrient source, especially with regard to proteins which are limited in the brown seaweed *Ecklonia maxima* that is commonly fed to abalone farmed on the west and southern coast of South Africa.

*Vibrio midae* SY9 is able to degrade alginate, gelatin, protein and starch and produces an extracellular alginate lyase and protease. However, the probiotic mechanism of *V. midae* SY9 remains largely unknown. It would be interesting to determine the role of the *V. midae* SY9 alginate lyase enzyme/s with regard to the probiotic effect of *V. midae* SY9 on farmed *Haliotis midae*. However, in order to investigate this, several other objectives must first be met.

It is important to know the characteristics and properties of the *V. midae* SY9 alginate lyase/s and how they function in alginate degradation by *V. midae* SY9 prior to attempting to assign their role in seaweed degradation by *H. midae*. How the alginate lyase/s function to degrade alginate in *V. midae* SY9 might provide clues as to how they might be important in seaweed degradation by *H. midae*. Such properties may include the number of alginate lyases produced by *V. midae* SY9, substrate specificity of the enzyme/s, cellular location/s, whether more than one enzyme act co-operatively to degrade alginate and the regulation of the enzyme/s. The primary aim of this study was to clone, sequence and characterise the alginate lyase/s of *V. midae* SY9 and to begin to understand their role in alginate degradation by this bacterium.

# Chapter 2

## Cloning and Molecular Characterisation of Four Alginate Lyase Genes from *Vibrio midae* SY9

### CONTENTS

## 2.1. SUMMARY

A *Vibrio midae* SY9 genomic library was screened for alginolytic *Escherichia coli* clones by visually detecting zones of alginate degradation on alginate agar after flooding with 70% ethanol. Plasmid pAlg15 was chosen for further analysis and was subsequently sequenced. The sequence of pAlg15 revealed three open reading frames (ORFs) which had similarity to known alginate lyase genes as determined by a BLAST search of the GENBANK database. The middle ORF encoded a full-length alginate lyase gene and the two flanking ORFs were partial gene sequences. Two *V. midae* SY9 genomic libraries were constructed and screened in order to complete the sequence of the two partial alginate lyase genes of pAlg15. Plasmids pAlg65 and pAlg86 were identified and sequenced. The assembled sequence of pAlg15, pAlg65 and pAlg86 revealed seven ORFs, six encoded for full-length genes and one was a partial gene sequence. The complete genes were four alginate lyase genes, a putative pectin degrading enzyme and a putative galacturonate specific porin. The partial gene coded for a putative deoxygluconokinase. The four alginate lyase genes were designated *alyVMI*, *alyVMII*, *alyVMIII* and *alyVMIV*, respectively. AlyVMI and AlyVMII contain a chondroitin AC/alginate lyase domain as well as a heparinase II/III-like domain. AlyVMI and AlyVMII do not contain any of the conserved alginate lyase sequence motifs. AlyVMIII and AlyVMIV contain an alginate lyase 2 domain and a concanavalin A-like lectin/glucanase domain. AlyVMIII and AlyVMIV contain the conserved alginate lyase sequence motifs RXELR, QIH and YFKAGXYXQ. This is the first study to report four alginate lyase genes adjacent to each other on a bacterial chromosome as well as adjacent to other genes involved in carbohydrate metabolism.

## 2.2. INTRODUCTION

Alginate is a linear, un-branched polysaccharide of (1-4)-linked β-$_D$-mannuronate (M) and its C-5 epimer, α-$_L$-guluronate acid (G) (Gacesa, 1988). These uronic acids are arranged in block structures in the alginate polymer. The blocks can be homopolymeric; i.e. poly-(M) or poly-(G), or they can be heteropolymeric; i.e. poly-(MG).

Alginate is found in the cell walls and intercellular material of brown seaweeds where they lend strength, structure and shape (Draget *et al.* 2005). Some bacteria can also produce alginate. These include the opportunistic pathogen *Pseudomonas aeruginosa* (Linker and Jones, 1966) and *Azotobacter vinelandii* (Gorin and Spencer, 1966). Alginate produced by *Pseudomonas aeruginosa* is a key virulence factor in cystic fibrosis (Govan and Deretic, 1996; May *et al.* 1991). *Azotobacter vinelandii* forms metabolically dormant cysts which are surrounded by a coat of alginate (Ertesvag *et al.* 1998; Sadoff, 1975). Bacterial alginates differ from algal alginates in that they are usually *O*-acetylated at the O-2 or/and O-3 position of the $_D$-mannuronate residue, with the O-2 position being preferred (Gacesa, 1988; May *et al.* 1991).

Alginate lyases have been isolated from a wide range of sources. These include brown algae, marine fungi, marine invertebrates, Gram-positive soil bacteria, Gram-negative bacteria, marine bacteria, *Chlorella* virus and bacteriophage (Wong *et al.* 2000). It is common for bacteria to have more than one alginate lyase enzyme. Two alginate lyases, *alyVOA* and *alyVOB*, were cloned and sequenced from *Vibrio* sp. O2 (Kawamoto *et al.* 2006), while *Vibrio halioticoli* from the gut of the Japanese abalone *Haliotis discus hannai* has been reported to produce 6 alginate lyases, 2 poly-mannuronate lyases and 4 poly-guluronate lyases (Sawabe *et al.* 1995). Three of the poly-guluronate lyases, *alyVG1*, *alyVG2* and *alyVG3*, have been cloned and sequenced (Sugimura *et al.* 2000).

Alginate lyases are enzymes that are able to degrade alginate. The reaction catalyzed is a β-elimination reaction whereby the (1-4)-*O*-glycosidic linkage is cleaved. The resulting oligosaccharides contain the unsaturated uronic acid, 4-deoxy-$_L$-*erythro*-hex-4-ene pyranosyluronate, at the new non-reducing terminus (Gacesa 1988, 1992; Wong *et al.* 2000).

Alginate lyases are single subunit enzymes consisting of one polypeptide chain and are typically endolytic (Gacesa, 1992). Few exolytic enzymes have been described (Brown *et al.* 1991; Doubet and Quatrano, 1984; Nakada and Sweeny, 1967). An example is HdAlex from *Haliotis discus hannai* that cleaves alginate at the second glycosidic linkage from the reducing terminus producing an unsaturated disaccharide (Suzuki *et al*. 2006).

Alginate lyases are classified based on their dominant cleaving action (Gacesa, 1988; Wong *et al*. 2000). Some are specific for the β-$_D$-mannuronate linkage and others are specific for the α-$_L$-guluronate linkage meaning they can degrade the poly(M) or poly(G) regions of alginate, respectively. Others are non-specific and are able to degrade both blocks. Poly(M) specific lyases are given the enzyme commission number EC 4.2.2.3 and poly(G) specific lyases the number EC 4.2.2.11. Enzymes active on carbohydrates are classified into families based on amino acid sequence and structure similarities (Cantarel *et al*. 2009). Alginate lyases belong to polysaccharide lyase families PL-5, -6, -7, -14, -15, -17 and -18 (http://www.cazy.org). Alginate lyases are typically not well conserved and do not share a high sequence homology (Wong *et al*. 2000). However, all members of the PL-7 lyase family contain the highly conserved sequence motifs RXELR, QIH and YFKAGXYXQ, where X is any residue, in the N-terminus, middle and C-terminus of the protein, respectively. These residues are suggested to be responsible for the catalytic reaction of the enzymes. Alginate lyases are also classified into molecular weight classes.

Secondary and tertiary structures of alginate lyases tend to differ. They consist predominantly of α-helices or β-sheets in an α/α barrel or β-sandwich conformation. The mannuronate lyase A1-III from *Sphingomonas* sp. is composed of 12 α-helices arranged in an $\alpha_6/\alpha_5$ helix barrel which contains a deep cleft that comprises the substrate binding site (Yoon *et al.* 1999; 2001), whereas Aly-1 from *Corynebacterium* sp. is comprised almost entirely of β-sheets (Osawa *et al.* 2005). Aly-1 is made up of two α-helices and 14 β-strands which form two curved anti-parallel β-sheets. This latter structure is known as a jelly roll β-sandwich. Although these two proteins have different structures, the putative catalytic residues of both enzymes are positioned in nearly identical spatial arrangements (Osawa *et al*. 2005).

*Vibrio midae* SY9 was isolated from the gut of the South African abalone *Haliotis midae* and shown to produce a number of extracellular enzymes including a protease, an amylase and an alginate lyase (Macey and Coyne, 2005). *V. midae* SY9 has been shown to increase the growth rate of farmed *H. midae* and has an immunostimulatory effect when included as a cocktail of three probiotics in commercially produced abalone feed (Macey and Coyne, 2005). However, the probiotic mechanism remains unknown.

Following on from the work of Erasmus and Macey (Chapter 1, Section 1.4.2), it would be interesting to determine the role of the *V. midae* SY9 alginate lyase enzyme/s with regard to the probiotic effect of *V. midae* SY9 on farmed *Haliotis midae*. However, in order to investigate this, it is important to know the characteristics and properties of the *V.* midae SY9 alginate lyase/s and how they function in alginate degradation by *V.* midae SY9 prior to attempting to assign their role in seaweed degradation by *H. midae*. The primary aim of this study was to clone, sequence and characterise the alginate lyase/s of *V. midae* SY9. This chapter describes the screening of a *V. midae* SY9 genomic library for *Escherichia coli* clones with the ability to degrade alginate. The sequencing and characterisation of four *V. midae* SY9 alginate lyase genes is also described.

## 2.3. MATERIALS AND METHODS

All media and solutions used in this study are listed in Appendix A.

### 2.3.1. Bacterial strains and plasmids used

Bacterial strains and plasmids used in this study are listed in Table 2.1.

### 2.3.2. Culture conditions and media used

*Escherichia coli* JM107 was cultured either in Luria broth (LB) or on Luria Agar (LA) at 37°C. *E. coli* JM107 transformants harboring recombinant pBluescript SK (+)$^{TM}$ plasmids were cultured either in LB or on LA containing 100 µg/ml ampicillin. *Vibrio midae* SY9 was cultured either in marine broth (MB) or on marine agar (MA) at room temperature.

**Table 2.1.** Bacterial strains and plasmids

| Strain/plasmid | Genotype/relevant characteristic(s)[a] | Reference |
|---|---|---|
| **Strains** | | |
| *E. coli* JM107 | *end*A1 *gln*V44 *thi*-1 *rel*A1 *gyr*A96 Δ(*lac-pro*AB) F' (*tra*D36 *pro*AB$^+$ *lac*I$^q$ *lac*ZΔM15) *hsd*R17($R_K^-$ $m_K^+$) $\lambda^-$ | Yanisch-Perron *et al.* (1985) |
| *Vibrio midae* SY9 | Putative probiont isolated from *Haliotis midae,* South Africa | Macey and Coyne (2005) |
| **Plasmids** | | |
| pBluescript SK (+)$^{TM}$ | Amp$^r$, β-galactosidase | Short *et al.* (1988) |
| pAlg15 | pBluescript SK (+)$^{TM}$ containing ~5.7 kb of *V. midae* SY9 genomic DNA | This study |
| pAlg65 | pBluescript SK (+)$^{TM}$ containing ~3.3 kb of *V. midae* SY9 genomic DNA | This study |
| pAlg86 | pBluescript containing (+)$^{TM}$ ~4.8 kb of *V. midae* SY9 genomic DNA | This study |

[a] Amp$^r$, ampicillin resistant

### 2.3.3. Isolation of an alginolytic clone

Taylor (2002) constructed a *Vibrio midae* SY9 genomic library. Briefly, *V. midae* SY9 genomic DNA was partially restricted with *Sau*3A and electrophoresed through a 0.7% agarose gel. Fragments, 6-8 kb in size, were excised from the gel and purified using the QIAquick Gel Extraction Kit (Qiagen). Purified fragments were ligated to pBluescript SK (+)$^{TM}$ which had been restricted with *Bam*HI and dephosphorylated. Ligated plasmids were transformed into competent *E. coli* JM109 cells and spread onto LA using sterile technique. Colonies were pooled and plasmid DNA isolated. Pools of plasmid DNA were stored at -70°C.

For the study reported in this chapter, the *Vibrio midae* SY9 genomic library described above was screened for alginolytic clones. Five microlitres of each pool of plasmid DNA was transformed into competent *E. coli* JM107 cells (Appendix B.1) as described in Appendix B.2. Transformation mixes were spread-plated onto LA media containing 0.5 mM IPTG, 0.004% X-gal and 100 µg/ml ampicillin using sterile technique and incubated overnight at 37°C.

White colonies were transferred onto alginate lyase detection agar containing 100 µg/ml ampicillin using sterile toothpicks. Petri dishes were incubated at 37°C overnight followed by incubation at room temperature for a further five days. Transformants were replica plated using sterile pieces of velvet on a wooden block and imprinting them onto fresh LA containing 100 µg/ml ampicillin. Petri dishes were incubated at 37°C overnight and stored at 4°C.

Transformants on alginate lyase detection agar were flooded with 70% ethanol for 30 min at room temperature, the ethanol was poured off and the petri dishes allowed to stand for 30-60 min at room temperature before examination (Sawabe *et al*. 1995). Intact alginate precipitates in the presence of ethanol giving a cloudy appearance and degraded alginate appears as a clear zone. Alginate lyase activity was therefore detected as clear zones around colonies. Colonies positive for alginate hydrolysis were recovered from the replica LA petri dishes.

### 2.3.4. Restriction endonuclease analysis of pAlg15

Plasmid pAlg15 was characterised by the number and position of restriction endonuclease sites. Initially enzymes that cleave once or not at all within pBluescript SK (+)$^{TM}$ were used. These included *Bam*HI, *Bgl*II, *Eco*RI, *Eco*RV, *Not*I and *Sac*I. Once the number of recognition sites for these enzymes was determined, additional sites could be identified through double digestions with combinations of the above enzymes. Other enzymes used for restriction mapping included *Hind*III, *Kp*nI, *Sma*I, *Sty*I, *Xba*I, *Xho*I and *Xmn*I.

Large scale plasmid isolation of pAlg15 was performed using the Qiagen Midi-Prep Plasmid Purification Kit (Qiagen) according to the manufacturer's instructions. Plasmid DNA was resuspended in sterile $dH_2O$ and quantitated using a Nanodrop spectrophotometer (Thermo Scientific). Restriction digests were performed as described in Appendix B.4 and digestion products separated on 1% TAE agarose gels as described in Appendix B.5.

### 2.3.5. Nucleotide sequencing of pAlg15

The sequence of the insert of plasmid pAlg15 was completed in both directions by primer walking. Using identified sequence, new oligonucleotide primers were designed and additional sequence information obtained from sequencing with the new primer. The insert of pAlg15 was sequenced with the following primers: universal M13 forward, universal M13 reverse, 15Fc, 15Rd, 15Re, 15Rf, 15Rg, 15Fh, 15Ri, 15Rj, 15Fk, 15Fl and 15Fm. All primer pairs were tested by PCR amplification to verify reaction specificity and fragment sizes prior to sequencing. PCR products were separated on a 1% TAE agarose gel as described in Appendix B.5. Oligonucleotide primers, primer pairs and PCR conditions are described in Appendix C.1.

Where PCR products were to be sequenced, PCR products were purified using the BioFlux Gel Extraction Kit (Bioer Technology) according to the manufacturer's instructions after electrophoresis through a 0.8% TAE agarose gel (Appendix B.5). The BioSpin Plasmid DNA extraction Kit (Bioer Technology) was used to isolate plasmids where plasmid templates were to be sequenced. Sequencing reactions were performed using the Big Dye Terminator Version 3.1

Cycle Sequencing Kit (Applied Biosystems) as per the manufacturer's instructions and sequence of the samples obtained using the 3130 Genetic Analyser (Applied Biosystems, DNA Automated Capillary Sequencer).

### 2.3.6. Construction and screening of two genomic libraries

In order to complete the sequence of two alginate lyase genes present on the insert of pAlg15, two genomic libraries were constructed and screened as follows.

Two Southern hybridisation experiments were performed to determine which size fragments to clone into pBluescript SK (+)$^{TM}$ to construct the genomic libraries. Genomic DNA was isolated from *Vibrio midae* SY9 and *E. coli* JM107 as described in Appendix B.8. Plasmid pAlg15 was isolated using the BioSpin Plasmid DNA extraction Kit (Bioer Technology) according to the manufacturer's instructions. The resulting plasmid DNA was used as a template for PCR amplification of a 558 bp *alyVMI* or a 700 bp *alyVMIII* fragment using primer pairs 15Fn and 15Rj or 15Fc and 15Ro, respectively. Oligonucleotide primers and PCR conditions are described in Appendix C.2. Amplified PCR products were analysed by agarose gel electrophoresis (Appendix B.5) to verify reaction specificity and fragment size. PCR products were purified using the BioFlux Gel Extraction Kit (Bioer Technology) according to the manufacturer's instructions after electrophoresis through a 0.8% TAE agarose gel (Appendix B.5). The purified DNA fragments were labeled with digoxigenin (DIG) using the DIG High Prime DNA labeling and Detection Starter Kit II (Roche) according to the manufacturer's instructions (Appendix B.9.2). The DIG-labeled DNA fragments were used as probes against equal amounts (8 µg) of *V. midae* SY9 genomic DNA digested with the restriction enzymes *Xmn*I, *Hind*III or *Eco*RV for the *alyVMI* Southern hybridisation experiment or with *Hind*III, *Bgl*II or *Kpn*I for the *alyVMIII* Southern hybridisation experiment and separated on a 0.7% TAE agarose gel (Appendix B.5). *E. coli* JM107 genomic DNA digested with *Eco*RV was used as a negative control for each experiment. The Southern hybridisation procedure that was followed is described in Appendix B.9. The DIG Easy Hyb hybridisation buffer containing the DIG-labeled probes was stored at -20°C for re-use.

The 3.3 kb *Eco*RV fragment from the *alyVMI* Southern hybridisation experiment and the 4.8 kb *Hind*III fragment from the *alyVMIII* Southern hybridisation experiment were cloned into pBluescript SK (+)$^{TM}$. *Vibrio midae* SY9 genomic DNA was digested with either *Eco*RV or *Bgl*II (Appendix B.4) and the digestion products were separated on a 0.7% TA low-melting point agarose gel in 1X TA buffer (Appendix B.5). Approximately 3.3 kb *Eco*RV fragments and ~4.8 kb *Hind*III fragments were excised from the gel. Large scale preparation of pBluescript SK (+)$^{TM}$ was performed using the Qiagen Midi-Prep Plasmid Purification Kit (Qiagen) according to the manufacturer's instructions. The resulting plasmid DNA was restricted with either *Eco*RV or *Bam*HI (Appendix B.4). The restricted plasmids were treated with calf intestine alkaline phosphatase (CIP, Fermentas) according to the manufacturer's instructions. *Eco*RV restricted genomic fragments were ligated with plasmids linearized with *Eco*RV to obtain an *alyVMI* mini-genomic library. *Bgl*II restricted genomic fragments were ligated with plasmids linearized with *Bam*HI to construct the *alyVMIII* mini-genomic library. Ligation reactions were performed as described in Appendix B.7. Ligation mixes were transformed into competent *E. coli* JM107 cells (Appendix B.2) and spread-plated onto LA containing 0.5 mM IPTG, 0.004% X-gal and 100 µg/ml ampicillin. Petri dishes were incubated overnight at 37°C.

The genomic libraries were arranged into pools of plasmid DNA and screened using DNA hybridisation. Plasmid DNA (12 µg) was transferred to Hybond $N^+$ nylon membrane (Amersham) using the slot blot procedure described in Appendix B.10. Plasmid pAlg15 was used as a positive control and *E. coli* JM107 genomic DNA was used as a negative control. Colony hybridisation was used to screen individual colonies. Colony lifts were prepared as described in Appendix B.11. The Southern hybridisations were performed as described in Appendix B.9. The hybridisation buffer containing the DIG-labeled probes used in the Southern hybridisation experiments was re-used for the slot blot and colony hybridisation experiments.

Insert sizes of identified plasmids were confirmed by restriction analysis with *Pvu*II (Appendix B.4). Digestion products were separated on a 1% TAE agarose gel (Appendix B.5). Clones were tested for the ability to hydrolyse alginate as described in Section 2.3.3. Plasmids pAlg65 and pAlg86 isolated from the *alyVMI* and *alyVMIII* genomic libraries, respectively, were chosen for further analysis.

Sequencing of the inserts of plasmids pAlg65 and pAlg86 was completed in both directions by primer walking as described in Section 2.3.5. The insert of pAlg65 was sequenced with the universal M13 forward and reverse primers and primers 65Fa, 65Fb and 65Rc. The insert of pAlg86 was sequenced with the universal M13 forward and reverse primers and primers 86Fa, 86Rb, 86Fc, 86Rd, 86Fg, 86Rh, 86Fi, 86Rj, 86Rk and 86Rl. Primer pairs were tested by PCR amplification to verify reaction specificity and fragment sizes prior to sequencing. PCR products were separated on a 1% TAE agarose gel as described in Appendix B.5. Oligonucleotide primers, primer pairs and PCR conditions are described in Appendix C.3. PCR products and plasmids were purified as described in Section 2.3.5. Sequencing and sequence analysis was performed as described in Section 2.3.5.

### 2.3.7. Analysis of sequencing results and bioinformatics approaches

The complete sequences of pAlg15, pAlg65 and pAlg86 were assembled using DNAMAN Version 4.13 (Lynnon Biosoft). Open reading frames were identified using DNAMAN Version 4.13 (Lynnon Biosoft). Homology searches of deduced amino acid sequences were carried out using the BLASTx and Protein BLAST algorithms at the National Center for Biotechnology Information (NCBI; http://www.ncbi.blast.nlm.nih.gov/BLAST; Altschul *et al*. 1997, 2005).

The molecular weight and pI of the translated gene products were predicted using the ExPASy Compute pI/MW tool (http://ca.expasy.org/tools/pi_tools.html; Gasteiger *et al.* 2005). Putative signal peptides were identified using the SignalP Version 3.0 server (http://www.cbs.dtu.dk.services/SignalP; Bendtsen *et al*. 2004). Protein domains and signal peptides were predicted by InterProScan (http://www.ebi.ac.uk/InterProScan; Quevillon *et al*. 2005). Subcellular localizations of the translated proteins were predicted using PSORTb Version 2.0. (http://www.psort.org/psortb; Gardy *et al.* 2003). Putative transmembrane domains and secondary structure were predicted using MINNOU (http://minnou.cchmc.org; Cao *et al.* 2006) and viewed in POLYVIEW (http://polyview.cchmc.org; Porollo *et al*. 2004).

### 2.3.8. Southern hybridisation analysis of *alyVMII* and *alyVMIV*

Southern hybridisation analysis of *alyVMII* and *alyVMIV* was performed to confirm that these two genes originated from *Vibrio midae* SY9. Genomic DNA was isolated from *Vibrio midae* SY9 and *E. coli* JM107 as described in Appendix B.8. Plasmid pAlg15 and pAlg86 were isolated using the BioSpin Plasmid DNA extraction Kit (Bioer Technology) according to the manufacturer's instructions. The resulting plasmid DNA was used as a template for PCR amplification of either a ~2161 bp *alyVMII* or a ~1566 bp *alyVMIV* fragment, respectively, using primer pairs EVF1 and EVR1 or EVF4 and EVR4, respectively. Oligonucleotide primers and PCR conditions are described in Appendix C.4. Amplified PCR products were analysed by agarose gel electrophoresis (Appendix B.5) to verify reaction specificity and fragment size. PCR products were purified using the BioFlux Gel Extraction Kit (Bioer Technology) according to the manufacturer's instructions after electrophoresis through a 0.8% TAE agarose gel (Appendix B.5). The purified DNA fragments were labeled with digoxigenin (DIG) using the DIG High Prime DNA labeling and Detection Starter Kit II (Roche) according to the manufacturer's instructions (Appendix B.9.2). The DIG-labeled DNA fragments were used as probes against equal amounts (9 µg) of *V. midae* SY9 genomic DNA that had been digested with the restriction enzymes *Pst*I, *Hind*III or *Sty*I for the *alyVMII* Southern hybridisation experiment or with *Bgl*I, *Pst*I or *Hind*III for the *alyVMIV* Southern hybridisation experiment and separated on a 0.7% TAE agarose gel (Appendix B.5). *E. coli* JM107 genomic DNA digested with *Eco*RV was used as a negative control for each experiment. The Southern hybridisation procedure described in Appendix B.9 was followed.



### 2.3.9. Determination of co-transcription of *alyVMI* and *alyVMII*

DNA sequence analysis revealed that 6 base pairs separate *alyVMII* from *alyVMI*. The question was asked whether *alyVMI* and *alyVMII* are co-transcribed as a single mRNA transcript.

*Vibrio midae* SY9 was inoculated into 5 ml alginate media and incubated at room temperature with shaking at 100 rpm on an orbital shaker for 8 h. This starter culture was used to inoculate 100 ml of fresh new alginate media to a starting absorbance of 0.02 at 600 nm. The inoculated

media was incubated at room temperature with shaking overnight. This overnight culture was used to inoculate 500 ml of fresh alginate media to a starting absorbance of 0.02 at 600 nm. The inoculated media was incubated at room temperature with shaking at 100 rpm. Eight hours after inoculation, 2 ml samples of the culture were collected in RNAse free eppendorf tubes. Samples were centrifuged at 8000 rpm for 2 min. Culture supernatant was discarded and cells were stored at -20°C until RNA was extracted.

Total RNA was isolated from *V. midae* SY9 cells and treated with DNAse I (Promega) as described in Appendix B.12. Total RNA was quantitated using a Nanodrop spectrophotometer (Thermo Scientific). Two micrograms of total RNA were separated on a 1.2% formaldehyde/MOPS agarose gel (Appendix B.13) to determine the integrity of the RNA. Total RNA was assessed for traces of genomic DNA using PCR amplification and primers specific for the *Vibrio midae* SY9 16S rRNA gene. The oligonucleotide primers and amplification conditions are described in Appendix C.5. First strand cDNA synthesis was performed on total RNA (5 µg) using random hexamers and the ImPromII™ Two Step Reverse Transcriptase Kit (Promega) as described in Appendix B.14. The resulting cDNA was stored at -70°C.

Plasmid pAlg15 and cDNA were subjected to PCR amplification using primer pairs as indicated in Figure 2.11 (a). Primer pair 15Fh and 15Ri amplify an internal fragment of *alyVMI*, 15Fm and 15Re amplify an internal fragment of *alyVMII* and 15Fk and 15Rg amplify the region connecting *alyVMI* and *alyVMII*. Oligonucleotide primers and PCR conditions are described in Appendix C.6. PCR products were separated on a 1% TAE agarose gel as described in Appendix B.5.

## 2.4. RESULTS

### 2.4.1. Isolation of and restriction endonuclease mapping of pAlg15

A *Vibrio midae* SY9 genomic library constructed by Taylor (2002) was transformed into competent *E. coli* JM107 cells and screened for clones that degrade alginate. Plasmid pAlg15 was chosen for further analysis since the *E. coli* clone carrying this plasmid displayed the ability to degrade alginate. Plasmid pAlg15 was mapped with respect to the number and position of restriction enzyme sites (Figure 2.1) and was sequenced by primer walking.

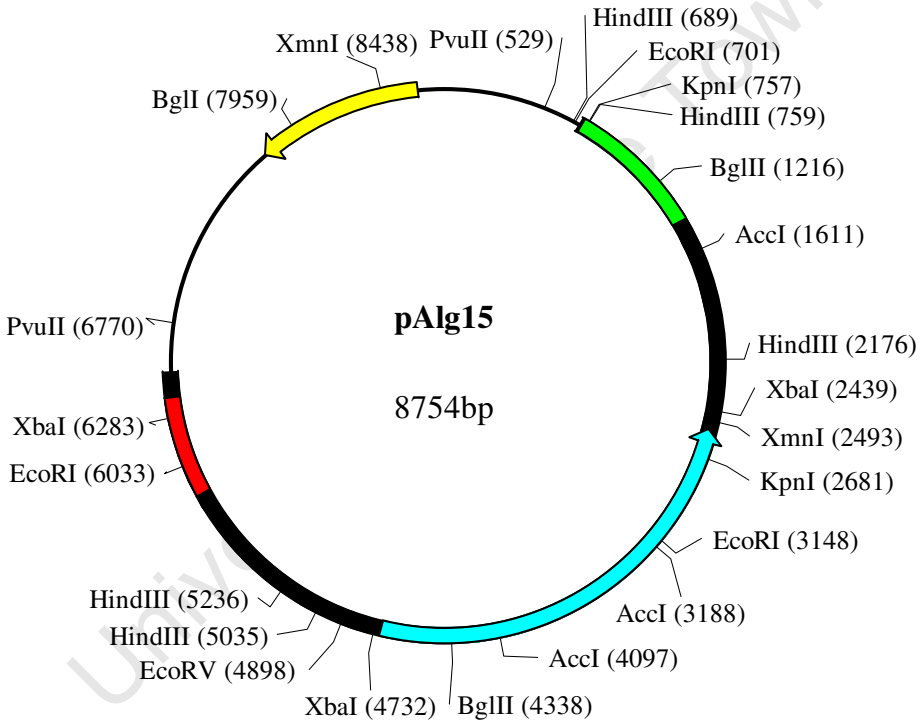


**Figure 2.1.** Restriction enzyme map of pAlg15. The thick line represents cloned *Vibrio midae* SY9 genomic DNA whereas the thin line represents pBluescript SK (+)$^{TM}$ DNA. The size of the recombinant plasmid is shown in base pairs (bp). The position of the various restriction enzyme sites is shown in bp in brackets and the position of the β-lactamase gene is indicated by the yellow arrow. The red and green blocks represent the probes specific for *alyVMI* and *alyVMIII* genes, respectively, used in the Southern hybridisation experiments. The blue arrow represents the *alyVMII* gene in 5' to 3' orientation in the direction of the arrow.

Sequence analysis of pAlg15 revealed the presence of three open reading frames (ORFs). All three ORFs had similarity to known alginate lyase genes as determined by a BLAST search of the GENBANK database. One of the ORFs encoded a full-length alginate lyase gene and two ORFs were partial gene sequences. The full-length alginate lyase gene was named *alyVMII*. The two partial genes were named *alyVMI* and *alyVMIII*. Alginate lyase gene *alyVMI* is upstream of *alyVMII* and *alyVMIII* is downstream of *alyVMII* (Figure 2.3).

### 2.4.2. Construction and screening of two genomic libraries

Two *Vibrio midae* SY9 genomic libraries were constructed and screened to obtain full length sequences of alginate lyase genes *alyVMI* and *alyVMIII*. A Southern hybridisation experiment was performed to determine which *Vibrio midae* SY9 genomic DNA fragments to clone to construct each library. DIG-labeled DNA probes specific for *alyVMI* or *alyVMIII* were used in two separate experiments (Figure 2.2 a and c). Both probes hybridised to *V. midae* SY9 genomic DNA indicating that both genes originated from *V. midae* SY9. Neither probe hybridised to *E. coli* JM107 DNA which was used as a negative control.

The *alyVMI* probe hybridised to a ~3.3 kb *Eco*RV fragment of *V. midae* SY9 genomic DNA (Figure 2.2 a). This fragment was chosen for cloning since it was upstream of the probe (on the 5' end of the red block in Figure 2.1) used for the Southern hybridisation experiment and upstream of the *Eco*RV (4898bp) restriction site present in *alyVMI* on pAlg15 and thus would complete the sequence of *alyVMI*. The *alyVMIII* probe hybridised to a ~4.8 kb *Bgl*II fragment of *V. midae* SY9 genomic DNA (Figure 2.2 c). This fragment was chosen for cloning since it was downstream of the probe (on the 3' end of the green block in Figure 2.1) used for the Southern hybridisation experiment and downstream of the *Bgl*II restriction site (1216bp) present in *alyVMIII* on pAlg15 and therefore would complete the sequence of *alyVMIII*.

The two genomic libraries were screened by DNA hybridisation using the DIG-labeled probes employed in the Southern hybridisation experiment described above. Plasmid pAlg65 was generated from the genomic library probed with the *alyVMI* specific probe and plasmid pAlg86 was constructed from the genomic library probed with the *alyVMIII* specific probe. The genomic

DNA inserts of pAlg65 and pAlg86 were sequenced in both directions by primer walking. Plasmids pAlg65 and pAlg86 contained inserts of 3.335 kb and 4.824 kb, respectively, and completed the sequence of *alyVMI* and *alyVMIII*, respectively. A fourth alginate lyase gene was identified on pAlg86 downstream of *alyVMIII* and was designated *alyVMIV* (Figure 2.3).

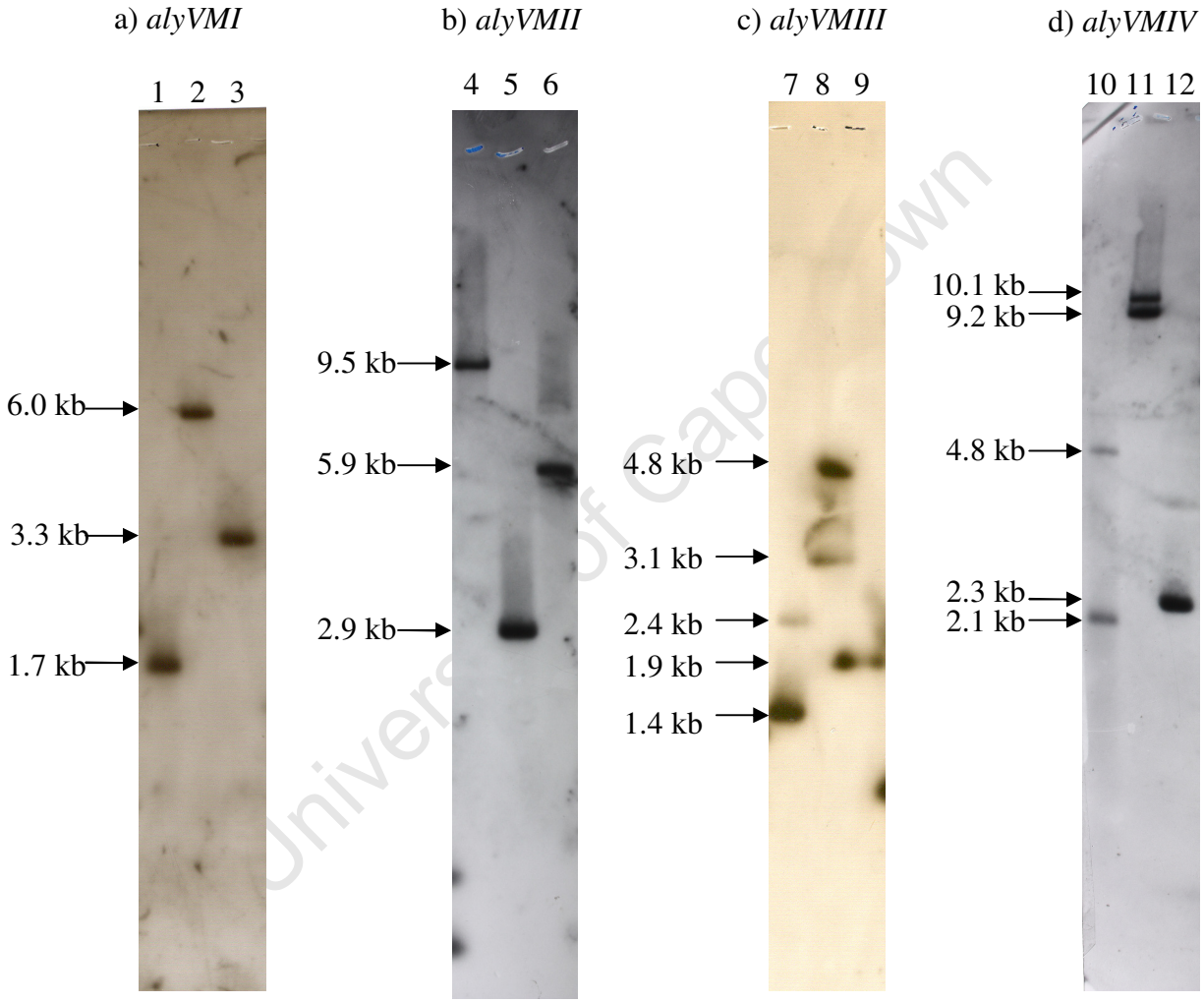


**Figure 2.2** Southern hybridisation of a DIG-labeled probe specific for *alyVMI* (a), *alyVMII* (b), *alyVMIII* (c) and *alyVMIV* (d) against *Vibrio midae* SY9 genomic DNA. *V. midae* SY9 genomic DNA was digested with *Xmn*I (Lane 1), *Hind*III (Lanes 2, 5, 7 and 12), *Eco*RV (Lane 3), *Pst*I (Lanes 4 and 11), *Sty*I (Lane 6), *Bgl*II (Lane 8), *Kpn*I (Lane 9) and *Bgl*I (Lane 10). The arrows indicate the approximate sizes of the DNA fragments to which the DIG-labeled probes hybridised in kb.

### 2.4.3. Sequence and homology analysis

The sequenced inserts of pAlg15, pAlg65 and pAlg86 were assembled using DNAMAN Version 4.13 (Lynnon BioSoft) resulting in 11.842 kb of sequence (Figure 2.3 a). Seven ORFs were revealed in the translation overview of the assembled sequence (Figure 2.3 b). A Protein BLAST search of the GENBANK database revealed the seven ORFs to be four alginate lyase genes, a putative oligogalacturonate specific porin, a putative pectin degrading protein and a putative deoxygluconokinase.

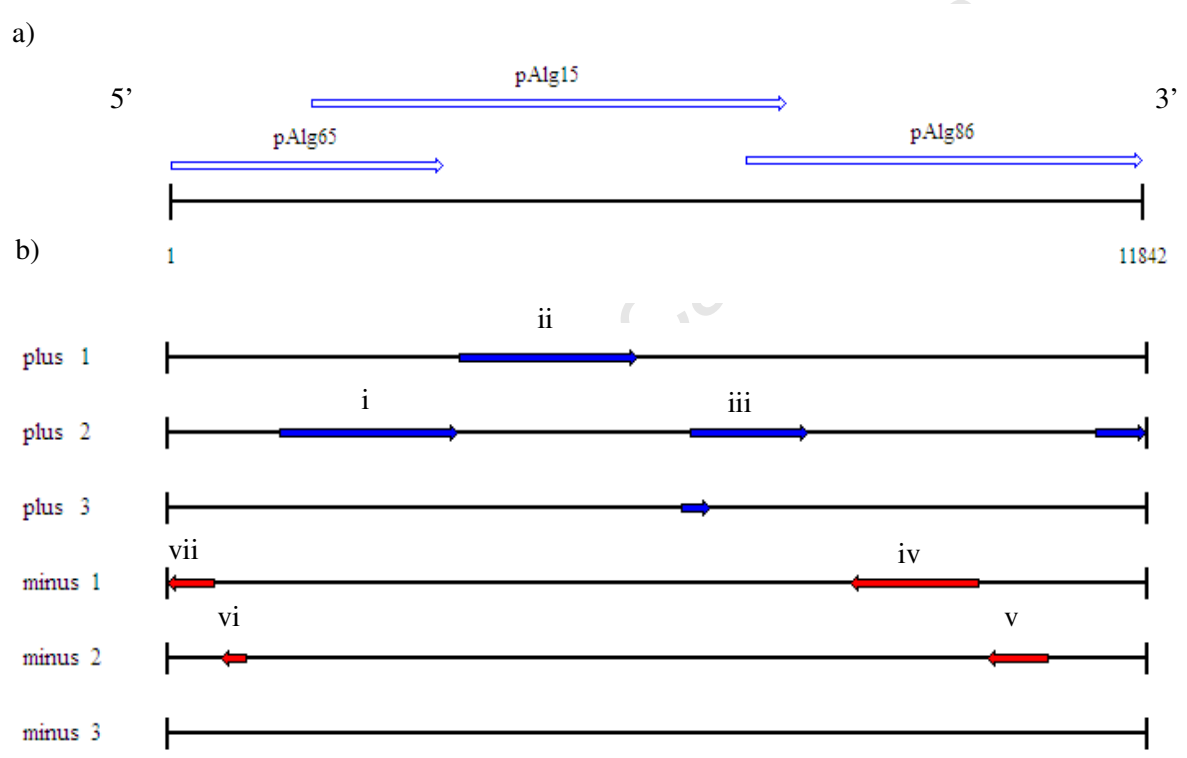


**Figure 2.3** a) Assembly overview of DNA inserts from pAlg15, pAlg65 and pAlg86. b) Overview of the seven open reading frames (ORFs) predicted for the assembled sequence. ORFs in the plus or minus reading frame are indicated by blue or red arrows, respectively. Arrow heads and tails represent stop and start codons, respectively. The ORFs are: i) *alyVMI*, ii) *alyVMII*, iii) *alyVMIII*, iv) *alyVMIV*, v) putative oligogalacturonate specific porin, vi) putative pectin degrading protein and vii) putative deoxygluconokinase.

### 2.4.3.1. Sequence analysis of *alyVMI*

The partial sequence of alginate lyase gene *alyVMI* occurred on pAlg15 and the remainder of the gene was situated on pAlg65. The full-length sequence of *alyVMI* is 2163 bp and a putative -10 region (TTATA) was identified (Figure 2.4). The deduced protein is 721 amino acids with a calculated molecular weight of 81.63 kDa and a predicted pI of 5.07. InterProScan identified two protein domains in AlyVMI: a Chondroitin AC/Alginate lyase domain as well as a Heparinase II/III-like domain (Table 2.2). PSORTb did not predict a cellular location for AlyVMI. The amino-terminus (residue 1 to ~340) of AlyVMI was predicted to include α-helices and the carboxy-terminus (residues ~361 to 721) β-strands by MINNOU (Figure 2.4). Two short transmembrane regions were also predicted from amino acid residue 116-127 and 228-246 (Figure 2.4). A Protein BLAST search of the GENBANK database revealed that AlyVMI had similarity to chondroitin AC/alginate lyases and heparinase II/III-like proteins (Table 2.3). AlyVMI does not contain any alginate lyase conserved sequence motifs.



**Table 2.2.** Predicted protein domains of the *Vibrio midae* SY9 alginate lyase enzymes

| | Position of domain (amino acid residues) | | | |
|---|---|---|---|---|
| Domain | AlyVMI | AlyVMII | AlyVMIII | AlyVMIV |
| Size of protein (amino acids) | 721 | 721 | 483 | 521 |
| Signal peptide | -[a] | - | - | 1-24 |
| Chondroitin AC/alginate lyase | 54-339 | 59-275 | - | - |
| Alginate lyase 2 | - | - | 2-186 and 195-482 | 252-516 |
| Heparinase II/III-like | 363-523 | 365-535 | - | - |
| Concanavalin A-like lectins/glucanases | - | - | 7-187 and 195-483 | 252-517 |
| Coagulation factor 5/8 type (FA58C) | - | - | - | 27-168 |
| Galactose binding-like | - | - | - | 19-170 |

[a]-, domain not present

```
          TTGTATTACTTTATAGAAAGTTTAAGAAAACAACACACTTCATCTGAGGGTGGTAAAGAA
                    -10
1         ATGACTACACAACCGATTTTGTTGACTGAAGCAGAAGTCGAACTACTAAGAAAGGAAGTC
1          M  T  T  Q  P  I  L  L  T  E  A  E  V  E  L  L  R  K  E  V

61        GGAAAGCCGAGCTTAATGGGCAAATCCATTGAAGCGAATCGCAAGGAACTTGAAGCCTTC
21         G  K  P  S  L  M  G  K  S  I  E  A  N  R  K  E  L  E  A  F

121       ATGCGTCTGCCTCTAGACGTACCAGGTCACGGCGAAGCGGGTGGCTACGAGCACAACCGC
41         M  R  L  P  L  D  V  P  G  H  G  E  A  G  G  Y  E  H  N  R

181       CACAAACAAAACTATACCTACATGAACTTAGCGGGTCGTTTGTTCTTGATCACTCAAGAA
61         H  K  Q  N  Y  T  Y  M  N  L  A  G  R  L  F  L  I  T  Q  E

241       GAAAAGTACGCGCAGTTCGTTAAAGATCTTCTCGCTATTTACGCAGAGAAGTACCTAACT
81         E  K  Y  A  Q  F  V  K  D  L  L  A  I  Y  A  E  K  Y  L  T

301       TTTGATTTCCACGTACAGAAAAACACTAACCCAACAGGTCGTCTTTTCCACCAGATCCTT
101        F  D  F  H  V  Q  K  N  T  N  P  T  G  R  L  F  H  Q  I  L

361       AATGAACACTGTTGGTTAATGTTTACTAGCCTTGCTTACTCTTGCGTGGCATCAGTGATG
121        N  E  H  C  W  L  M  F  T  S  L  A  Y  S  C  V  A  S  V  M

421       ACAGAAGAAGAGCGCACAGCCGTTGTTGAGCGCATTTTCGAACCAATGCTAGACATGTTC
141        T  E  E  E  R  T  A  V  V  E  R  I  F  E  P  M  L  D  M  F

481       ACAGTGAAATATGCGCACGATTTCGACCGTATTCACAACCACGGTATCTGGGCAGTTGCG
161        T  V  K  Y  A  H  D  F  D  R  I  H  N  H  G  I  W  A  V  A

541       GCTGTTGGTATTTGTGGTCTAGCTATTGGTAAACCTGAATATCTAGAGATGTCGGTATAC
181        A  V  G  I  C  G  L  A  I  G  K  P  E  Y  L  E  M  S  V  Y

601       GGTCAAGACCGTGATGATACTGGTGGCTTCCTAGCGCAAATCTCACAGCTGTTCGCCCCT
201        G  Q  D  R  D  D  T  G  G  F  L  A  Q  I  S  Q  L  F  A  P

661       TCTGGTTACTACATGGAAGGTCCGTACTACCATCGTTATGCGATTCGTCCAACTTGTGTA
221        S  G  Y  Y  M  E  G  P  Y  Y  H  R  Y  A  I  R  P  T  C  V

721       TTTGCAGAAGTAGTACACCGTCACATGCCTGAAGTAGACATCTACAACTACAAAGACAAA
241        F  A  E  V  V  H  R  H  M  P  E  V  D  I  Y  N  Y  K  D  K

781       GTGATTGGCAACACAGTACAAGCGATGCTGGCAACGGCTTACCCGAATGGTGAATTCCCT
261        V  I  G  N  T  V  Q  A  M  L  A  T  A  Y  P  N  G  E  F  P

841       GCCCTAAACGACGCATCTCGTACCATGAGCATTACCGACATGGGCGTTCAAGTTGCAGTG
281        A  L  N  D  A  S  R  T  M  S  I  T  D  M  G  V  Q  V  A  V

901       AGCGTTTACAGCAAACACTACGGTATGGATGACAACATCCTTGGCATGGCGAAGATTCAA
301        S  V  Y  S  K  H  Y  G  M  D  D  N  I  L  G  M  A  K  I  Q

961       AACGCCGTTTGGATGCACCCATGTGGTCTTGAGCTTTCTCAAGCCTACGATAAAGCCATC
321        N  A  V  W  M  H  P  C  G  L  E  L  S  Q  A  Y  D  K  A  I

1021      GCAGACCGTGAAATCGGCATGCCTTTCTGGCCGAGCGTGGAGCTCAACGAAGGTCCAACT
341        A  D  R  E  I  G  M  P  F  W  P  S  V  E  L  N  E  G  P  T

1081      GGTAACAACGGAGCACAAGGCTTTATCCGTATGCAGGATAAAACCGGTGATGTGTCACAG
361        G  N  N  G  A  Q  G  F  I  R  M  Q  D  K  T  G  D  V  S  Q

1141      CTTGTGATGAACTACGGTCAACACGGCATGGGACATGGTAACTTCGATACGCTTGGCATT
381        L  V  M  N  Y  G  Q  H  G  M  G  H  G  N  F  D  T  L  G  I

1201      ACCTTCTTCAACCGTGGTCAAGAAGTGCTGCGTGAATACGGCTTCTGTCGCTGGGTAAAC
401        T  F  F  N  R  G  Q  E  V  L  R  E  Y  G  F  C  R  W  V  N

1261      GTTGAGCCTAAATTCGGCGGTCGTTACCTAGACGAAAACAAATCGTACGCACGTCAAACT
421        V  E  P  K  F  G  G  R  Y  L  D  E  N  K  S  Y  A  R  Q  T

1321      ATCGCGCACAACGCGGTAACGATTGATGAGCAATGTCAGAACGGTTTCGATGTAGACCGC
441        I  A  H  N  A  V  T  I  D  E  Q  C  Q  N  G  F  D  V  D  R
```

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1381    GCTGATTCAGTGCACGGTTTGCCTCACTTCTTCAAAGTAGAAGGCACTGAAATCAACGGT
461      A  D  S  V  H  G  L  P  H  F  F  K  V  E  G  T  E  I  N  G

1441    ATGAGCGCGTTTGCGAACGACCATTACCCGAACACAGACATGCAGCGCAGTGTGTTCATG
481      M  S  A  F  A  N  D  H  Y  P  N  T  D  M  Q  R  S  V  F  M

1501    CTTAACCTCGATGAGCTTGAAGCACCGCTATTGCTAGACCTTTACCGCATCGAAGGTGAA
501      L  N  L  D  E  L  E  A  P  L  L  L  D  L  Y  R  I  E  G  E

1561    GACGAACATCAGTACGACTACTCTCATCAATACGATGGTCAAATCGTACGTACTAACTTT
521      D  E  H  Q  Y  D  Y  S  H  Q  Y  D  G  Q  I  V  R  T  N  F

1621    GATTACCAAAGCTTTGGTGAACTGAACACGCTTGGCGATGACTTCGGTTACCAACATCTT
541      D  Y  Q  S  F  G  E  L  N  T  L  G  D  D  F  G  Y  Q  H  L

1681    TGGAAAGTAGCGAGCGGCAAAGTGCAAGATACGGCGCTGGTTAGCTGGCTACAAAACAAC
561      W  K  V  A  S  G  K  V  Q  D  T  A  L  V  S  W  L  Q  N  N

1741    ACCTACTACACTTGGTTAGGCACAAGCAGCAGCACGAAACAGAACGGCGATAATGAAGTG
581      T  Y  Y  T  W  L  G  T  S  S  S  T  K  Q  N  G  D  N  E  V

1801    ATCTTCACTCGCACTGGCGCCAATGACCCAAGCTTTAACCTACGTAGCGAACCGGCATTC
601      I  F  T  R  T  G  A  N  D  P  S  F  N  L  R  S  E  P  A  F

1861    ATTCTACGCAGCAAGGGCGAATCGACGCTATTTGCTTCTGTGCTAGAAACACACGGCTAC
621      I  L  R  S  K  G  E  S  T  L  F  A  S  V  L  E  T  H  G  Y

1921    TTCAACGAAGAGTTTGAGCAGTCGGTGAATGCTCGTGGTCAGGTGAAAGATATCCGCGTC
641      F  N  E  E  F  E  Q  S  V  N  A  R  G  Q  V  K  D  I  R  V

1981    GTGGGTTACAACGCCGTTGGCAGCATCGTAGAAATCACGACTGAAAAATCACTGGTTACT
661      V  G  Y  N  A  V  G  S  I  V  E  I  T  T  E  K  S  L  V  T

2041    GTGATGATCAGCAATGTGCTAGGCGCTGACGACCAAACCCCACACCAAGTAGAATTGAAC
681      V  M  I  S  N  V  L  G  A  D  D  Q  T  P  H  Q  V  E  L  N

2101    GGTAAAACCTACAGCTGGAATGGCTTCTACTCTCTAGAAGTAAACGCATTCGGGCAGGAG
701      G  K  T  Y  S  W  N  G  F  Y  S  L  E  V  N  A  F  G  Q  E

2161    AAATAA
721      K
```

**Figure 2.4.** Nucleotide and deduced amino acid sequence of the cloned *alyVMI* gene. The putative -10 region (underlined) and the initiation codon (ATG, bold) are shown. Amino acids highlighted in green form α-helices and amino acids highlighted in red form β-sheets as predicted by MINNOU. The amino acids highlighted in grey represent the predicted transmembrane domains. The above sequence was submitted to GENBANK and assigned the accession number HQ328551.

**Table 2.3.** Sequence similarities obtained from a protein BLAST search of the GENBANK database with the deduced amino acid sequence of AlyVMI

| GENBANK Accession Number | Protein | Organism | % Similarity | % Identity | E-Value |
|---|---|---|---|---|---|
| EAP93062 | Putative Chondroitin AC/Alginate lyase | *Vibrio splendidus* 12B01 | 91 | 83 | 0.0 |
| EAQ52860 | Putative Chondroitin AC/Alginate lyase | *Vibrio* sp. MED222 | 91 | 83 | 0.0 |
| EDK27096 | Heparinase II/III-like protein | *Vibrionales* bacterium SWAT-3 | 90 | 83 | 0.0 |
| EDL54548 | Heparinase II/III-like protein | *Vibrio shilonii* AK1 | 87 | 74 | 0.0 |
| EAQ52861 | Putative Chondroitin AC/Alginate lyase | *Vibrio* sp. MED222 | 67 | 50 | 0.0 |
| EAP93063 | Putative Chondroitin AC/Alginate lyase | *Vibrio splendidus* 12B01 | 67 | 50 | 0.0 |
| EDK27097 | Heparinase II/III-like protein | *Vibrionales* bacterium SWAT-3 | 67 | 49 | 0.0 |
| EDL54549 | Heparinase II/III-like protein | *Vibrio shilonii* AK1 | 66 | 46 | 0.0 |
| EDP70456 | Heparinase II/III-like protein | *Flavobacteriales* bacterium ALC-1 | 55 | 35 | 7 e-121 |
| EAQ43012 | Heparinase II/III-like protein | *Polaribacter* sp. MED152 | 54 | 34 | 5 e-121 |
| EAP86660 | Putative Chondroitin AC/Alginate lyase | *Croceibacter atlanticus* HTCC2559 | 52 | 33 | 9 e-116 |



### 2.4.3.2. Sequence analysis of *alyVMII*

The full-length sequence (2163 bp) of *alyVMII* was present on pAlg15, 6 bp downstream of *alyVMI* (Figure 2.5). The deduced AlyVMII protein is 721 amino acids with a calculated molecular weight of 81.06 kDa and a predicted pI of 5.02. A 7 bp inverted repeat is present ~50bp after the stop codon of *alyVMII* and has a calculated ΔG of -2.80 Kcal/mol. This region of DNA has the ability to form a hairpin loop and could function as a Rho-independent transcription terminator. It has a negative Gibbs free energy making the hairpin formation favourable; however, it is a low value and thus might not be a very strong terminator.

InterProScan identified a Chondroitin AC/Alginate lyase domain and a Heparinase II/III-like domain (Table 2.2). PSORTb did not predict the cellular location of AlyVMII. The amino-terminus (residues 1 to ~345) of AlyVMII was predicted to include α-helices and the carboxy-terminus (residues ~370 to 721) β-strands by MINNOU (Figure 2.5). Three transmembrane regions were also predicted at amino acid residues 122-134, 179-195 and 235-254. A Protein BLAST search of the GENBANK database revealed that AlyVMII was similar to chondroitin AC/alginate lyases and heparinase II/III-like proteins (Table 2.4). AlyVMII does not contain any alginate lyase consensus sequence motifs.

### 2.4.3.3. Sequence analysis of *alyVMIII*

The partial sequence of *alyVMIII* was found on pAlg15 and the remainder of the sequence was located on pAlg65. Alginate lyase gene *alyVMIII* is situated 625 bp downstream of *alyVMII*. The full-length sequence of *alyVMIII* is 1449 bp (Figure 2.6). A putative -10 region (TATAA) and ribosome binding site (RBS, AGGA) were identified. The deduced protein is 483 amino acids with a calculated molecular weight of 54.78 kDa and a predicted pI of 4.98. PSORTb did not make any predictions as to the cellular location of AlyVMIII. InterProScan identified two concanavalin A-like lectin/glucanase domains and two alginate lyase 2 domains (Table 2.2). The alginate lyase 2 and concanavalin A-like lectin/glucanase domain present on the amino-terminus of AlyVMIII is truncated at the amino-end since ~80-100 amino acids are missing when compared to the domain present on the carboxy-terminus of AlyVMIII and in AlyVMIV. AlyVMIII was predicted to be predominantly comprised of β-strands with only a few α-helices (Figure 2.6). No transmembrane regions were predicted to be present in AlyVMIII. A Protein BLAST search of the GENBANK database revealed that AlyVMIII had sequence similarity to several alginate lyase enzymes (Table 2.5). AlyVMIII contains the alginate lyase consensus sequence motifs RXELR, QIH and YFKAGXYXQ where X is any residue. RSELR is present at amino acid residues 274-282, QIH at residues 49-56 and 337-339, YFKAGVYNQ at residues 161-169 and YFKAGIYPQ at residues 451-459 (Figure 2.6). The conserved sequence motif RXELR is present at the amino-terminus of the alginate lyase 2 superfamily conserved domain whereas QIH and YFKAGXYXQ are present at the carboxy-terminus of the domain. QIH and YFKAGXYXQ are present twice in AlyVMIII whereas RXELR occurs only once. The amino-

terminus of the alginate lyase 2 superfamily conserved domain present on the amino-end of AlyVMIII is missing which could be the reason that only one RXELR consensus sequence motif is present in AlyVMIII.

```
1          ATGAGCTACCAAACCCAGTCTTACCAACCGCTATTAATGAACTTTGAAGAAGCGGCAGAA
1           M  S  Y  Q  T  Q  S  Y  Q  P  L  L  M  N  F  E  E  A  A  E

61         CTAAGCAAAGCACTTGGCACGGATAGCCTATTAGGAAACGCACTAGCACGTGACATCAAA
21          L  S  K  A  L  G  T  D  S  L  L  G  N  A  L  A  R  D  I  K

121        CAAACTGACGCTTACATGGCCGAGGTAGGTATTGAAGTTCCAGGTCACGGCGAAGGCGGC
41          Q  T  D  A  Y  M  A  E  V  G  I  E  V  P  G  H  G  E  G  G

181        GGTTACGAGCATAACCGTCACAAGCAAAACTACATCCACATTGATCTAGCGGGTCGCCTA
61          G  Y  E  H  N  R  H  K  Q  N  Y  I  H  I  D  L  A  G  R  L

241        TTCCTTATCACAGGCGAACAGAAGTACCGCGATTACATCGTGGACATGCTAACGGCTTAC
81          F  L  I  T  G  E  Q  K  Y  R  D  Y  I  V  D  M  L  T  A  Y

301        GCGAAGGTATACCCGACGCTAGAAAGCAACACCAGCCGTGACTCTAACCCTCCGGGTAAG
101         A  K  V  Y  P  T  L  E  S  N  T  S  R  D  S  N  P  P  G  K

361        ATCTTCCACCAAACTCTGAACGAGAACATGTGGATGCTATACGCGTCTTGTGCGTACAGC
121         I  F  H  Q  T  L  N  E  N  M  W  M  L  Y  A  S  C  A  Y  S

421        TGCATTTACCACACGTTGGAAGAAGAGCAAAAAACACTGATTGAAAACGACCTGTTCAAA
141         C  I  Y  H  T  L  E  E  E  Q  K  T  L  I  E  N  D  L  F  K

481        CAAATGATTGAGCTGTTCGTAGTCACTTACGGTCACGACTTCGACATCGTACACAACCAC
161         Q  M  I  E  L  F  V  V  T  Y  G  H  D  F  D  I  V  H  N  H

541        GGTCTGTGGGCAGTAGCAGCAGTAGGTATCTGTGGTTACGCAATTAACGATCAAGATGCG
181         G  L  W  A  V  A  A  V  G  I  C  G  Y  A  I  N  D  Q  D  A

601        GTAGACAAAGCGCTTTACGGCTTGAAACTGGACAAAGTGAGCGGCGGCTTCCTAGCTCAG
201         V  D  K  A  L  Y  G  L  K  L  D  K  V  S  G  G  F  L  A  Q

661        CTAGACCAACTGTTCTCGCCAGATGGCTACTACATGGAAGGTCCTTACTACCACCGTTTC
221         L  D  Q  L  F  S  P  D  G  Y  Y  M  E  G  P  Y  Y  H  R  F

721        TCGCTGCGTCCAATCTACCTGTTCGCAGAAGCGATTGAGCGTCGTCAGCCTGAGCTTGGT
241         S  L  R  P  I  Y  L  F  A  E  A  I  E  R  R  Q  P  E  L  G

781        ATTTACGAGTTCAACGACTCGGTAATCAAGACCACGTCTTACGCCGTATTCAAGACAGCA
261         I  Y  E  F  N  D  S  V  I  K  T  T  S  Y  A  V  F  K  T  A

841        TTCCCAGATGGTACTTTGCCTGCACTGAACGATTCATCGAAGACGATTTCGATTAACGAT
281         F  P  D  G  T  L  P  A  L  N  D  S  S  K  T  I  S  I  N  D

901        GAAGGCGTAATCATGGCTACGTCAGTATGTTTCCACCGTTACGAGCAGTCTGAAACGCTA
301         E  G  V  I  M  A  T  S  V  C  F  H  R  Y  E  Q  S  E  T  L

961        CTTGGCATGGCAGACCACCAGCAAGACGTTTGGGTTCATATCTCAGGTAAAACATTGTCT
321         L  G  M  A  D  H  Q  Q  D  V  W  V  H  I  S  G  K  T  L  S

1021       GACGCGGTTGCAGCAGCAGACAACATCAAACCATTCAACTGGGGTAGCCTATTCGTAACA
341         D  A  V  A  A  A  D  N  I  K  P  F  N  W  G  S  L  F  V  T

1081       GACGGCCCAGAAGGCGAAAAAGGCGGCGTAAGCATTCTTCGTCACCGTGACGAGCAAGAC
361         D  G  P  E  G  E  K  G  G  V  S  I  L  R  H  R  D  E  Q  D

1141       GACGATACGATGGCGCTTATCTGGTTTGGTCAACACGGCAGCGATCACCAGTACCACTCA
381         D  D  T  M  A  L  I  W  F  G  Q  H  G  S  D  H  Q  Y  H  S
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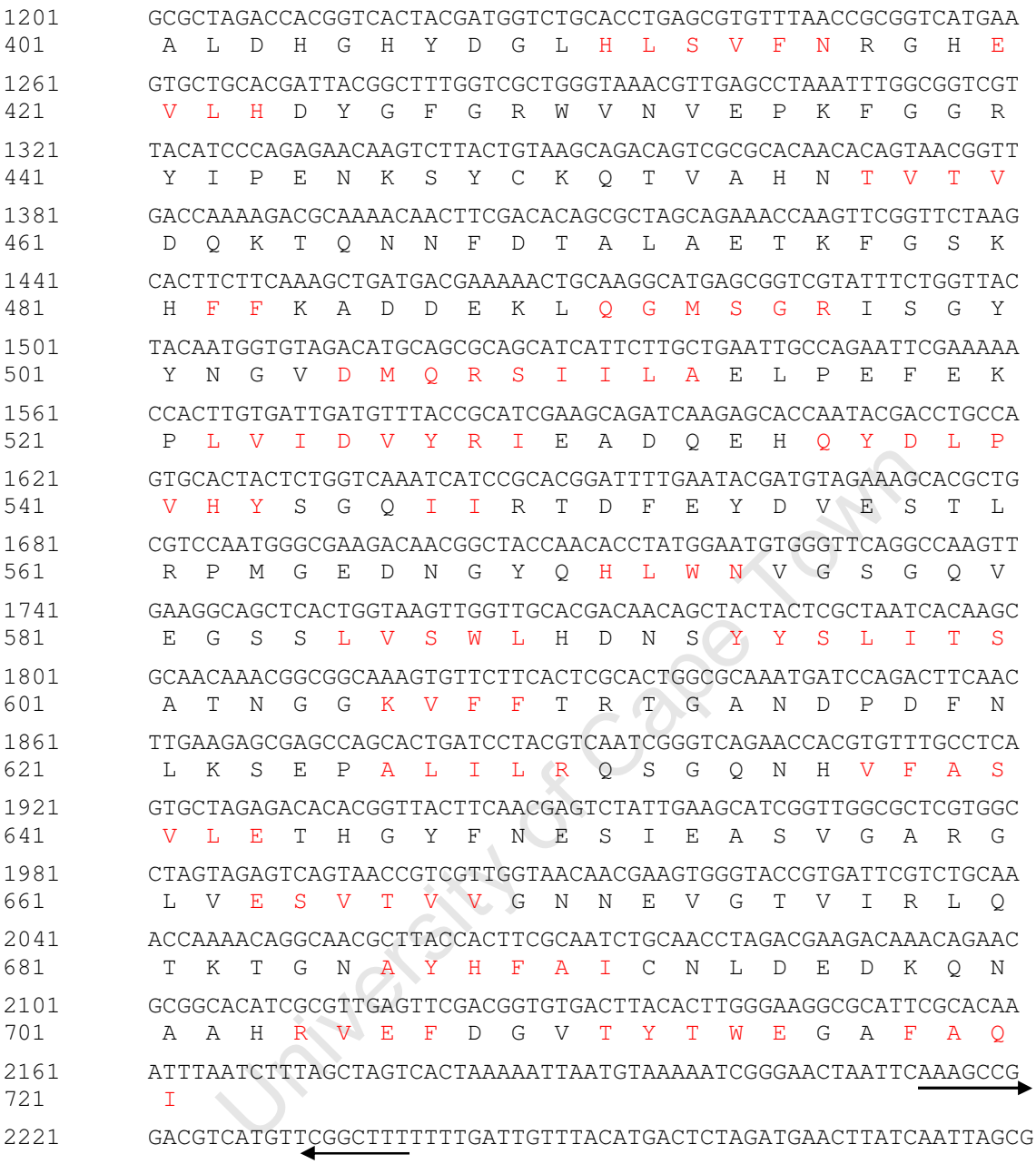





**Figure 2.5.** Nucleotide and deduced amino acid sequence of the cloned *alyVMII* gene. The initiation codon (ATG, bold) and putative transcriptional terminator (inverted arrows) are shown. Amino acids highlighted in green form α-helices and amino acids highlighted in red form β-sheets as predicted by MINNOU. The amino acids highlighted in grey represent the predicted transmembrane domains. The above sequence was submitted to GENBANK and assigned the accession number HQ328552.

**Table 2.4**. Sequence similarities obtained from a protein BLAST search of the GENBANK database with the deduced amino acid sequence of AlyVMII

| GENBANK Accession Number | Protein | Organism | % Similarity | % Identity | E-Value |
|---|---|---|---|---|---|
| EDK27097 | Heparinase II/III-like protein | *Vibrionales* bacterium SWAT-3 | 94 | 89 | 0.0 |
| EAQ52861 | Putative Chondroitin AC/Alginate lyase | *Vibrio* sp. MED222 | 93 | 88 | 0.0 |
| EAP93063 | Putative Chondroitin AC/Alginate lyase | *Vibrio splendidus* 12B01 | 93 | 87 | 0.0 |
| EDL54549 | Heparinase II/III-like protein | *Vibrio shilonii* AK1 | 90 | 83 | 0.0 |
| EDL54548 | Heparinase II/III-like protein | *Vibrio shilonii* AK1 | 67 | 46 | 0.0 |
| EAQ52860 | Putative Chondroitin AC/Alginate lyase | *Vibrio* sp. MED222 | 66 | 46 | 0.0 |
| EAP93062 | Putative Chondroitin AC/Alginate lyase | *Vibrio splendidus* 12B01 | 66 | 46 | 0.0 |
| EDK27096 | Heparinase II/III-like protein | *Vibrionales* bacterium SWAT-3 | 65 | 47 | 0.0 |
| EDP70456 | Heparinase II/III-like protein | *Flavobacteriales* bacterium ALC-1 | 56 | 33 | 3 e-123 |
| EAP86660 | Putative Chondroitin AC/Alginate lyase | *Croceibacter atlanticus* HTCC2559 | 54 | 35 | 4 e-126 |
| EAQ43012 | Heparinase II/III-like protein | *Polaribacter* sp. MED152 | 54 | 33 | 2 e-124 |

```
                GGGAAGGAGGGAAATAAAACCAGTGACGTCAAAAATGGAGCATTCTCGGATTATAAGAGT
                                                                  -10
                GACCACTTTTACGCAGAAAAGGATTCCAATTACCTTGTCTTTAAAATGTCGAACTACAAA
                                      RBS
1               ATGTCGAACTACAAAATGCGCTCAGAAGTTCGTGAGCGAGAAAACTTCAACATTTCAGAA
1                M  S  N  Y  K  M  R  S  E  V  R  E  R  E  N  F  N  I  S  E

61              CAAGGCGTCTTTAGAGCTTTGTATGCAGATGTTCGATTACCAGAGATTAACCTTGCAATG
21               Q  G  V  F  R  A  L  Y  A  D  V  R  L  P  E  I  N  L  A  M

121             GCTAGCTCCCCTGCAAACCATGATGAAGTCACTTTCCTGCAAATTCATAACAAAGGCACA
41               A  S  S  P  A  N  H  D  E  V  T  F  L  Q  I  H  N  K  G  T

181             GATACTTCTGGTACAGGTGCTATCCCACACCCTTTGTTACGAATCGTTTGGGAACAAGAA
61               D  T  S  G  T  G  A  I  P  H  P  L  L  R  I  V  W  E  Q  E

241             CGAAACAGTATCACTGGTCATTACTGGGCGGTAGTAAAGAACAATGCCATAGATTGCAGT
81               R  N  S  I  T  G  H  Y  W  A  V  V  K  N  N  A  I  D  C  S

301             CTACCTTCTAGCGCATCAGATTGTTATGCAACATCATATGATCGTTATGACTTAGGAAAA
101              L  P  S  S  A  S  D  C  Y  A  T  S  Y  D  R  Y  D  L  G  K

361             GCCGATCTCAACGCATTCACGCGATTCGAAGTAAAAATCGGAGAAAACACATTAACCATT
121              A  D  L  N  A  F  T  R  F  E  V  K  I  G  E  N  T  L  T  I

421             AAAGTGAACGATGAGCAAAAGGTAAATGTAGATGTATCCTACTGGCAGCACCTCCTGAGC
141              K  V  N  D  E  Q  K  V  N  V  D  V  S  Y  W  Q  H  L  L  S

481             TACTTTAAAGCCGGAGTTTACAACCAGTTTGAAAACGGTGAAGCAAAGGTACAATTTAAG
161              Y  F  K  A  G  V  Y  N  Q  F  E  N  G  E  A  K  V  Q  F  K

541             CAGTTAGGACTAACCAAAACTGACCATACTGATTCAATAGCTTGGAATATTGACGATTGG
181              Q  L  G  L  T  K  T  D  H  T  D  S  I  A  W  N  I  D  D  W

601             AAATTAACCATTCCTACAAGCAAAAATGATTGGTATGGTTTTGGCGGTGACAGCGCGGCT
201              K  L  T  I  P  T  S  K  N  D  W  Y  G  F  G  G  D  S  A  A

661             GAATTAGAGCCTGAGCGTTGTAATTCAAGTAAAGATCTTCTGTCTAACGAAGAGAGCGTT
221              E  L  E  P  E  R  C  N  S  S  K  D  L  L  S  N  E  E  S  V

721             TACCAACGCGAGATTGATTTATCATACTTCAATGTTATTGACGGTAGCATGCATTTCCGC
241              Y  Q  R  E  I  D  L  S  Y  F  N  V  I  D  G  S  M  H  F  R

781             GCCGATATGGGTTACGGCACGTCAACGGCCAACTCCAGTTACATCCGTTCAGAATTGCGA
261              A  D  M  G  Y  G  T  S  T  A  N  S  S  Y  I  R  S  E  L  R

841             GAGCTCTACATCAGTACTAACTCCCCGGATTGCAGCACCAGCGATGAAGAGACAAGTTGG
281              E  L  Y  I  S  T  N  S  P  D  C  S  T  S  D  E  E  T  S  W

901             TATATTGAAGATAGTCGTACCGGTGCAACTTCGCATACGCTAAACGCAACACTAAGAATT
301              Y  I  E  D  S  R  T  G  A  T  S  H  T  L  N  A  T  L  R  I

961             AACGAATACCCTAAAATCGACGGTCAATTACCAAAAGTCGTGGTAGGCCAGATACATGGC
321              N  E  Y  P  K  I  D  G  Q  L  P  K  V  V  V  G  Q  I  H  G

1021            TGGAAAATCAGCCAAGCACTCGTGAAGCTACTTTGGGAAGGTGACAATAAGCCAGTCAGA
341              W  K  I  S  Q  A  L  V  K  L  L  W  E  G  D  N  K  P  V  R

1081            GTTATTCTGAACGATAACTACAAACTTGATAACAACAAAGACTGTACTGATTGCAACGCA
361              V  I  L  N  D  N  Y  K  L  D  N  N  K  D  C  T  D  C  N  A

1141            TTCAGCGTTAAGCTTGGTACCTACGCGGTAAACGAAGACTGGCAATATACGATCCGTGCC
381              F  S  V  K  L  G  T  Y  A  V  N  E  D  W  Q  Y  T  I  R  A

1201            GATAAGGAAGGACTGTTTTTAGCCTCCTACGATGCAGATGGCAGTAATATGGTCTCGCAC
401              D  K  E  G  L  F  L  A  S  Y  D  A  D  G  S  N  M  V  S  H

1261            ACACTGAAGTGGGGAGAAGCATACTCAGACACCGCTAACAACAAGTCCTATACTCTGACT
421              T  L  K  W  G  E  A  Y  S  D  T  A  N  N  K  S  Y  T  L  T
```

```
1321      GAAAGATGGGCGTCGCCTGATATTGCGTTTTACTTCAAAGCCGGAATTTACCCTCAGTTT
441        E  R  W  A  S  P  D  I  A  F  Y  F  K  A  G  I  Y  P  Q  F
1381      AAACCTGATAATGCATATCGAGGAGAAATCTTTGATGTGAGCTTTAGTGCTTTGAGCACT
461        K  P  D  N  A  Y  R  G  E  I  F  D  V  S  F  S  A  L  S  T
1441      CTTCATCAATAG
481        L  H  Q
```

**Figure 2.6.** Nucleotide and deduced amino acid sequence of the cloned *alyVMIII* gene. The putative -10 region (underlined), ribosome binding site (RBS, underlined) and the initiation codon (ATG, bold) are shown. Amino acids highlighted in green form α-helices and amino acids highlighted in red form β-sheets as predicted by MINNOU. Underlined amino acids in bold italics represent alginate lyase conserved sequence motifs. The above sequence was submitted to GENBANK and assigned the accession number HQ328553.

**Table 2.5** Sequence similarities obtained from a protein BLAST search of the GENBANK database with the deduced amino acid sequence of AlyVMIII

| GENBANK Accession Number | Protein | Organism | % Similarity | % Identity | E-Value |
|---|---|---|---|---|---|
| EAP94921 | Putative lyase | *Vibrio splendidus* 12B01 | 82 | 67 | 0.0 |
| BAH79132 | Alginate lyase | *Vibrio* sp. A9m | 82 | 67 | 2 e-81 |
| CAA49630 | Alginate lyase | *Photobacterium* ATCC43367 | 62 | 44 | 4 e-40 |
| AAB36771 | Alginate lyase AlyVOA | *Vibrio* sp. O2 | 62 | 43 | 7 e-39 |
| BAH70324 | Alginate lyase | *Agarivorans* sp. JAM-A1m | 61 | 44 | 2 e-39 |
| BAH79131 | Alginate lyase | *Vibrio* sp. A9m | 59 | 43 | 2 e-37 |
| AAP45155 | Alginate lyase | *Vibrio* sp. QY101 | 59 | 42 | 2 e-50 |
| AAF22510 | Alginate lyase AlyVGII | *Vibrio halioticoli* | 55 | 40 | 4 e-41 |
| ABB36772 | Alginate lyase AlyVOB | *Vibrio* sp. O2 | 55 | 36 | 4 e-18 |
| ACO77598 | Alginate lyase AlyA3 | *Azotobacter vinelandii* DJ | 42 | 28 | 1 e-04 |

### 2.4.3.4. Sequence analysis of *alyVMIV*

The full-length sequence (1563 bp) of *alyVMIV* was present on pAlg86, 473 bp downstream of *alyVMIII* (Figure 2.7). A 10 bp inverted repeat is present ~30 bp after the stop codon of *alyVMIV* which has a calculated ΔG of -12.80 Kcal/mol and could act as a Rho-independent transcription terminator by forming a hairpin loop. The deduced protein is 521 amino acids with a calculated molecular weight of 57.63 kDa and a predicted pI of 4.96. InterProScan predicted the first 24 amino acid residues ($M^1$-$A^{22}$) of AlyVMIV to be a signal peptide. The calculated molecular weight and predicted pI of the mature protein is 55.09 kDa and 4.84, respectively. PSORTb predicted the cellular location of AlyVMIV to be extracellular. InterProScan identified a C-terminal coagulation factor 5/8 type (FA58C) domain and a galactose-binding like domain in the amino-terminus of AlyVMIV as well as a concanavalin A-like lectins/glucanases domain and an alginate lyase 2 domain in the carboxy-terminus (Table 2.2). AlyVMIV was predicted to be predominantly comprised of β-strands with few α-helices by MINNOU (Figure 2.7). No transmembrane regions were predicted. A Protein BLAST search of the GENBANK database revealed AlyVMIV to be similar to alginate lyase enzymes (Table 2.6). AlyVMIV contains the alginate lyase consensus sequence motifs RXELR, QIH and YFKAGXYXQ. RTELR is present at amino acid residues 314-322, QIH at residues 385-387 and YFKAGVYNQ at residues 488-495 (Figure 2.7).

### 2.4.3.5. Homology analysis of AlyVMI, AlyVMII, AlyVMIII and AlyVMIV

AlyVMI and AlyVMII share the highest sequence identity (49.07%) of the four alginate lyases of *Vibrio midae* SY9, followed by AlyVMI and AlyVMIII with 20.59% (Table 2.7). AlyVMII does not have a high homology with AlyVMIII or AlyVMIV, and AlyVMIV does not have a high homology with any of the other alginate lyases of *V. midae* SY9.

```
1          ATGAAGCATATTTTCTTCAAAAGCTTGTTAGCTTCTTCTATTTTACTTGCTGTGGGCTGT
1           M  K  H  I  F  F  K  S  L  L  A  S  S  I  L  L  A  V  G  C

61         AATAGTACGGCGACCATGGTAGATCCATTTCCAAACAACCAAGAAACTGGTGCTGATATT
21          N  S  T  A  T  M  V  D  P  F  P  N  N  Q  E  T  G  A  D  I

121        CTGACTCCCGTTACGATAACGGCGAGTAGTCATGATGGTAATGTTCCAGAGCAGTTGTTT
41          L  T  P  V  T  I  T  A  S  S  H  D  G  N  V  P  E  Q  L  F

181        GACCAAGATATTATGACACGTTGGTCAGCAAATGGTGATGGTGAGTGGGCGATGTTGGAT
61          D  Q  D  I  M  T  R  W  S  A  N  G  D  G  E  W  A  M  L  D

241        TACGGCTCGGTTTATGAGTTCGACGCAATTCAAGCCTCGTTCAGCAAAGGTAACGAACGC
81          Y  G  S  V  Y  E  F  D  A  I  Q  A  S  F  S  K  G  N  E  R

301        GTCAGCAAATTTGATGTTCAGTTTAGTACTGATGGTGAAAACTGGGTGACGGTTATTGAG
101         V  S  K  F  D  V  Q  F  S  T  D  G  E  N  W  V  T  V  I  E

361        GGTGCGCAAAGCTCTGGTCGCGCACTTGGCTTAGAGCGCTTCCAGTTTGAGCCTGCAGTA
121         G  A  Q  S  S  G  R  A  L  G  L  E  R  F  Q  F  E  P  A  V

421        AAAGCACGCTATGTTCGTTACGTTGGCCACGGTAATAGCAAAAACCAATGGAACTCAGTT
141         K  A  R  Y  V  R  Y  V  G  H  G  N  S  K  N  Q  W  N  S  V

481        ACTGAGTTAGCGGCAGTTAATTGCGGAATTAATGCCTGTCCGGCAAGTCACATCATTACT
161         T  E  L  A  A  V  N  C  G  I  N  A  C  P  A  S  H  I  I  T

541        GATGACGTCGTTAACGCTGAAGCGGCTATGATTGCGCAAATGAAAGCGGAGCAAAAGGCA
181         D  D  V  V  N  A  E  A  A  M  I  A  Q  M  K  A  E  Q  K  A

601        CAAAAGGATTTACTCAAAAAGAATCGTAAAGGCGACTTTGGAGCACCAATCGTCCGTCCT
201         Q  K  D  L  L  K  K  N  R  K  G  D  F  G  A  P  I  V  R  P

661        TGTGAAACGACGGTGACGTGTGATCTTTCTAAAGCAATGCCTTACCCTACGTTACCGAAA
221         C  E  T  T  V  T  C  D  L  S  K  A  M  P  Y  P  T  L  P  K

721        GAGCCACTCGCTACAAATGCACCGGGTGAAAACTTTGACTTAACACGTTGGAAACTAACG
241         E  P  L  A  T  N  A  P  G  E  N  F  D  L  T  R  W  K  L  T

781        ACACCTTTCGACCATGACAAAGATGGTCGTGCTGATGATATTGATGAGTGGGACTTGGCT
261         T  P  F  D  H  D  K  D  G  R  A  D  D  I  D  E  W  D  L  A

841        AACGGCTTCCAACACTCTGATATATTCTACACAGCAGATGATGGCGGAATGGTATTCAAG
281         N  G  F  Q  H  S  D  I  F  Y  T  A  D  D  G  G  M  V  F  K

901        AGCTATGTGAAAGGCGCTCGAACTTCTGAAAACACTAAATACGCGCGAACTGAGTTACGT
301         S  Y  V  K  G  A  R  T  S  E  N  T  K  Y  A  R  T  E  L  R

961        ACCATGCTTCGTGCTGGTGATAAGTCTTACAGCACAAAGGGTGTAAATCCTAATAACTGG
321         T  M  L  R  A  G  D  K  S  Y  S  T  K  G  V  N  P  N  N  W

1021       GTATTCAGTTCTGCACCAGTTGAAGATCAAAAAGAAGCGGGTGGTGTAGACGGTACGCTT
341         V  F  S  S  A  P  V  E  D  Q  K  E  A  G  G  V  D  G  T  L

1081       GAAGCGACTCTGAAGATTGACCACGCAACCACAACTGGTCAGTCGCATGAAGTTGGCCGC
361         E  A  T  L  K  I  D  H  A  T  T  T  G  Q  S  H  E  V  G  R

1141       TTTATTATCGGCCAGATTCATGACAAAGATGATGAGCCAATTCGTCTTTATTATCGTAAG
381         F  I  I  G  Q  I  H  D  K  D  D  E  P  I  R  L  Y  Y  R  K

1201       CTACCAGACCAACCAACAGGTACAGTTTACTTTGCTCACGAAAAAACCAAAACTGGAACA
401         L  P  D  Q  P  T  G  T  V  Y  F  A  H  E  K  T  K  T  G  T

1261       GAGGATTACTACAGCTTAGTGGGTGATATGACTGGTGAAATTGGTGACGATGGCATCGCG
421         E  D  Y  Y  S  L  V  G  D  M  T  G  E  I  G  D  D  G  I  A

1321       CTAGGTGAAAAATTCAGCTACATCATTGATGTGAAAGGCAACACAATGACAGTTACGGTA
441         L  G  E  K  F  S  Y  I  I  D  V  K  G  N  T  M  T  V  T  V
```


**Figure 2.7.** Nucleotide and deduced amino acid sequence of the cloned *alyVMIV* gene. The initiation codon (ATG, bold) and the putative transcriptional terminator (inverted arrows) are shown. The dashed line represents a putative signal sequence identified by InterProScan. Underlined amino acids in bold italics represent alginate lyase conserved sequence motifs. Amino acids highlighted in green form α-helices and amino acids highlighted in red form β-sheets as predicted by MINNOU. The reverse complement sequence is shown. The above sequence was submitted to GENBANK and assigned the accession number HQ328554.

### 2.4.3.6. Sequence analysis of the putative oligogalacturonate specific porin

The full-length sequence (771 bp) of a putative oligogalacturonate specific porin was present on pAlg86, 79 bp downstream of *alyVMIV* (Figure 2.8). The porin is 257 amino acids with a calculated molecular weight of 29.55 kDa and a predicted pI of 5.65. InterProScan predicted the first 22 amino acid residues ($M^1$ to $A^{22}$) to be a signal peptide (Figure 2.8). The calculated molecular weight and predicted pI of the mature porin is 27.38 kDa and 5.47, respectively. InterProScan identified an oligogalacturonate specific porin domain (residues 16-257) as well as a porins domain (residues 41-257). PSORTb predicted the cellular localization of the galacturonate specific porin to be in the outer-membrane. The porin was predicted to consist predominantly of β-strands with only a few α-helices by MINNOU (Figure 2.8). No transmembrane regions were predicted. A Protein BLAST search of the GENBANK database revealed the porin to be similar to oligogalacturonate specific porin proteins (Table 2.8).

**Table 2.6** Sequence similarities obtained from a protein BLAST search of the GENBANK database with the deduced amino acid sequence of AlyVMIV

| GENBANK Accession Number | Protein | Organism | % Similarity | % Identity | E-Value |
|---|---|---|---|---|---|
| ACM89454 | Alginate lyase | *Pseudoalteromonas* sp. CY24 | 83 | 73 | 0.0 |
| BAH79133 | Alginate lyase | *Vibrio* sp. A9m | 83 | 72 | 0.0 |
| EAP94922 | Putative alginate lyase | *Vibrio splendidus* 12B01 | 82 | 71 | 0.0 |
| AAA25049 | Alginate lyase | *Klebsiella pneumoniae* | 72 | 58 | 2 e-84 |
| EAQ42287 | Alginate lyase precursor | *Polaribacter* sp. MED152 | 67 | 51 | 8 e-70 |
| BAG70358 | Alginate lyase | *Agarivorans* sp. JAM-A1m | 65 | 50 | 9 e-69 |
| ABD82130 | Poly (beta-D-Mannuronate) lyase | *Saccharophagus degradans* 2-40 | 63 | 47 | 1 e-55 |
| ABD82541 | Poly (beta-D-Mannuronate) lyase | *Saccharophagus degradans* 2-40 | 61 | 43 | 2 e-58 |
| AAF22512 | Alginate lyase AlyVGI | *Vibrio halioticoli* | 50 | 35 | 5 e-32 |
| ABD82096 | Poly (beta-D-Mannuronate) lyase | *Saccharophagus degradans* 2-40 | 50 | 32 | 2 e-27 |
| EAP94925 | Putative alginate lyase | *Vibrio splendidus* 12B01 | 49 | 33 | 2 e-31 |
| EAP94396 | Putative alginate lyase | *Vibrio splendidus* 12B01 | 49 | 32 | 3 e-27 |



**Table 2.7.** Homology analysis of AlyVMI, AlyVMII, AlyVMIII and AlyVMIV

| % Identity | AlyVMII | AlyVMIII | AlyVMIV |
|---|---|---|---|
| AlyVMI | 49.07% | 20.59% | 16.37% |
| AlyVMII | - | 17.99% | 16.08% |
| AlyVMIII | - | - | 18.96% |

```
1          ATGAAAGCAGTTACAACAATGTCCCTATTCGTTGCTTCCACATTAATGGCGGGAGCGGTT
1           M  K  A  V  T  T  M  S  L  F  V  A  S  T  L  M  A  G  A  V
61         TCCGCAGCAACTTTAGACTATCGCGCAGAATATAAGCATGAGAGTGAAACCTATGCCCAA
21          S  A  A  T  L  D  Y  R  A  E  Y  K  H  E  S  E  T  Y  A  Q
121        CGTATCAAAATTAGTGGTAGTTCTAAAATTAACGATCAGACCAAACTGAACTTTGGTGTT
41          R  I  K  I  S  G  S  S  K  I  N  D  Q  T  K  L  N  F  G  V
181        GAACAAAAATTCCATAGTTACGACNACTCGCATTTTTGGGATCAACTCGTTGCTGGTGAT
61          E  Q  K  F  H  S  Y  D  X  S  H  F  W  D  Q  L  V  A  G  D
241        TCTGAGTTTGATTGGGGAGTTCGCTACCAAATAAATAAACAATGGTTTATCCAACCAGGG
81          S  E  F  D  W  G  V  R  Y  Q  I  N  K  Q  W  F  I  Q  P  G
301        ATGCCTATTACTTTTGGCGATGATGACGAAAAAACCACTTATAAACCACAAGTTCGTGTT
101         M  P  I  T  F  G  D  D  D  E  K  T  T  Y  K  P  Q  V  R  V
361        GGTTACAAATCCTCTTTTGGCTTAAGTACCGCTTTACGTTACCGACACGAATTTCAAGTT
121         G  Y  K  S  S  F  G  L  S  T  A  L  R  Y  R  H  E  F  Q  V
421        TATTCAGATGGTGCAGGTAACAAAACACTAACGGATGGTACTTCGGTAAGCGTTGCTGGG
141         Y  S  D  G  A  G  N  K  T  L  T  D  G  T  S  V  S  V  A  G
481        AAAACAGTTGAACAAGGTAAGTGGACACTGACAGGCTCTTACGATATGCGTCAGTTTGAT
161         K  T  V  E  Q  G  K  W  T  L  T  G  S  Y  D  M  R  Q  F  D
541        AACGAGTACCTTGATAACGTCAAGCTGAGCTATGAGATCAACTACAACAAAAACTATGAC
181         N  E  Y  L  D  N  V  K  L  S  Y  E  I  N  Y  N  K  N  Y  D
601        GAAGTGCGTTTATCTGATTGGAAAGATTCTGAGTGGGATGCTGGCTTAATTGTTGGTTAC
201         E  V  R  L  S  D  W  K  D  S  E  W  D  A  G  L  I  V  G  Y
661        CAAATGGGAGATTTCCGTCCTTACTTTGAATTGTGGAATGTCAAAGGTAAGGATGGTTCA
221         Q  M  G  D  F  R  P  Y  F  E  L  W  N  V  K  G  K  D  G  S
721        ACCACGGATGACCGCCAATTGAGAACACGCCTTGGCCTTAAATACAGCTTTTAA
241         T  T  D  D  R  Q  L  R  T  R  L  G  L  K  Y  S  F
```

**Figure 2.8.** Nucleotide and deduced amino acid sequence of the gene coding for a putative oligogalacturonate specific porin. The initiation codon (ATG, bold) is shown. The dashed line represents a signal peptide predicted by InterProScan and SignalP. Amino acids highlighted in green form α-helices and amino acids highlighted in red form β-sheets as predicted by MINNOU. The reverse complement sequence is shown. The above sequence was submitted to GENBANK and assigned the accession number HQ328555.

**Table 2.8.** Sequence similarities obtained from a protein BLAST search of the GENBANK database with the deduced amino acid sequence of a putative galacturonate specific porin from *Vibrio midae* SY9

| GENBANK Accession Number | Protein | Organism | % Similarity | % Identity | E-Value |
|---|---|---|---|---|---|
| AAF22513 | Alginate lyase AlyVGIII | *Vibrio halioticoli* | 56 | 36 | 7 e-35 |
| EAP94924 | Putative Oligogalacturonate specific porin | *Vibrio splendidus* 12B01 | 53 | 35 | 5 e-38 |
| EEQ07639 | Oligogalacturonate specific porin KdgM | *Yersinia bercovieri* ATCC 43970 | 50 | 29 | 5 e-14 |
| EEQ10654 | Oligogalacturonate specific porin KdgM | *Yersinia mollaretii* ATCC 43969 | 50 | 29 | 1 e-13 |
| EEQ16489 | Oligogalacturonate specific porin KdgM | *Yersinia frederiksenii* ATCC33641 | 49 | 28 | 4 e-14 |
| EEQ20360 | Oligogalacturonate specific porin KdgM | *Yersinia intermedia* ATCC 2909 | 49 | 28 | 8 e-14 |
| EEQ04378 | Oligogalacturonate specific porin KdgM | *Yersinia rohdei* ATCC 43380 | 49 | 26 | 2 e-13 |
| CAC86224 | Oligogalacturonate specific porin KdgM | *Erwinia chrysanthemi* | 47 | 27 | 2 e-11 |
| ACS85828 | Oligogalacturonate specific porin | *Dickeya dadantii* Ech703 | 47 | 27 | 1 e-12 |
| EFE95258 | Oligogalacturonate specific porin KdgM | *Serratia odorifera* DSM4582 | 43 | 27 | 8 e-15 |



### 2.4.3.7. Sequence analysis of the putative pectin degrading protein

The full-length sequence (339 bp) for a putative pectin degrading protein was present on pAlg65, 417 bp upstream of *alyVMI* (Figure 2.9). The deduced amino acid sequence is 113 amino acids with a calculated molecular weight of 12.75 kDa and a predicted pI of 5.09. InterProScan identified a cupin RmlC-type domain (residues 1-110), a Cupin 2 conserved barrel (residues 35-104) and a RmlC-like jelly roll fold (residues 32-101). PSORTb predicted the cellular localization of the pectin degrading protein to be cytoplasmic. The pectin degrading protein was predicted to consist predominantly of β-strands with few α-helices by MINNOU (Figure 2.9). No transmembrane regions were predicted. A Protein BLAST search of the GENBANK database revealed the protein to have similarity to pectin degrading proteins (Table 2.9).

```
1          ATGACGATGAATTCTTTCTTTGTATTAGATGATAACCCGTGGGAAGAGCTAGGCGGCGGT
1           M  T  M  N  S  F  F  V  L  D  D  N  P  W  E  E  L  G  G  G
61         ATCAAGCGTAAAATTGTTGCTTACACAGATGACCTAATGGCGGTTCACCTATGTTTTGAT
21          I  K  R  K  I  V  A  Y  T  D  D  L  M  A  V  H  L  C  F  D
121        AAAGGCGCGATTGGCGCTCCACATACTCACGAAATCCACGATCAAATCGGTTACGTAGTA
41          K  G  A  I  G  A  P  H  T  H  E  I  H  D  Q  I  G  Y  V  V
181        CGTGGCAGCTTTGAAGCGGAAATCGAGGGCGAGAAGAAAGTACTAAAAGAAGGCGACGCA
61          R  G  S  F  E  A  E  I  E  G  E  K  K  V  L  K  E  G  D  A
241        TACTTTGCTCGTAAGCACATGATGCACGGTGCGGTTGCACTAGAGCAAGACAGCATTCTT
81          Y  F  A  R  K  H  M  M  H  G  A  V  A  L  E  Q  D  S  I  L
301        CTAGACATCTTCAACCCAGCACGTGAAGATTTCTTAAAATAA
101         L  D  I  F  N  P  A  R  E  D  F  L  K
```

**Figure 2.9.** Nucleotide and deduced amino acid sequence of the gene coding for a putative pectin degrading protein. The initiation codon (ATG, bold) is shown. Amino acids highlighted in green form α-helices and amino acids highlighted in red form β-sheets as predicted by MINNOU. The reverse complement sequence is shown. The above sequence was submitted to GENBANK and assigned the accession number HQ328556.

**Table 2.9.** Sequence similarities obtained from a protein BLAST search of the GENBANK database with the deduced amino acid sequence of a putative pectin degrading protein from *Vibrio midae* SY9

| GENBANK Accession Number | Protein | Organism | % Similarity | % Identity | E-Value |
|---|---|---|---|---|---|
| CAV18380 | Pectin degradation protein | *Vibrio splendidus* LGP32 | 99 | 97 | 5 e-85 |
| EAQ52859 | Pectin degradation protein | *Vibrio* sp. MED222 | 99 | 97 | 5 e-85 |
| EAP93061 | Pectin degradation protein | *Vibrio splendidus* 12B01 | 99 | 96 | 1 e-57 |
| EDK27095 | Pectin degradation protein | *Vibrionales* bacterium SWAT-3 | 95 | 88 | 1 e-52 |
| EEQ04370 | Pectin degradation protein KdgF | *Yersinia rohdei* ATCC 43380 | 79 | 63 | 4 e-34 |
| EEQ16498 | Pectin degradation protein KdgF | *Yersinia frederiksenii ATCC* 33641 | 78 | 62 | 8 e-35 |
| CAA43987 | Pectin degradation protein KdgF | *Erwinia chrysanthemi* | 76 | 61 | 4 e-34 |

### 2.4.3.8. Sequence analysis of the putative deoxygluconokinase

The partial sequence (573 bp) of a putative deoxygluconokinase was present on pAlg86, 37 bp upstream of the putative pectin degrading protein (Figure 2.10). The incomplete protein is 191 amino acids with a calculated molecular weight of 21.98 kDa and a predicted pI of 5.50. InterProScan identified a carbohydrate/purine kinase or PfkB family domain (amino acid residues 3-191), a 2-dehydro-3-deoxygluconokinase domain (residues 7-182) and a ribokinase-like domain (residues 1-191). PsortB did not predict the cellular localization of the deoxygluconokinase. MINNOU showed that the deoxygluconokinase consists of nearly equal numbers of α-helices and β-strands which almost alternate with each other (Figure 2.10). No transmembrane regions were predicted. A Protein BLAST search of the GENBANK database revealed the protein to have similarity to ketodeoxygluconokinase and 2-dehydro-3-deoxygluconokinase proteins (Table 2.10).

### 2.4.4. Southern hybridisation analysis of *alyVMII* and *alyVMIV*

A Southern hybridisation experiment was performed to determine whether *alyVMII* and *alyVMIV* originated from *Vibrio midae* SY9. DIG-labeled DNA probes specific for *alyVMII* or *alyVMIV* were used in two separate experiments. Both probes hybridised to *V. midae* SY9 genomic DNA indicating that both genes originated from *V. midae* SY9 (Figure 2.2 b and d). Neither probe hybridised to *E. coli* JM107 DNA which was used as a negative control. The DIG-labeled probe specific for *alyVMII* hybridised to a 9.5 kb *Pst*I, a 2.9 kb *Hind* III and a 5.9 kb *Sty*I *V. midae* SY9 DNA fragment (Figure 2.2 b). The DIG-labeled probe specific for *alyVMIV* hybridised to a 2.1 kb and 4.8 kb *Bgl*I, a 9.2 kb and 10.1 kb *Pst*I and a 2.3 kb *Hind*III *V. midae* SY9 DNA fragment (Figure 2.2 d).

```
1          ATGAAATCATTAAACATCGCGGTCATTGGCGAATGTATGGTTGAGCTACAGAAAAAGGAA
1           M  K  S  L  N  I  A  V  I  G  E  C  M  V  E  L  Q  K  K  E

61         GGTCAGCTAAAGCAGTCATTCGGTGGTGATACGTTGAATACCGCACTGTATTTATCTCGT
21          G  Q  L  K  Q  S  F  G  G  D  T  L  N  T  A  L  Y  L  S  R

121        TTGACCAAAGCTCACGATATTAAAACCAGCTATGTAACGGCTCTGGGTAACGATCCTTTT
41          L  T  K  A  H  D  I  K  T  S  Y  V  T  A  L  G  N  D  P  F

181        AGCCAAGAAATGCTATCAGCATGGCAAGAAGAGGGCATCGATACTAGTCTGGTTCTTTCC
61          S  Q  E  M  L  S  A  W  Q  E  E  G  I  D  T  S  L  V  L  S

241        GTTAAAGAAAAGCAACCAGGTATCTACTACATCGAAACCGATGAAACTGGCGAACGTTAC
81          V  K  E  K  Q  P  G  I  Y  Y  I  E  T  D  E  T  G  E  R  Y

301        TTCCACTACTGGCGTAACGAAGCGGCCGCTAAGTTCCTATTCGAGCAAAACGAGTCTCCG
101         F  H  Y  W  R  N  E  A  A  A  K  F  L  F  E  Q  N  E  S  P

361        CTATTAGTCGACAAACTTTACTCGTACGATGCGGTTTACTTGAGCGGTATTACATTAGCG
121         L  L  V  D  K  L  Y  S  Y  D  A  V  Y  L  S  G  I  T  L  A

421        ATCCTTACTGAAGAAGGTAAGACGCAACTGTTTGGCTTCCTTGAGCGCTTCAAGGCACAA
141         I  L  T  E  E  G  K  T  Q  L  F  G  F  L  E  R  F  K  A  Q

481        GGTGGTAAGGTTATTTTCGATAACAACTACCGTCCTAAATTGTGGGAAAGCCGCGAGAAT
161         G  G  K  V  I  F  D  N  N  Y  R  P  K  L  W  E  S  R  E  N

541        GCCATGTCTTGGTACCTTAAGATCCTTAAGCACACA
181         A  M  S  W  Y  L  K  I  L  K  H  T
```

**Figure 2.10.** Nucleotide and deduced amino acid sequence of the gene coding for a putative deoxygluconokinase. The initiation codon (ATG, bold) is shown. Amino acids highlighted in green form α-helices and amino acids highlighted in red form β-sheets as predicted by MINNOU. The reverse complement sequence is shown. The above sequence was submitted to GENBANK and assigned the accession number HQ328557.



**Table 2.10.** Sequence similarities obtained from a protein BLAST search of the GENBANK database with the deduced amino acid sequence of a putative deoxygluconokinase of *Vibrio midae* SY9

| GENBANK Accession Number | Protein | Organism | % Similarity | % Identity | E-Value |
|---|---|---|---|---|---|
| EDK27094 | Ketodeoxygluconokinase | *Vibrionales* bacterium SWAT-3 | 89 | 76 | 8 e-84 |
| EAP93060 | Putative 2-dehydro-3-deoxygluconokinase | *Vibrio splendidus* 12B01 | 89 | 76 | 7 e-84 |
| CAV18379 | 2-dehydro-3-deoxygluconokinase | *Vibrio splendidus* LGP32 | 89 | 75 | 9 e-83 |
| EAQ52858 | 2-dehydro-3-deoxygluconokinase | *Vibrio* sp. MED222 | 88 | 75 | 9 e-83 |
| EDL55320 | Putative 2-dehydro-3-deoxygluconokinase | *Vibrio shilonii* AK1 | 80 | 66 | 4 e-71 |

### 2.4.5. Determination of co-transcription of *alyVMI* and *alyVMII*

It was investigated whether alginate lyase genes *alyVMI* and *alyVMII* are co-transcribed as one mRNA transcript as they are separated by 6 base pairs on the *Vibrio midae* SY9 chromosome. Total RNA was isolated from *Vibrio midae* SY9 after growth in alginate media for 8 hours. There were no amplification products present after PCR amplification of total RNA as template using primers specific for the *V. midae* SY9 16S rRNA gene, indicating that the isolated RNA was free of genomic DNA contamination.

The isolated RNA was converted to cDNA and used as a template for PCR amplification. Plasmid pAlg15 was amplified as a positive control. The PCR strategy employed is depicted in Figure 2.11. The expected amplification products are a 975 bp fragment with primer pair 15Fh and 15Ri, a 525 bp fragment with primers 15Fm and 15Re and a 900 bp fragment with primer pair 15Fk and 15Rg. Amplification products were observed when pAlg15 and cDNA were amplified with all three primer sets. The presence of amplification products following PCR with primers 15Fk and 15Rg using cDNA as template indicates that *alyVMI* and *alyVMII* are transcribed as a single mRNA transcript.

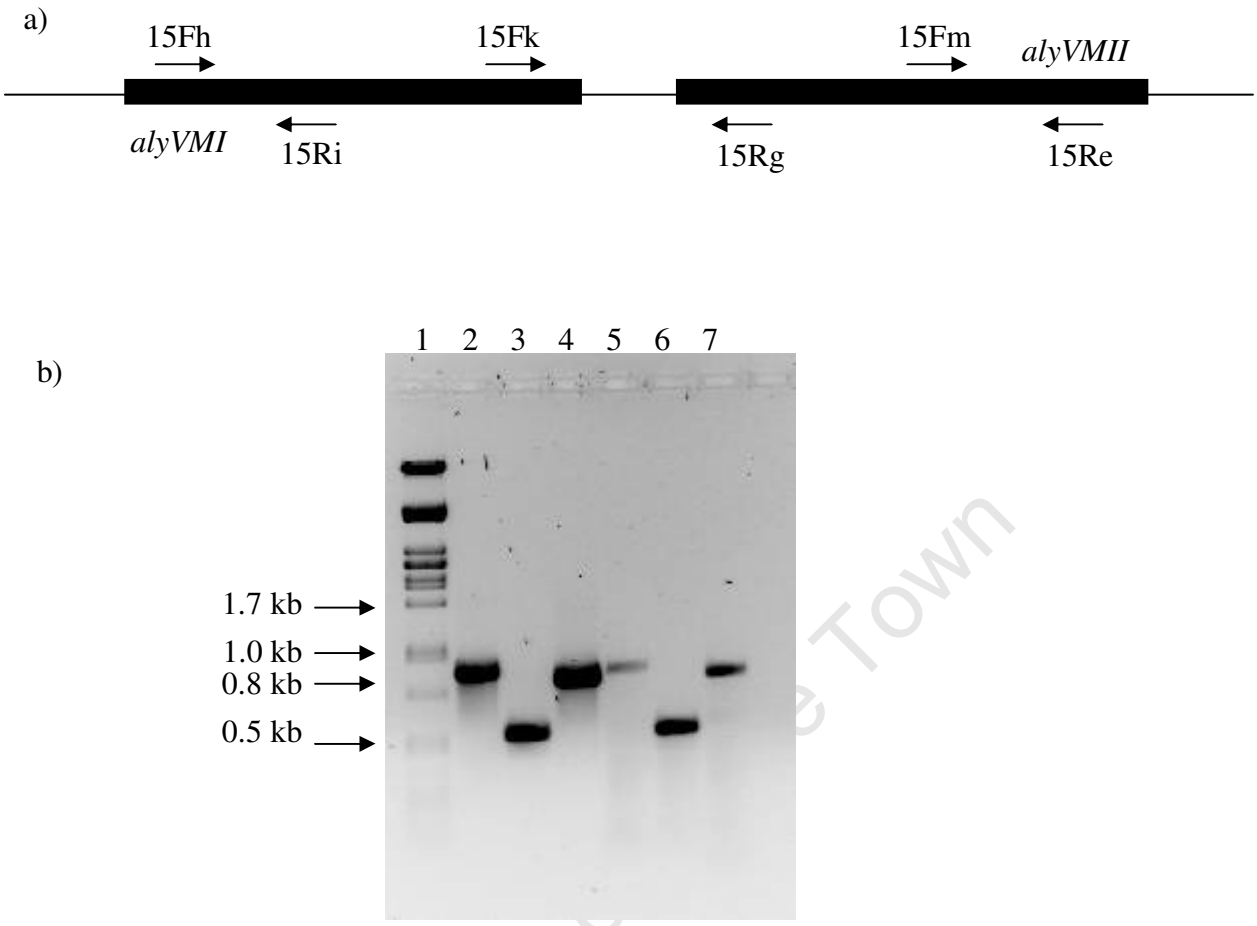





**Figure 2.11.** a) Diagram showing the PCR strategy employed to determine whether *alyVMI* and *alyVMII* are co-transcribed (diagram not drawn to scale). The thick lines represent *alyVMI* and *alyVMII* and the thin line represents the *Vibrio midae* SY9 chromosome. Fh, Fm and Fk represent the forward primers and Ri, Re and Rg represent the reverse primers. b) The amplified pAlg15 and cDNA products separated on a 1% TAE agarose gel. Lanes 2-4 are pAlg15 as template and lanes 5-7 are cDNA as template. Lane 1: lambda DNA digested with *Pst*I, Lanes 2 and 5: primer pair 15Fh and 15Ri, Lanes 3 and 6: primer pair 15Fm and 15Re, Lanes 4 and 7: primer pair 15Fk and 15Rg.

## 2.5. DISCUSSION

Alginate lyases have been isolated from many marine bacteria such as *Alteromonas* sp. strain H-4 (Sawabe *et al*. 1992, 1997, 1998a), *Photobacterium* sp. (Malissard *et al*. 1993), *Pseudoalteromonas elyakovii* (Sawabe *et al*. 2001), *Vibrio halioticoli* (Sugimura *et al*. 2000), *Vibrio* sp. O2 (Kawamoto *et al*. 2006) and *Vibrio* sp. QY101 (Han *et al*. 2004). Many strains produce more than one alginate lyase. For example, *Alteromonas* sp. strain H-4 was reported to produce an extracellular (Sawabe *et al*. 1997) and four intracellular alginate lyases (Sawabe *et al*. 1998a). *Vibrio halioticoli* has been reported to produce six alginate lyases of which three polyguluronate-specific enzymes were cloned and sequenced (Sugimura *et al*, 2000). Two alginate lyases, AlyVOA and AlyVOB, were cloned and sequenced from *Vibrio* sp. O2 (Kawamoto *et al*, 2006).

In the present study, four alginate lyase genes were cloned and sequenced after screening three genomic libraries of the abalone enteric bacterium, *Vibrio midae* SY9. The four alginate lyase genes were isolated on three overlapping genomic DNA fragments and are adjacent to each other on the *V. midae* SY9 chromosome. Genes encoding a putative oligogalacturonate specific porin, a putative pectin degrading protein and a putative deoxygluconokinase were present adjacent to the alginate lyase genes on the same DNA fragment (Figure 2.3).

To the best of our knowledge, this is the first study to report four alginate lyase genes adjacent to each other on a bacterial chromosome and adjacent to other genes involved in carbohydrate metabolism. Sugimura *et al* (2000) showed by Southern hybridisation that the genes encoding the three alginate lyases *alyVG1*, *alyVG2* and *alyVG3* from *Vibrio halioticoli* are not adjacent to each other on the chromosome. Alginate lyase genes *alyVOA* and *alyVOB* from *Vibrio* sp. O2 were isolated on different plasmids and were not shown to be connected on the chromosome (Kawamoto *et al*, 2006). The complete genome sequences of *Alteromonas macleodii*, *Azotobacter vinelandii* DJ ATCC BAA-1303 (GENBANK accession number CP001157), *Pseudoalteromonas atlantica* T6c (GENBANK accession number CP000388), *Pseudoalteromonas haloplanktis* TAC125 (GENBANK accession number CR954246 and CR954247), *Saccharophagus degradans* 2-40 (GENBANK accession number CP000282) and

*Vibrio splendidus* LGP32 (GENBANK accession number FM954972 and FM954973) were searched for alginate lyase, oligogalacturonate specific porins, pectin degrading proteins and deoxygluconokinases genes. None of the searched genomes contained any of these genes adjacent to each other.

Several protein domains were predicted to be present in the four alginate lyases of *Vibrio midae* SY9 (Table 2.2). AlyVMI and AlyVMII contained a chondroitin AC/alginate lyase domain and a heparinase II/III-like domain. AlyVMIII and AlyVMIV contained an alginate lyase 2 domain and a concanavalin A-like lectins/glucanases domain. AlyVMIV also contained a C-terminal coagulation factor 5/8 type (FA58C) and a galactose binding-like domain. AlyVMI and AlyVMII were predicted to contain transmembrane domains (Figure 2.4 and 2.5) but AlyVMIII and AlyVMIV were not. AlyVMIII and AlyVMIV contain alginate lyase consensus sequences YXRSELREM, QIH, YFKAGXYXQ (Figure 2.6 and 2.7) but AlyVMI and AlyVMII do not. Properties and possible functions of the protein domains and consensus sequences present in the alginate lyases of *V. midae* SY9 are discussed.

**Chondroitin AC/alginate lyase domain:**

Chondroitin sulphate consists of disaccharide repeating units of 2-deoxy-2-acetamide-$_D$-galactose 1-4 linked to uronic acids such as $_D$-glucuronic acid in chondroitin sulphate A and C (Zhang *et al.* 2008). Chondroitin AC lyases act on chondroitin sulphate A and C in either an endolytic or exolytic manner. The chondroitin AC/alginate lyase family is part of the alginate lyase 1 super family and these proteins exhibit an incomplete $(\alpha/\alpha)_5$ toroid or all α-fold (Huang *et al*. 2001). The amino-terminus of AlyVMI and AlyVMII were predicted to consist mostly of α-helices (Figure 2.4 and 2.5). This prediction is in agreement with the all α-fold of the chondroitin AC/alginate lyase domain. Many polysaccharide lyases which act on other uronic acid polymers display variants of the $(\alpha/\alpha)_6$ fold (Charnock *et al.* 2002). An example is family PL-5 lyases, which includes alginate lyases, and PL-8 lyases which include chondroitin and hyaluronate lyases. Charnock *et al.* (2002) suggested that the α-toroid fold is a powerful motif which can be adapted to accommodate a wide range of specificities and catalytic mechanisms of enzymes which act on carbohydrates.

**Heparinase II/III-like domain:**

Heparin and heparan sulphate are composed of repeating glucosamine or hexuronic acid residues, either iduronic or glucuronic acid. The 2, 3 or 6 position of glucosamine or the 2 position of the hexouronic acid may be sulphated (Su *et al.* 1996). Heparinase II degrades heparin and heparan sulphate whereas heparinase III predominantly degrades heparan sulphate. Both heparinases are secreted into the periplasmic space upon induction with heparin. Proteins in the heparinase II/III-like family feature sequences similar to those of heparinase II and heparinase III from *Flavobacterium heparinum*. However, unlike the typical heparinase II/III-like domain, the C-terminus of AlyVMI and AlyVMII, where this domain is predicted to be located, consists predominantly of β-strands and not α-helices. The presence of a heparinase II/III-like domain in the carboxy-terminus of AlyVMI and AlyVMII could simply represent similarity with the heparinase genes of *F. heparinum* at the amino acid level. Heparin and alginate consist of uronic acids and this could lead to similar catalytic sequences as discussed for the chondroitin AC/alginate lyase domain.

**Alginate lyase 2 domain:**

The difference between the alginate lyase and alginate lyase 2 super families is in the secondary structure of the proteins. The alginate lyase family adopts an all α-fold i.e. the secondary structure of the protein consists mostly of α-helices, whereas proteins in the alginate lyase 2 family form an all β-fold; i.e. the secondary structure of the proteins of this family consist mostly of β-sheets. AlyVMIII and AlyVMIV were predicted to consist predominantly of β-sheets (Figure 2.6 and 2.7). This prediction is in agreement with the all β-fold of the alginate lyase 2 super family.

**Concanavalin A-like lectins/glucanases domain:**

Concanavalin A is a lectin that was isolated from seeds of the legume *Canavalia ensiformis* (Wang *et al*. 1975). Concanavalin A is a tetramer of identical subunits each of which possess a single saccharide binding site. Concanavalin A possesses hemagglutinating activity, is able to precipitate various polysaccharides, is mitogenic for lymphocytes and binds to cells by interacting with saccharide-containing receptors (Wang *et al*. 1975). Bacterial and fungal β-glucanases carry out acid catalysis of β-glucans, such as cellulose and xylan, that occur in

microorganisms and plants (Hahn *et al*. 1995). Lectins and glucanases contain a concanavalin A-like domain and can reversibly bind to specific complex carbohydrates (Sanz-Aparicio *et al*. 1997). Many proteins involved in cell recognition and adhesion, such as bacterial and viral toxins, also contain concanavalin A-like domains. For example, the *Clostridium* neurotoxins responsible for the neuroparalytic effects of botulism and tetanus contain concanavalin A-like domains (Swaminathan and Eswaramoorthy, 2000). The concanavalin A domain consists of 12-14 β-strands arranged into two large anti-parallel pleated β-sheets. The role of a concanavalin A-like domain in AlyVMIII and AlyVMIV could be to confer reversible binding to a carbohydrate, possibly alginate, and would probably exhibit a β-fold.

**Galactose-binding like domain and coagulation factor 5/8-type C-terminal domain:**

Proteins containing a galactose-binding domain-like fold can be found in several different prokaryotic and eukaryotic protein families. The common function of this domain is to confer binding to specific ligands such as cell-surface attached carbohydrate substrates in the case of galactose oxidase (Ito *et al*. 1991) or phospholipids on the outer surface of coagulation factor Va (Macedo-Ribeiro *et al*. 1999). The structure consists of a β-sandwich, in which the strands making up the sheets exhibit a jelly roll fold.

The coagulation factor 5/8-type C-terminal or FA58C domain is related to the galactose-binding like domain. The FA58C domain occurs twice in blood coagulation factors V and VIII and forms part of a larger functional domain which promotes binding to anionic phospholipids on the surface of platelets and endothelial cells (Kane and Davie, 1988). A related domain exists in discoidin, a cell adhesion protein from the slime mold *Dictyostelium discoideum*, which binds galactose (Poole *et al*. 1981). The common functional theme of proteins harboring this domain is binding to cell surface-attached carbohydrate residues (Baumgartner *et al.* 1998).

AlyVMIV is predicted to contain a signal peptide suggesting it is exported from the cell and was predicted to consist predominantly of β-strands (Figure 2.7). This prediction is in agreement with the β-fold of the galactose-binding domain. PSORTb also predicted the cellular location of AlyVMIV to be extracellular. The role of the galactose binding-like and FA58C domain could be to anchor AlyVMIV to the cell via binding to a cell-surface attached carbohydrate. This would

prevent the enzyme diffusing away from the cell and would allow products of hydrolysis to remain in close proximity to the cell.

**AlyVMI and AlyVMII Transmembrane domains:**

AlyVMI and AlyVMII were predicted to include two and three transmembrane domains, respectively. Membrane bound alginate lyases have not been described, although a transmembrane search conducted on the alginate lyase sequences included in Tables 2.3 and 2.4 using MINNOU revealed putative transmembrane regions in the N-terminal domain of all these proteins. Whether AlyVMI and AlyVMII are indeed membrane bound would need to be verified.

**Alginate lyase consensus sequences:**

The carboxy-terminal alginate lyase consensus motif YFKAGXYXQ, where X is a variable residue, was first reported by Baron *et al*. (1994). Alginate lyases belonging to polysaccharide lyase family 5 (PL-5) have the conserved regions INNHSYW in the middle of the amino acid sequence and WLEPXCXLY at the carboxy-terminus (Wong *et al*. 2000). The motifs RXELR, QIH and YFKAGXYXQ are highly conserved in PL-7 lyases; however some PL-7 lyases contain YXRSELREM in place of RXELR. Examples of PL-7 alginate lyases containing RXELR include AlyVOA and AlyVOB from *Vibrio* sp. O2 (Kawamoto *et al*. 2006) and AlxM from *Photobacterium* sp. ATCC 433367 (Malissard *et al*. 1993). Examples of PL-7 alginate lyases containing YXRSELREM include AlyA from *Klebsiella pneumonia* (Baron *et al*. 1994) and A1-I, A1-II and A1-II' from *Sphingomonas* sp. A1 (Yoon *et al*. 2000; Miyake *et al*. 2004). AlyVI from *Vibrio* sp. QY101 (Han *et al*. 2004) and AlyVG1 and AlyVG2 from *V. halioticoli* (Sugimura *et al*. 2000) contain slightly altered YXRSELREM motifs.

AlyVMIII and AlyVMIV from *Vibrio midae* SY9 contain the alginate lyase consensus motifs RXELR, QIH and YFKAGXYXQ (Figure 2.6 and 2.7). AlyVMIII and AlyVMIV do not contain YXRSELREM but do contain the slightly altered motifs YIRSELREL and YARTELRTM, respectively. AlyVOA and AlyVOB from *Vibrio* sp. O2 and AlxM from *Photobacterium* sp. ATCC 433367 belong to PL-7 and contain KIDLG as well as RXELR and YFKAGXYXQ but EITFLQVH in place of QIH (Kawamoto *et al*. 2006). Although, AlyVMIII and AlyVMIV from *Vibrio midae* SY9 do not EITFLQVH or KIDLG, AlyVMIII contains EVTFLQIH, a slight

variation of EITFLQVH. AlyVMI and AlyVMII from *V. midae* SY9 do not contain any of these alginate lyase consensus motifs.

Yamasaki *et al*. (2005) and Osawa *et al*. (2005) found YXRSELREM, QIH and YFKAGXYXQ to be present in the active site of PL-7 alginate lyases. Yamasaki *et al*. (2005) found that the active cleft of family PL-7 lyases is extremely well ordered and that the arginine, glutamate, tyrosine, lysine, glycine, glutamine and histidine residues of YXRSELREM, QIH and YFKAGXYXQ were important in preserving the structure of the active cleft. Yamasaki *et al*. (2005) and Osawa *et al*. (2005) implicated these conserved sequences in the catalytic mechanism of PL-7 lyases, specifically the glutamine and histidine residues of QIH as well as the first arginine of YXRSELREM and the second tyrosine of YFKAGXYXQ. Given this, it is likely that the sequences RXELR, QIH and YFKAGXYXQ play a role in the active site and possibly the catalytic mechanism of AlyVMIII and AlyVMIV. No putative active site or catalytic residues were identified in AlyVMI and AlyVMII. The structure of these enzymes and putative catalytic residues would have to be confirmed experimentally.

Polysaccharide lyases are grouped into families based on their amino acid sequence and structural similarities (Cantarel *et al*. 2009). There are 21 families in total. Alginate lyases belong to polysaccharide lyase families PL-5, -6, -7, -14, -15, -17 and -18. (http://www.cazy.org). Family PL-7 enzymes are characterised by a β-jelly roll fold and include alginate lyases and $\alpha$-$_L$-guluronate lyases. AlyVMIII and AlyVMIV can be placed into family PL-7 based on their predicted secondary structures of predominantly β-strands as well as the presence of the conserved sequences RXELR, QIH and YFKAGXYXQ. Proteins which belong to PL-17 are alginate lyases which contain chondroitin AC/alginate lyase and heparinase II/III-like protein domains. The predicted structure of these proteins is α-helices in the amino-terminus, β-strands in the carboxy-terminus and between one and three transmembrane domains present in the amino-terminus. AlyVMI and AlyVMII have similar primary and secondary structures and can thus be placed in family PL-17.

**Protein domains of the putative oligogalacturonate-specific porin, pectin degrading protein and gluconokinase of *Vibrio midae* SY9:**

The putative oligogalacturonate-specific porin contains an oligogalacturonate-specific porin or KdgM domain. This family contains proteins which are homologous to KdgM, the oligogalacturonate specific porin from *Erwinia chrysanthemi*. This major outer membrane protein belongs to the OmpG superfamily, is monomeric and strongly induced in the presence of pectic derivatives (Blot *et al.* 2002). OmpG is also monomeric with a 14-stranded β-barrel structure (Yildiz *et al.* 2006). The putative porin has the features of an outer membrane protein (Figure 2.8): a 22 amino acid signal peptide, no long stretches of hydrophobic residues, no cysteine residues (Fajardo *et al.* 1998) and the translated amino acid sequence ends in a phenylalanine residue which is important for membrane insertion (Struyve *et al.* 1991). The putative porin is predicted to predominantly consist of β-helices (Figure 2.8). This porin would need to be validated in *Vibrio midae* SY9 to determine whether it does indeed transport galacturonate or other carbohydrates across the membrane into the cell and whether it plays a role in carbohydrate assimilation. The inability of a KdgM deletion mutant of *E. chrysanthemi* to grow on oligogalacturonides longer than trimers (Blot *et al.* 2002) suggests that the function of this porin would be significant.

The putative pectin degrading protein of *Vibrio midae* SY9 contains a RmlC-like domain, a Cupin 2 conserved barrel and a RmlC-like jelly roll fold. Pectin is a polymer of 1-4 linked galacturonate residues in which some of the residues have been methylated (Suberkropp, 2005). There are three classes of enzyme which degrade pectin: hydrolytic enzymes such as polygalacturonases; pectin lyases which cleave glycosidic bonds by β-elimination; and esterases which cleave the methyl group from galacturonic acid residues. The products of pectin degradation are mainly dimers to tetramers of galacturonides with some longer oligomers (Blot *et al.* 2002). RmlC is a dTDP-sugar isomerase involved in the synthesis of $_{L}$-rhamnose and is a dimer with each monomer consisting of two β-sheets arranged in a β-sandwich with the substrate binding site located between the two sheets of each monomer (Giraud *et al.* 2000). This structure is called a β-jelly roll fold. Other protein families that contain β-barrel domains include porins and pore-forming proteins, adhesions, lipases and proteases (Wimley, 2003). Cupins are characterised by a conserved β-barrel fold (Dunwell, 1998). The Latin word 'cupa' means small

barrel or cask. Cupins are a superfamily of prokaryotic and eukaryotic proteins which includes enzymes, co-factors and plant storage proteins that bind sugars and other compounds (Dunwell and Gane, 1998). The cupins are found in a wide range of cell types and have a wide range of biochemical functions. These include reactions which involve sugar binding and/or modification (Dunwell *et al*. 2000). The domains identified in the putative pectin degrading protein of *Vibrio midae* SY9 indicate that it would most likely be able to bind sugars and that it has a β-barrel structure. The pectin degrading protein is predicted to consist almost entirely of β-strands (Figure 2.9) which is in agreement with the secondary structure of the predicted domains. The ability of this protein to degrade pectin would have to be verified.

The deoxygluconokinase of *Vibrio midae* SY9 contains a carbohydrate purine/kinase or PfkB domain as well as a ribokinase-like domain. When $_{D}$-ribose enters the *E. coli* cell, it needs to be phosphorylated at O5' before it can be used in anabolism or catabolism (Sigrell *et al*. 1998). This reaction is carried out by ribokinase. The product, $_{D}$-ribose-5-phosphate, is then available for the synthesis of nucleotides, the amino acids tryptophan and histidine, or for entry into the pentose phosphate pathway (Sigrell *et al.* 1998). Metabolic enzymes are not usually found together with their transport components (Anderson and Cooper, 1969). However, the *E. coli* ribokinase gene is located on the same operon as the periplasmic ribose-binding protein and the membrane-bound permease system (Lida *et al.* 1984; Lopilato *et al.* 1984). This situation may reflect the essential role of the ribokinase in ribose metabolism in *E. coli* (Anderson and Cooper, 1969). The ribokinase family phosphorylates the hydroxymethyl groups of a variety of sugar moieties. The PfkB family includes 6-phosphofructokinase (PfkB), ribokinases and 2-dehydro-3-deoxygluconokinases (Sigrell *et al.* 1998). The phosphorylation of sugars is important in their role in energy production and biosynthesis. The addition of the charged phosphate group also prevents movement of uncharged sugars across the membrane. The presence of the kinase domains suggests that the putative deoxygluconokinase of *Vibrio midae* SY9 would be involved in the phosphorylation of carbohydrates in the cell. The substrates recognized by the *V. midae* SY9 deoxygluconokinase would need to be identified in order to determine whether this enzyme plays a role in carbohydrate metabolism. First, the full length sequence would need to be elucidated.

**Conclusions and future work:**

Except for AlyVMI and AlyVMII, the four alginate lyases of *Vibrio midae* SY9 do not share a high level of identity with each other (Table 2.7). The similarities and differences between the four enzymes possibly indicate a different role for each enzyme in the degradation of alginate by *Vibrio midae* SY9.

We hypothesise the following potential mechanism of alginate degradation by *V. midae* SY9: AlyVMIV is exported from the cell and degrades the alginate polymer to oligo-alginates which can enter the cell. The oligo-galacturonate specific porin may play a role in the uptake of oligo-alginates. AlyVMI, AlyVMII and AlyVMIII are intracellular enzymes and would degrade the alginate molecule to mono- or oligosaccharides which could enter cellular metabolism. However, the substrate specificity and sequence of involvement of these enzymes is unknown. The deoxygluconokinase could phosphorylate the mono- or oligosaccharides at this point allowing them to enter the general metabolism of the cell.



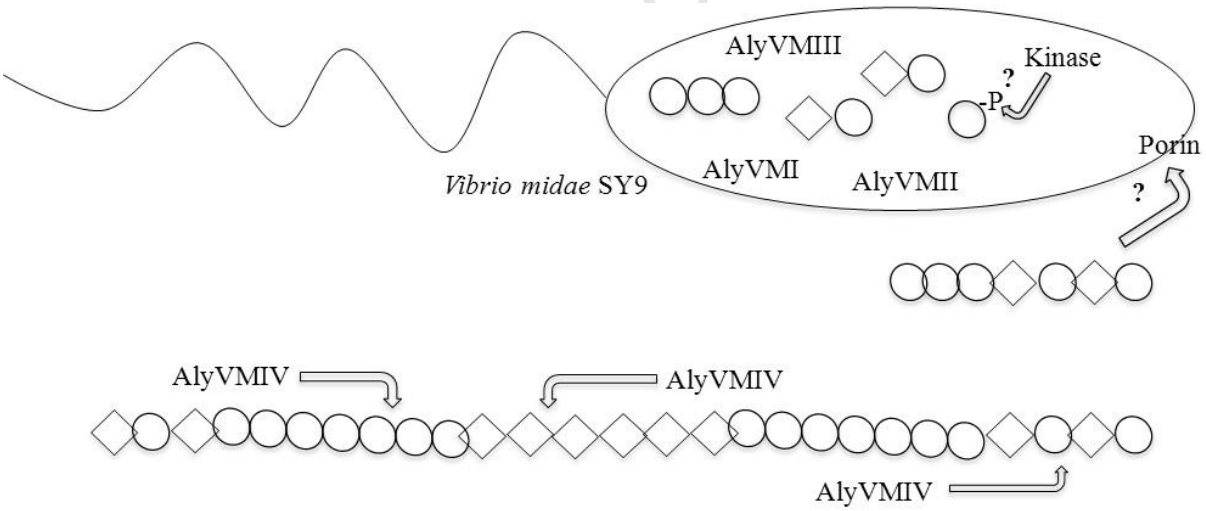



**Figure 2.12.** Schematic digram of the proposed mechanism of alginate degradation by *Vibrio midae* SY9. Circles and diamonds represent $\beta$-$_D$-mannuronate and $\alpha$-$_L$-guluronate residues, respectively, and arrows represent reactions. Kinase represents the putative *V. midae* deoxygluconokinase, porin represents the putative *V. midae* oligogalacturonate-specific porin and P represents a phosphate group. The substrate specificity and sequence of involvement of AlyVMI, AlyVMII and AlyVMIII is unknown.

The cellular location, substrate specificity, the minimum size of oligo-alginates required as a substrate for activity and the alginate degradation products produced by each enzyme would assist in elucidating the role of each enzyme in the degradation of alginate by *V. midae* SY9. Purified active enzyme would be required to determine the substrate specificity, the minimum size of oligo-alginates required as a substrate for activity and alginate degradation products produced. Antibodies specific for each enzyme would be required to perform immunolocalisation studies in order to determine cellular locations. The location of the enzymes relative to each other would also be interesting to determine. The enzyme kinetics of each alginate lyase could also be investigated as well as the optimal temperature and pH required for activity.

Alginate lyase genes *alyVMI* and *alyVMII* were shown to be co-transcribed. This could indicate a regulatory mechanism for expression of these genes by *Vibrio midae* SY9. It is possible that the actions of AlyVMI and AlyVMII are required together for the depolymerisation of alginate and the two genes are therefore co-expressed. It is also possible that other genes identified in this study are co-transcribed, such as *alyVMIV* and the putative oligogalacturonate specific porin which are separated by 79 bp, and the putative pectin degrading protein and the putative deoxygluconokinase which are separated by 37 bp. It would be interesting to determine which genes on this region of the chromosome are co-transcribed and how they are regulated. It seems that carbohydrate utilisation genes have been clustered together in one region of the *V. midae* SY9 chromosome. It would be interesting to know which genes are present on either side of the sequenced fragment of chromosomal DNA, whether they are co-transcribed and whether they are regulated in conjunction with the genes identified in this study.

In conclusion, screening *Vibrio midae* SY9 genomic libraries resulted in the successful cloning and sequencing of four alginate lyase genes, a putative oligogalacturonate-specific porin, a putative pectin degrading protein and the partial sequence of a putative deoxygluconokinase adjacent to each other on the chromosome. A total of 11.842 kb of *Vibrio midae* SY9 genomic DNA was sequenced in this study. This is the first study to report four alginate lyase genes adjacent to one another on a bacterial chromosome and adjacent to other proteins involved in carbohydrate utilisation.

# Chapter 3

# Purification of AlyVMI and AlyVMII and Production of Antibodies to These Alginate Lyases from *Vibrio midae* SY9

## CONTENTS

## 3.1. SUMMARY

Four alginate lyase genes from *Vibrio midae* SY9, namely *alyVMI*, *alyVMII*, *alyVMIII* and *alyVMIV*, were cloned into the expression vector pET-29a. *Escherichia coli* BL21 (DE3) pLysS was used as a host for expression of the alginate lyases. Western hybridisation analysis using rabbit anti 6x histidine antibody revealed an ~81 kDa protein in *E. coli* cell lysates containing either AlyVMI or AlyVMII, confirming that *alyVMI* and *alyVMII* were expressed in *E. coli* BL21 (DE3) pLysS and that the recombinant proteins were histidine tagged. Alginate lyase gene *alyVMIV* was also expressed in *E. coli* BL21 (DE3) pLysS and a histidine tagged protein of ~60 kDa was detected in the western hybridisation experiment. Expression of AlyVMIII in *E. coli* BL21 (DE3) pLysS was unsuccessful. Large scale purification of recombinant AlyVMI, AlyVMII and AlyVMIV was performed using nickel affinity chromatography. AlyVMIV could not be purified to homogeneity. Polyclonal antibodies were raised against purified recombinant AlyVMI and AlyVMII in rabbits. Specificity of the antibodies was determined by western hybridisation analysis and ensured by pre-absorption with *E. coli* cell lysates. The antibodies recognized both the recombinant and native form of the enzymes.

## 3.2. INTRODUCTION

Polysaccharide lyases are carbon-oxygen lyases that cleave polysaccharides containing (1-4)-linked uronic acids by a β-elimination reaction (Anderson, 1998). Alginate lyases utilise a β-elimination reaction to depolymerise the alginate polymer resulting in cleavage of the (1-4)-*O*-glycosidic linkage between monomers and the formation of an unsaturated uronic acid, 4-deoxy-$_L$-*erythro*-hex-4-ene pyranosyluronate, at the new non-reducing terminus (Gacesa 1988, Gacesa 1992; Wong *et al.* 2000). A three step mechanism for the enzymatic depolymerization of alginate by alginate lyases has been proposed (Gacesa 1987, 1992). The mechanism involves neutralisation of the negatively charged substrate, general base-catalysis and the formation of a double bond (Chapter 1, Section 1.2.2). Alginate lyases can be classified or grouped based on their substrate specificity, molecular mass or amino acid sequence and structural similarity (Chapter 1, Section 1.2.4).

Various of methods for purifying proteins have been developed and many use a combination of techniques. Wild type alginate lyases have been purified from a crude extract of the native organism using classical purification techniques (Kobayashi *et al*. 2009; Shimizu *et al*. 2003; Suzuki *et al*. 2006). Recombinant enzymes have been purified by classical techniques (Hashimoto *et al*. 2000b; Yoon *et al*. 2000) or by affinity chromatography if the protein was fused with an affinity tag (Suzuki *et al*. 2006; Hashimoto *et al*. 2005; Miyake *et al*. 2004). A cold-inducible yeast expression system has also been developed to express and subsequently purify active recombinant alginate lyase (Yamamoto *et al*. 2008) when insoluble inclusion bodies were formed in *E. coli* (Shimizu *et al*. 2003). Thus, there is no singular method for purifying alginate lyases.

Although many alginate lyases have been purified, antibodies have been raised against only three purified proteins: A1-II' and A1-IV from *Sphingomonas* sp. A1 (Hashimoto *et al*. 2005; Miyake *et al*. 2004) and HdAly from *Haliotis discus hannai* (Hata *et al*. 2009). The antibodies raised against A1-II' and A1-IV were used in western hybridisation analysis to determine whether A1-II, A1-II' or both and A1-IV, A1-IV' or both, respectively, were expressed in *Sphingomonas* sp A1 cells grown in alginate. The anti-HdAly antibodies were used in western hybridisation

analysis to determine structural similarities between 5 other molluscan alginate lyases. We are not aware of other reports of antibodies raised against any other purified alginate lyase.

A range of commercial systems are available for the expression of recombinant proteins. One system is the pET expression system from Novagen which uses the powerful bacteriophage T7 promoter and RNA polymerase to express recombinant genes. The pET system employs various affinity tags that can be fused to the N- or C-terminus of recombinant proteins and thus facilitate purification of these proteins by affinity chromatography. Expression and solubility of the target protein in *E. coli* can be optimized with this system.

This chapter describes cloning the four alginate lyase genes of *Vibrio midae* SY9 into the expression vector pET-29a and subsequent purification of histidine tagged AlyVMI and AlyVMII using nickel affinity chromatography. Polyclonal antibodies were raised against purified AlyVMI and AlyVMII in rabbits. Western hybridisation analysis was used to determine the specificity of the raised antibodies. Activity of the recombinant enzymes in *E. coli* cell extracts was determined using the TBA assay.

## 3.3. MATERIALS AND METHODS

All media and solutions used in this study are listed in Appendix A.

### 3.3.1. Bacterial strains and plasmids used

The bacterial strains and plasmids used in this study are listed in Table 3.1.

### 3.3.2. Culture conditions and media used

*Escherichia coli* DH5α was cultured in either Luria broth (LB) or on Luria Agar (LA) at 37°C. *E. coli* BL21 (DE3) pLysS was cultured either in LB or on LA containing 20 µg/ml chloramphenicol at 37°C. *E. coli* DH5α transformants harboring recombinant pBluescript SK (+)$^{TM}$ plasmids were cultured in LB or on LA containing 100 µg/ml ampicillin at 37°C. *E. coli* DH5α transformants harboring pET-29a or recombinant pET-29a plasmids were cultured in LB or on LA containing 30 µg/ml kanamycin at 37°C. *E. coli* BL21 (DE3) pLysS transformants harboring pET-29a or recombinant pET-29a plasmids were cultured in LB or on LA containing 20 µg/ml chloramphenicol and 30 µg/ml kanamycin at 37°C. *Vibrio midae* SY9 was cultured either in marine broth (MB) or on marine agar (MA) at room temperature.

### 3.3.3. Cloning *alyVMI*, *alyVMII*, *alyVMIII* and *alyVMIV* into pET-29a

In order to purify AlyVMI, AlyVMII, AlyVMIII and AlyVMIV, a histidine tag was fused to each protein using the pET system (Novagen). Each gene was amplified with a high fidelity DNA polymerase and cloned into pBluescript SK (+)$^{TM}$. Recombinant plasmids were screened for insert size and presence of the correct restriction enzyme sites. Each gene was then sub-cloned into pET-29a.

**Table 3.1.** Bacterial strains and plasmids

| Strain/plasmid | Genotype/relevant characteristic(s)[a] | Reference |
|---|---|---|
| **Strains** | | |
| *E. coli* DH5α | F$^-$ *end*A1 *gln*V44 *thi*-1 *rec*A1 *rel*A1 *gyr*A96 *deo*R *nup*G Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *hsd*R17($r_K^-$ $m_K^+$) $\lambda^-$ | Grant *et al.* (1990) |
| *E. coli* BL21 (DE3) pLysS | F$^-$ *omp*T *gal dcm lon hsd*SB(rB- mB-) λ(DE3 [*lac*I *lac*UV5-T7 gene 1 *ind*1 *sam*7 *nin*5]) pLysS(cm$^r$) | Moffatt and Studier (1987) |
| *Vibrio midae* SY9 | Putative probiont isolated from *Haliotis midae,* South Africa | Macey and Coyne (2005) |
| **Plasmids** | | |
| pBluescript SK (+)$^{TM}$ | Amp$^r$, β-galactosidase | Short *et al.* (1988) |
| pET-29a | Kan$^r$, T7*lac*, C-terminal His-tag, N-terminal S-tag, Thrombin cleavage site | Rosenberg et al. (1987) |
| pAlg15 | pBluescript SK (+)$^{TM}$ containing ~5.7 kb of *V. midae* SY9 genomic DNA | Chapter 2 |
| pAlg86 | pBluescript containing (+)$^{TM}$ ~4.8 kb of *V. midae* SY9 genomic DNA | Chapter 2 |
| pAlyVMI | pBluescript SK (+)$^{TM}$ carrying the 2163 bp *alyVMI* gene | This study |
| pAlyVMII | pBluescript SK (+)$^{TM}$ carrying the 2163 bp *alyVMII* gene | This study |
| pAlyVMIII | pBluescript SK (+)$^{TM}$ carrying the 1449 bp *alyVMIII* gene | This study |
| pAlyVMIV | pBluescript SK (+)$^{TM}$ carrying the 1563 bp *alyVMIV* gene | This study |
| pETalyVMI | pET-29a carrying the 2163 bp *alyVMI* gene | This study |
| pETalyVMII | pET-29a carrying the 2163 bp *alyVMII* gene | This study |
| pETalyVMIII | pET-29a carrying the 1449 bp *alyVMIII* gene | This study |
| pETalyVMIV | pET-29a carrying the 1563 bp *alyVMIV* gene | This study |

[a] Cm$^r$, chloramphenicol resistant; amp$^r$, ampicillin resistant; kan$^r$, kanamycin resistant.

#### 3.3.3.1. Amplification of *alyVMI*, *alyVMII*, a*lyVMIII*, *alyVMIV* and cloning into pBluescript SK (+)™

The oligonucleotide primers designed to amplify the alginate lyases included the start codon of each gene, removed the stop codon to ensure read-through into the C-terminal histidine tag of pET-29a, incorporated restriction enzyme sites for cloning into pET-29a and allowed the recombinant gene to be inserted in-frame with the T7 promoter and C-terminal histidine tag. The restriction enzymes chosen must be present in the multiple cloning site of pET-29a but not in the gene of interest. *Nde*I and *Xho*I were used for *alyVMI*, a*lyVMII* and *alyVMIV* whereas *Xba*I and *Xho*I were used for *alyVMIII*. A restriction map of pET-29a is shown in Figure 3.1.

Genomic DNA was isolated from *Vibrio midae* SY9 (Appendix B.8) and pAlg15 and pAlg86 were isolated using the BioSpin Plasmid DNA extraction Kit (Bioer Technology) according to the manufacturer's instructions. Alginate lyase genes *alyVMI* and *alyVMIII* were amplified from *V. midae* SY9 genomic DNA using the primer pairs EVF2 and EVR2 or EVF3 and EVR3, respectively (Table 3.2). Alginate lyase gene *alyVMII* was amplified from pAlg15 using primers EVF1 and EVR1 and *alyVMIV* was amplified from pAlg86 using primers EVF4 and EVR4 (Table 3.2). The high fidelity DNA polymerase, *Pfu* (Fermentas), was used for the PCR reactions. PCR conditions are described in Appendix C.7.

An aliquot of the PCR products were separated on a 1% TAE agarose gel (Appendix B.5) to verify product size and reaction specificity. The remainder of the PCR products were separated on a 0.8% TA low-melting point agarose gel in 1x TA buffer (Appendix B.5). Amplified fragments were excised from the gel and gel slices stored at 4°C until used in ligation reactions.

Large scale preparation of pBluescript SK (+)™ was performed using the Midi Plasmid Isolation Kit (Qiagen) according to the manufacturer's instructions. Approximately 5 µg of the resulting plasmid DNA was linearized with *Eco*RV (Appendix B.4). Linear plasmids were treated with calf intestine alkaline phosphatase (CIP, Fermentas) according to the manufacturer's instructions. The reaction was stopped by inactivating the enzyme by heating at 85°C for 15 min.

**Table 3.2.** Primer pairs and templates used to amplify the four alginate lyase genes of *Vibrio midae* SY9 for cloning into pBluescript SK (+)$^{TM}$ and subsequently pET-29a

| Gene | Primer | Primer sequence (5'-3')[a] | Ta[b] (°C) | DNA template |
|---|---|---|---|---|
| *alyVMI* | EVF2 | GTGGTAAACATATGACTACACAACCGAT | 54 | *V. midae* SY9 genomic DNA |
| | EVR2 | CTCATTACTCGAGCTCCTGCCCGAATG | | |
| *alyVMII* | EVF1 | CATATGAGCTACCAAACCCAGTCTTACC [c] | 61 | pAlg15 |
| | EVR1 | CTCGAGAATTTGTGCGAATGCGCCTTCC [c] | | |
| *alyVMIII* | EVF3 | CCTTGTCTCTAGAATGTCGAACTAC | 55 | *V. midae* SY9 genomic DNA |
| | EVR3 | GACTGAAATGCTCGAGGATGAAGAGTG | | |
| *alyVMIV* | EVF4 | GGTGTTAGATCATATGAAGCATATTTTCTTC | 60 | pAlg86 |
| | EVR4 | GTGATACTCGAGGCCTTGGTACTTACC | | |

[a] Underlined sequences represent the restriction enzyme sites included in the primers. *Nde*I, CATATG; *Xho*I, CTCGAG; *Xba*I, TCTAGA; [b] Ta, annealing temperature; [c] EVF1 and EVR1 were HPLC purified to retain only full length primers. The other primers were extended on the 5' end of the restriction site.

Amplification products were ligated (Appendix B.7) with pBluescript SK (+)$^{TM}$ plasmids that had been linearized with *Eco*RV and treated with CIP. Ligation reactions were conducted in low-melting point agarose to prevent the loss of insert DNA. Ligation mixes were transformed into competent *E. coli* DH5α cells (Appendix B.1) as described in Appendix B.2. Transformed cells were spread-plated onto LA containing 0.5 mM IPTG, 0.004% X-gal and 100 µg/ml ampicillin and incubated overnight at 37°C.

Eighteen transformants from each transformation were transferred onto fresh LA containing 100 µg/ml ampicillin using a sterile toothpick and incubated at 37°C overnight. Plasmid DNA was isolated from each transformant according to the small scale isolation method described in Appendix B.3. The resulting plasmids were digested with the restriction endonuclease *Pvu*II (Appendix B.4) to determine the size and orientation of inserts. Recombinant plasmids carrying *alyVMI*, *alyVMII* and *alyVMIV* were also digested with *Nde*I and *Xho*I, or with *Xba*I and *Xho*I if carrying *alyVMIII*, to ascertain whether the restriction sites included in the PCR primers were present. Digestion products were separated on an 0.8% agarose TAE gel (Appendix B.5).

### 3.3.3.2. Sub-cloning *alyVMI*, *alyVMII*, a*lyVMIII* and *alyVMIV* from recombinant pBluescript SK (+)$^{TM}$ into pET-29a

Recombinant plasmids that included 5'-3' oriented insert DNA with the expected restriction endonuclease sites that had been incorporated by PCR were chosen for further analysis. The recombinant pBluescript SK (+)$^{TM}$ plasmids carrying *alyVMI*, *alyVMII*, *alyVMIII* and *alyVMIV* were designated pAlyVMI, pAlyVMII, pAlyVMIII and pAlyVMIV, respectively.

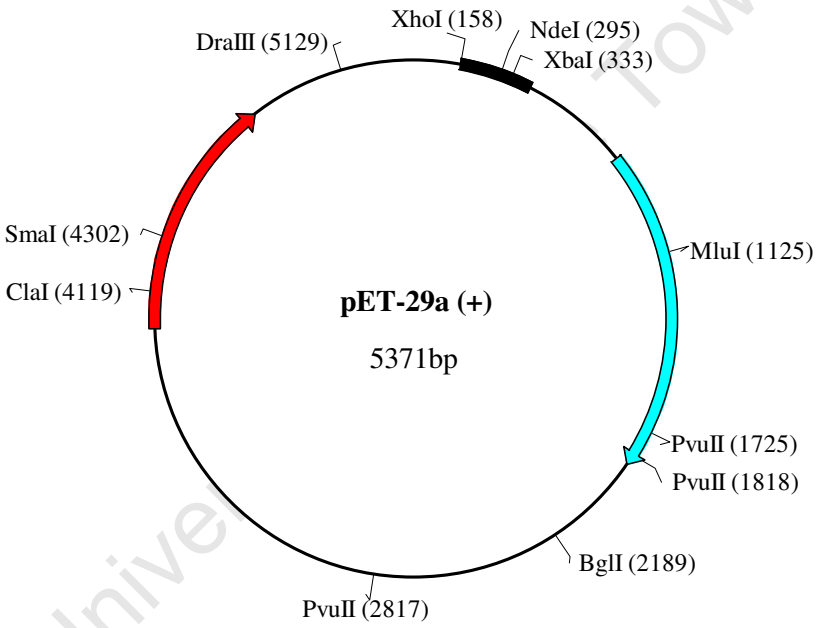



**Figure 3.1.** Restriction enzyme map of pET-29a (+). The size of the plasmid is shown in base pairs (bp) and the positions of the restriction sites are shown in bp in brackets. The position of the kanamycin resistance gene and the *lacI* gene is indicated by the red and blue arrows, respectively. The bold black bar indicates the cloning and expression region of the plasmid and includes the T7 promoter, transcription start site and terminator, multiple cloning region, and the His and S tag coding sequences.

Plasmids pAlyVMI, pAlyVMII, pAlyVMIII and pAlyVMIV were isolated according to the small scale isolation method described in Appendix B.3. Plasmids pAlyVMI, pAlyVMII and pAlyVMIV were digested with *Nde*I and *Xho*I and pAlyVMIII was digested with *Xba*I and *Xho*I as described in Appendix B.4. Large scale preparation of pET-29a was performed using the Midi Plasmid Isolation Kit (Qiagen) according to the manufacturer's instructions. Approximately 2 µg of the resulting pET-29a DNA was digested with *Nde*I and *Xho*I or *Xba*I and *Xho*I (Appendix B.4). Digestion products were separated on a 0.8% TA low-melting point agarose gel in 1x TA buffer (Appendix B.5). Digested insert DNA and plasmids were excised from the gel.

DNA fragments excised from pAlyVMI, pAlyVMII or pAlyVMIV were ligated (Appendix B.7) into pET-29a that had been digested with *Nde*I and *Xho*I, while the insert obtained from pAlyVMIII was ligated (Appendix B.7) into pET-29a digested with *Xba*I and *Xho*I. Ligation mixes were transformed into competent *E. coli* DH5α cells (Appendix B.1) as described in Appendix B.2. Transformants were spread-plated onto LA containing 30 µg/ml kanamycin and incubated at 37°C overnight.

Thirty six transformants from each transformation were transferred onto fresh LA containing 30 µg/ml kanamycin using a sterile toothpick and incubated at 37°C overnight. Plasmid DNA was isolated from each transformant according to the small scale isolation method described in Appendix B.3. The resulting plasmids were digested with *Pvu*II (Appendix B.4) to determine insert size. Isolated plasmids were also digested with *Nde*I and *Xho*I for *alyVMI*, *alyVMII* and *alyVMIV* recombinant plasmids or with *Xba*I and *Xho*I for *alyVMIII* recombinant plasmids to ascertain whether the restriction sites used for sub-cloning from recombinant pBluescript SK (+)$^{TM}$ plasmids were present in the recombinant pET-29a plasmids. Digestion products were separated on a 0.8% agarose TAE gel (Appendix B.5).

#### 3.3.3.3. Sequencing of recombinant pET-29a plasmids

Inserts of the recombinant pET-29a plasmids carrying *alyVMI*, *alyVMII*, *alyVMIII* or *alyVMIV* were sequenced using the T7 promoter and terminator primers in order to determine whether the nucleotide sequences were correct, that the restriction sites used for cloning were conserved, and

consequently, that the recombinant genes were in-frame with the T7 promoter and C-terminal histidine tag. The sequence of the T7 promoter primer is 5' GTAATACGACTCACTATAGGGC 3' and the sequence of the T7 terminator primer is 5' GCTAGTTATTGCTCAGCGG 3' (Dunn *et al*. 1983).

Recombinant pET-29a plasmids carrying *alyVMI*, *alyVMII*, *alyVMIII* or *alyVMIV* were isolated using the QIAprep Spin Mini Prep Kit (Qiagen) according to the manufacturer's instructions. The inserts of the resulting plasmids were sequenced using the Big Dye Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems) as per the manufacturer's instructions and the sequences obtained with a 3130 Genetic Analyser (Applied Biosystems, DNA Automated Capillary Sequencer). Nucleotide sequences were edited and assembled using Chromas Version 2.01 (Technelysium) and DNAMAN Version 4.13 (Lynnon Biosoft). The sequences were compared to the original *alyVMI*, *alyVMII*, *alyVMIII* and *alyVMIV* sequences reported in Chapter 2.

### 3.3.4. Expression of *alyVMI*, *alyVMII*, *alyVMIII* and *alyVMIV* in *E. coli*

Recombinant pET-29a plasmids carrying *alyVMI*, *alyVMII*, *alyVMIII* and *alyVMIV* were designated pETalyVMI, pETalyVMII, pETalyVMIII and pETalyVMIV, respectively. *E. coli* DH5α was used as a cloning host, while *E. coli* BL21 (DE3) pLysS was used as a host for expression of the alginate lyase genes. Competent *E. coli* BL21 (DE3) pLysS cells were prepared as described in Appendix B.1. Plasmids pETalyVMI, pETalyVMII, pETalyVMIII, pETalyVMIV and pET-29a were isolated according to the small scale isolation method described in Appendix B.3. The resulting plasmids were transformed into competent *E. coli* BL21 (DE3) pLysS cells (Appendix B.2).

*E. coli* BL21 (DE3) pLysS transformed with pET-29a, pETalyVMI, pETalyVMII, pETalyVMIII or pETalyVMIV were inoculated into 5 ml LB containing 30 µg/ml kanamycin and 20 µg/ml chloramphenicol and incubated at 37°C with shaking for 8 h. The starter cultures were used to inoculate 100 ml pre-warmed LB containing 30 µg/ml kanamycin and 20 µg/ml chloramphenicol to a starting absorbance of 0.02 at 600 nm. The cultures were incubated overnight at 37°C with shaking. These 100ml overnight cultures were used to inoculate fresh 100 ml pre-warmed LB

containing 30 µg/ml kanamycin and 20 µg/ml chloramphenicol to a starting absorbance of 0.02 at 600 nm. The cultures were incubated at 37°C with shaking and the absorbance of the culture at 600 nm monitored. Once the culture had reached an absorbance of between 0.5 and 0.6 at 600 nm, a 1 ml sample was taken (T0) and then sterile IPTG added to a final concentration of 1 mM. A 10 ml sample was taken 4 h after the addition of IPTG (T4). Samples were centrifuged at 14000 rpm for 2 min, the supernatant was discarded and the cells stored at -20°C.

### 3.3.5. Western hybridisation analysis of alginate lyase gene expression from recombinant pET-29a

Expression of *alyVMI*, *alyVMII*, *alyVMIII* and *alyVMIV* from recombinant pET-29a plasmids was analysed by western hybridisation using anti-histidine antibodies.

*E. coli* BL21 (DE3) pLysS transformed with pET-29a, pETalyVMI, pETalyVMII, pETalyVMIII or pETalyVMIV were cultured and induced with IPTG as described in Section 3.3.4. *E. coli* lysates containing soluble cellular proteins were prepared by sonication as follows (The QIAexpressionist Handbook, Qiagen, USA). Frozen cell samples were thawed by re-suspension in 1 ml lysis buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, 10 mM imidazole) containing 1 mg/ml lysozyme and incubated on ice for 30 min. The cells were sonicated (Misonix sonicator 3000 fitted with a microtip) with sixteen 15 sec bursts at power setting 1.5 (3W) and a 15 sec cooling period between bursts. Samples were centrifuged at 10 000 rpm for 30 min at room temperature and the supernatant fraction retained. The protein concentration of each lysate was determined by the Bradford method (Appendix B.16.1).

Twenty micrograms of total protein of the cell lysates prepared above were separated on an 8% denaturing SDS-PAGE gel (Appendix B.15). The proteins were transferred to a nitrocellulose membrane using a Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad) as described in Appendix B.17. To prevent non-specific binding of antibody, the membrane was incubated in blocking solution for 30 min with gentle agitation at room temperature. Rabbit anti-6x histidine antibodies (Gene Tex, Inc.) were diluted 1:5000 in blocking solution and incubated with the membrane overnight at 4°C with gentle agitation. The membrane was washed 4x in blocking

solution for 15 min per cycle. Peroxidase labeled goat anti-rabbit IgG (Kirkegaard and Perry Laboratories, KPL) was diluted 1:5000 in blocking solution and incubated with the membrane at 4°C for 2 h. The membrane was washed 4x in washing solution for 15 min per cycle. One milliliter of TMB membrane peroxidase substrate (KPL) was added to the membrane. Colour development was allowed to proceed for a few minutes. The reaction was stopped by rinsing the membrane in water.

### 3.3.6. Large scale protein purification

Preliminary experiments were performed to optimize the expression of recombinant pET-29a constructs in *E. coli* BL21 (DE3) pLysS by induction with IPTG as well as the purification of recombinant proteins using affinity chromatography prior to large scale purification. Large scale purification of AlyVMI, AlyVMII and AlyVMIV from *E. coli* BL21 (DE3) pLysS lysates was performed using His-Select® Nickel Affinity Gel (Sigma).

*E. coli* BL21 (DE3) pLysS clones transformed with pETalyVMI, pETalyVMII or pETalyVMIV were cultured and induced with IPTG as described in Section 3.3.4 except that 500 ml LB was used for the IPTG induced culture and the entire culture was harvested by centrifugation. Soluble cellular lysates were prepared as described in Section 3.3.5 except that cells were resuspended in 15 ml lysis buffer and sonicated at power setting 4 (9W). Samples were centrifuged twice at 10 000 rpm for 20 min at 4°C.

Nickel affinity gel columns were prepared by blocking the bottom of a 5 ml syringe with sterile glass wool. One milliliter of His-Select® Nickel Affinity Gel (Sigma) was applied to the column. The column was washed with water and equilibration buffer (10 mM imidazole). Cell lysates were loaded onto the column and allowed to stand for 5 min. Lysates were passed through the column 5x by gravity flow. Samples of cell lysates were collected before loading onto the column and after passing through the column. The column was washed with 15 ml wash buffer (20 mM imidazole) and fractions collected. Proteins were eluted in 2.5 ml elution buffer (250 mM imidazole) which was passed through the column 20x. Columns were washed with elution buffer followed by sterile water. Columns were stored under 30% ethanol and re-used

several times. Separate columns were prepared for each protein and used for that protein only. Cell lysates collected before and after passing through the column, wash fractions and purified protein were separated on an 8% denaturing SDS-PAGE gel to determine the purity of the purified proteins (Appendix B.15). A dilution series of purified protein was separated alongside known concentrations of BSA on an 8% denaturing SDS-PAGE gel (Appendix B.15) in order to quantitate the purified alginate lyases.

### 3.3.7. Alginate lyase activity of *E. coli* and *Vibrio midae* SY9 cell lysates

*E. coli* BL21 (DE3) pLysS clones transformed with pETalyVMI, pETalyVMII or pETalyVMIV were cultured and induced with IPTG as described in Section 3.3.4. *Vibrio midae* SY9 was inoculated into 5 ml alginate media and incubated at room temperature with shaking for 8 h. This starter culture was used to inoculate 100 ml alginate media to a starting absorbance of 0.02 at 600 nm which was incubated at room temperature with shaking overnight. Cells were harvested by centrifugation at 8000 rpm for 10 min at 4°C and culture supernatants were retained. *E. coli* and *V. midae* SY9 cell lysates were prepared as described in Section 3.3.5 except that the cells were resuspended in 10 ml lysis buffer lacking imidazole. Alginate lyase activity of the prepared cell lysates and culture supernatants was determined by the thiobarbituric acid (TBA) assay (Appendix B.6).

### 3.3.8. Antibody production against purified AlyVMI and AlyVMII

Polyclonal antibodies against purified AlyVMI and AlyVMII were raised by immunising New Zealand White rabbits with a total of 11 µg purified AlyVMI or 580 µg purified AlyVMII in the presence of Freund's incomplete adjuvant (Ausubel et al. 1989). Purified AlyVMI and AlyVMII were injected into separate rabbits which were housed at the Animal Unit of the Medical School at the University of Cape Town. Each rabbit was bled prior to administration of the antigen to obtain pre-immune serum (bleed 1). Three injections containing 2 µg AlyVMI or 100 µg AlyVMII were administered every 7 days over a period of 3 weeks. A fourth injection of 2 µg AlyVMI or 100 µg of AlyVMII was administered 2 weeks after the third injection. Beginning the week after the third injection, rabbits were bled every 14 days over an 8 week period to

obtain serum in order to monitor antibody production against AlyVMI and AlyVMII (bleeds 2-5). A booster immunisation of 3 µg AlyVMI or 180 µg AlyVMII was administered three weeks after bleed 5. The rabbits were bled immediately before (bleed 6) and two weeks after (bleed 7) the booster immunisation. The final bleed was taken 4 weeks after the booster immunisation (bleed 8).

### 3.3.9. Indirect ELISA to determine antibody titre in immune sera

Anti-serum from each bleed was tested for the presence of anti-AlyVMI and anti-AlyVMII antibodies using indirect ELISA (Ausubel *et al*. 1989 unit 11.4). A single experiment was performed.

The wells of a PolySorp™ microtitre plate (Nunc) were coated with 10 ng of purified AlyVMI or AlyVMII in 100 µl PBS. The microtitre plates were covered with plastic wrap and incubated overnight at 4°C. Excess antigen was removed and the wells washed three times with TBS containing 0.1% Tween 20. To prevent non-specific binding of the primary antibody to the microtitre plate, the wells were blocked for 1 h at room temperature with 200 µl of 3% BSA in TBS containing 0.1% Tween 20. Excess blocking solution was removed and the wells washed three times with TBS containing 0.1% Tween 20. Anti-serum from each bleed was diluted 1:128 in TBS containing 0.1% Tween 20. One hundred microlitres of diluted anti-serum was added to the wells and incubated for 1 h at room temperature. Excess antibody was removed and the wells washed three times with TBS containing 0.1% Tween 20. Alkaline phosphatase conjugated goat anti-rabbit secondary antibody (Sigma) was diluted 1:10 000 in TBS containing 0.1% Tween 20. One hundred microlitres of secondary antibody was added to the wells and incubated for 1 h at room temperature. Excess secondary antibody was removed and the wells washed three times with TBS containing 0.1% Tween 20. The wells were equilibrated with two 200 µl washes of equilibration buffer. One hundred microlitres of freshly prepared substrate, 1 mg/ml 4-nitrophenyl disodium-orthophospate (Merck) in equilibration buffer, was added to the wells. After 30 min incubation at room temperature, the absorbance was read at 405 nm on a Titretek Multiscan Plus spectrophotometer. A blank or negative control accounting for non-specific interactions of the secondary antibody with sample proteins was prepared by adding TBS

containing 0.1% Tween 20 to the well instead of primary antibody. The absorbance of the blank well was subtracted from the absorbance of the experimental wells.

### 3.3.10. Western hybridisation analysis to determine antibody specificity

The anti-AlyVMI and anti-AlyVMII antibodies were tested for specificity to recombinant AlyVMI and AlyVMII, respectively, by western hybridisation analysis with cell lysates of *E. coli* BL21 (DE3) pLysS transformed with either pET-29a, pETalyVMI, pETalyVMII or pETalyVMIV. *Vibrio midae* SY9 cell lysates containing native AlyVMI and AlyVMII were also analysed.

Anti-AlyVMI and anti-AlyVMII polyclonal antibodies were precipitated from bleed 7 immune sera using a modified polyethylene glycol (PEG) method (Polson *et al*. 1964). Two volumes of borate-buffered saline were added to one volume immune serum. Finely ground PEG 6000 was added to a final concentration of 14% and dissolved by inversion. The samples were centrifuged at 14 000 rpm for 10 min at 4°C. The supernatant was discarded and the pellet resuspended in 1x PBS in the original serum volume. Finely ground PEG 6000 was added to a final concentration of 14% and dissolved by inversion. The samples were centrifuged at 14 000 rpm for 10 min at 4°C. The pellet was resuspended in 1x PBS containing 60% glycerol in half the original serum volume. PEG-precipitated antibodies were stored at -20°C.

*E. coli* BL21 (DE3) pLysS cells transformed with pET-29a, pETalyVMI, pETalyVMII or pETalyVMIV were cultured as described in Section 3.3.4 except that 100 ml LB was used for the IPTG induced culture. *Vibrio midae* SY9 was cultured as described in Section 3.3.7. Cell lysates were prepared as described in Section 3.3.5 except that cells were resuspended in 10 ml lysis buffer lacking imidazole. The protein concentration of each lysate was determined by the Bradford method (Appendix B.16.1).

PEG-precipitated anti-AlyVMI and anti-AlyVMII antibodies were pre-absorbed against *E. coli* cell lysate proteins as described by Rybicki *et al.* (1990) with modifications. Nitrocellulose membranes (4 cm x 4 cm) were incubated with 2 ml *E. coli* cell lysates for ~8 h with gentle

agitation at room temperature. The *E. coli* cell lysates used were *E. coli* BL21 (DE3) pLysS transformed with either pET-29a, pETalyVMI or pETalyVMII. The membranes were rinsed in 1x PBS and incubated in blocking solution for 30 min with gentle agitation at room temperature to prevent non-specific binding of the antibody to the membrane. PEG-precipitated anti-AlyVMI or anti-AlyVMII antibodies were diluted 1:2500 or 1:3000, respectively, in blocking solution and added to individual membranes. The membranes were incubated at 4°C overnight with gentle agitation. The pre-absorbed antibodies were stored at -20°C and re-used.

Three western hybridisation analyses were performed for each antibody. Twenty micrograms of total protein of cell lysates from *E. coli* BL21 (DE3) pLysS transformed with either pET-29a, pETalyVMI, pETalyVMII or pETalyVMIV were separated on an 8% denaturing SDS-PAGE gel (Appendix B.15) in triplicate for each antibody. The proteins were transferred to a nitrocellulose membrane using a Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad) as described in Appendix B.17. Western hybridisation analysis was performed as described in Section 3.3.5 differing only in the primary antibody that was used. The first two membranes were incubated with AlyVMI or AlyVMII pre-immune sera (bleed 1) diluted 1:2500 or 1:3000, respectively, in blocking buffer. The second set of membranes was incubated with PEG-precipitated anti-AlyVMI or anti-AlyVMII antibodies (bleed 7) diluted 1:25000 or 1:3000, respectively, in blocking buffer. The third set of membranes was incubated with PEG-precipitated anti-AlyVMI or anti-AlyVMII antibodies pre-absorbed with cell lysates of *E. coli* BL21 (DE3) pLysS transformed with pET-29a.

An additional western hybridisation analysis was performed to determine whether anti-AlyVMI and anti-AlyVMII recognized native AlyVMI and AlyVMII, respectively. Twenty micrograms of total protein of *E. coli* BL21 (DE3) pLysS cell lysates containing AlyVMI or AlyVMII and 20 µg of *Vibrio midae* SY9 cell lysates were separated on an 8% denaturing SDS-PAGE gel (Appendix B.15) in duplicate. The proteins were transferred to a nitrocellulose membrane using a Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad) as described in Appendix B.17. Western hybridisation analysis was performed as described in Section 3.3.5 except that PEG-precipitated anti-AlyVMI or anti-AlyVMII antibodies pre-absorbed against *E. coli* BL21 (DE3) pLysS cell lysates containing AlyVMII or AlyVMI, respectively, were used.

## 3.4. RESULTS

### 3.4.1. Cloning *alyVMI*, *alyVMII*, *alyVMIII* and *alyVMIV* into pET-29a

Alginate lyase genes *alyVMI*, *alyVMII*, *alyVMIII* and *alyVMIV* were successfully cloned into pET-29a and sequenced. Recombinant pET-29a plasmids were designated pETalyVMI, pETalyVMII, pETalyVMIII and pETalyVMIV, respectively. Analysis of the sequence data showed that the nucleotide sequence of each gene was correct, each gene possessed a start codon and each gene had been cloned in frame with the T7 promoter and C-terminal 6x histidine tag. The restriction enzyme sites present on the primers used for the original PCR amplification and required for sub-cloning from pBluescript SK (+)$^{TM}$ into pET-29a were present on the nucleotide sequences.

### 3.4.2. Expression of recombinant alginate lyase genes in *E. coli*

Absorbance of *E. coli* cultures at 600 nm was measured during IPTG induction in order to monitor cell growth. *E. coli* BL21 (DE3) pLysS cells transformed with pETalyVMI, pETalyVMII or pETalyVMIII continued to grow exponentially after the addition of IPTG as indicated by an increase in absorbance at 600 nm (data not shown). *E. coli* BL21 (DE3) pLysS cells transformed with pETalyVMIV did not grow as well as *E. coli* cells transformed with pETalyVMI, pETalyVMII or pETalyVMIII after the addition of IPTG as indicated by a ~50-60% reduction in absorbance at 600 nm (data not shown).

#### 3.4.2.1. Western hybridisation analysis of alginate lyase gene expression from recombinant pET-29a

Expression of AlyVMI, AlyVMII, AlyVMIII and AlyVMIV from recombinant pET-29a constructs in *E. coli* BL21 (DE3) pLysS was tested by western hybridisation of soluble cellular lysates using rabbit anti-6x histidine antibody. The predicted molecular weight of AlyVMI and AlyVMII is ~81 kDa, AlyVMIII is ~54 kDa and AlyVMIV is ~57 kDa (Chapter 2, Section 2.4.3). *E. coli* BL21 (DE3) pLysS transformed with pET-29a was included as a negative control.

In the western hybridisation experiment, a protein of ~81 kDa was detected in cell lysates prepared from *E. coli* BL21 pLysS transformed with pETalyVMI or pETalyVMII (Figure 3.2, lane 2 and 3). Proteins were not detected in cell lysates prepared from *E. coli* BL21 pLysS transformed with pETalyVMIII (Figure 3.2, lane 5), indicating that AlyVMIII was not expressed from pETalyVMIII. A protein of ~60 kDa was detected in the cell lysate prepared from *E. coli* BL21 pLysS transformed with pETalyVMIV confirming expression of AlyVMIV in *E. coli* (Figure 3.2, lane 4). As expected, no proteins were detected in the cell lysate prepared from *E. coli* BL21 (DE3) pLysS transformed with pET-29a (Figure 3.2, lane 6). *E. coli* BL21 (DE3) pLysS transformed with pETalyVMIII was cultured and induced with IPTG at 25°C and 30°C in an attempt to express *alyVMIII*. However, expression of *alyVMIII* remained unsuccessful.

#### 3.4.2.2. Alginate lyase activity of *E. coli* and *Vibrio midae* SY9 cell lysates

Alginate lyase activity was investigated in *Vibrio midae* SY9 cell lysates, cell lysates prepared from *E. coli* BL21 (DE3) pLysS transformed with pETalyVMI, pETalyVMII or pETalyVMIV and culture supernatants. There was no activity in the *E. coli* cell lysates containing AlyVMI or AlyVMII, even when assayed at 20°C. Alginate lyase activity was detected in the *E. coli* cell lysate containing AlyVMIV, the *V. midae* SY9 cell lysate, the culture supernatant of *E. coli* transformed with pETalyVMIV and the *V. midae* SY9 culture supernatant.

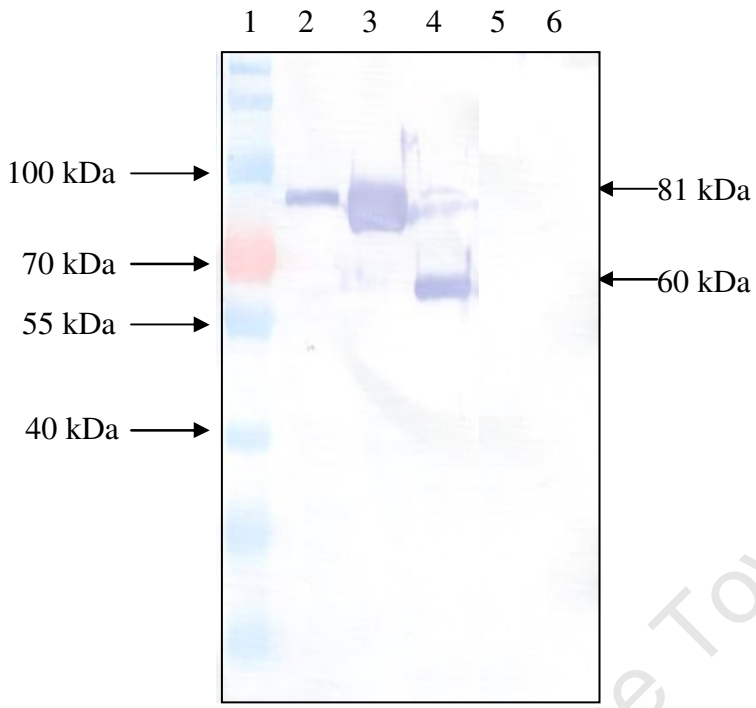



**Figure 3.2.** Western hybridisation analysis of alginate lyase gene expression from recombinant pET-29a constructs in *E. coli* BL21 (DE3) pLysS using anti-histidine antibodies. Lanes: 1) Fermentas pre-stained molecular weight ladder, 2) *E. coli* cell lysate containing AlyVMI, 3) *E. coli* cell lysate containing AlyVMII, 4) *E. coli* cell lysate containing AlyVMIV, 5) *E. coli* cell lysate containing AlyVMIII and 6) *E. coli* cell lysate containing pET-29a . Proteins were electrophoresed on an 8% PAGE gel, transferred onto a nitrocellulose membrane and probed with an anti-histidine antibody.



### 3.4.3. Purification of AlyVMI and AlyVMII

Large scale purification of AlyVMI, AlyVMII and AlyVMIV was performed from *E. coli* BL21 (DE3) pLysS cell lysates using His-Select® Nickel Affinity Gel. Cell lysates were passed over nickel affinity columns, washed and the proteins eluted using 250 mM imidazole. There is no difference between the profile of proteins in the *E. coli* cell lysates before application to the affinity column and after passing through the affinity column (Figure 3.3 lanes 2 and 3) and all three proteins were partially eluted in the wash fractions (Figure 3.3 lanes 4-7). A greater amount

of AlyVMII was present in the *E. coli* cell lysates and more AlyVMII was eluted in the wash fractions and in the final elution fraction than AlyVMI or AlyVMIV.

Recombinant AlyVMI (Figure 3.3a lane 8) and AlyVMII (Figure 3.3b lane 8) were successfully purified. However, non-specific *E. coli* proteins continued to co-elute with AlyVMIV (Figure 3.3c lane 8). Attempts at optimising AlyVMIV purification included varying the growth temperature of the *E. coli* culture, varying the concentration of IPTG added to induce expression, varying the concentration of imidazole in the wash buffer and attempting purification under protein denaturing conditions. Unfortunately, all the above mentioned strategies were unsuccessful in preventing non-specific *E. coli* protein bands co-eluting with AlyVMIV. Thus, only AlyVMI and AlyVMII could be purified to homogeneity using affinity chromatography.

### 3.4.4. Determination of antibody titre in immune sera

Polyclonal antibodies were raised against AlyVMI and AlyVMII by immunising rabbits with purified recombinant AlyVMI or AlyVMII. An indirect ELISA assay was used to determine which bleed collected from the immunised rabbits had the highest titre of anti-AlyVMI or anti-AlyVMII antibodies.

Anti-serum from the pre-immune bleed (bleed 1) contained no anti-AlyVMI or anti-AlyVMII antibodies since there was no positive reaction and no absorbance reading obtained from the wells to which pre-immune sera had been added (Figure 3.4). The titre of anti-AlyVMI antibodies increased steadily from bleeds 2 to 7 (Figure 3.4). Bleed 7 had the highest anti-AlyVMI antibody titre of the bleeds tested. The titre of anti-AlyVMII antibodies increased dramatically between the pre-immune bleed (bleed 1) and bleed 2 (Figure 3.4). The anti-AlyVMII antibody titre seemed to decrease in bleeds 3 to 6 when compared to bleed 2, but was still greater than bleed 1. There was an increase in antibody titre from bleeds 6 to 7 after the booster immunisation. Bleed 2 had the highest anti-AlyVMII antibody titre of the bleeds tested followed by bleeds 7 and 4.

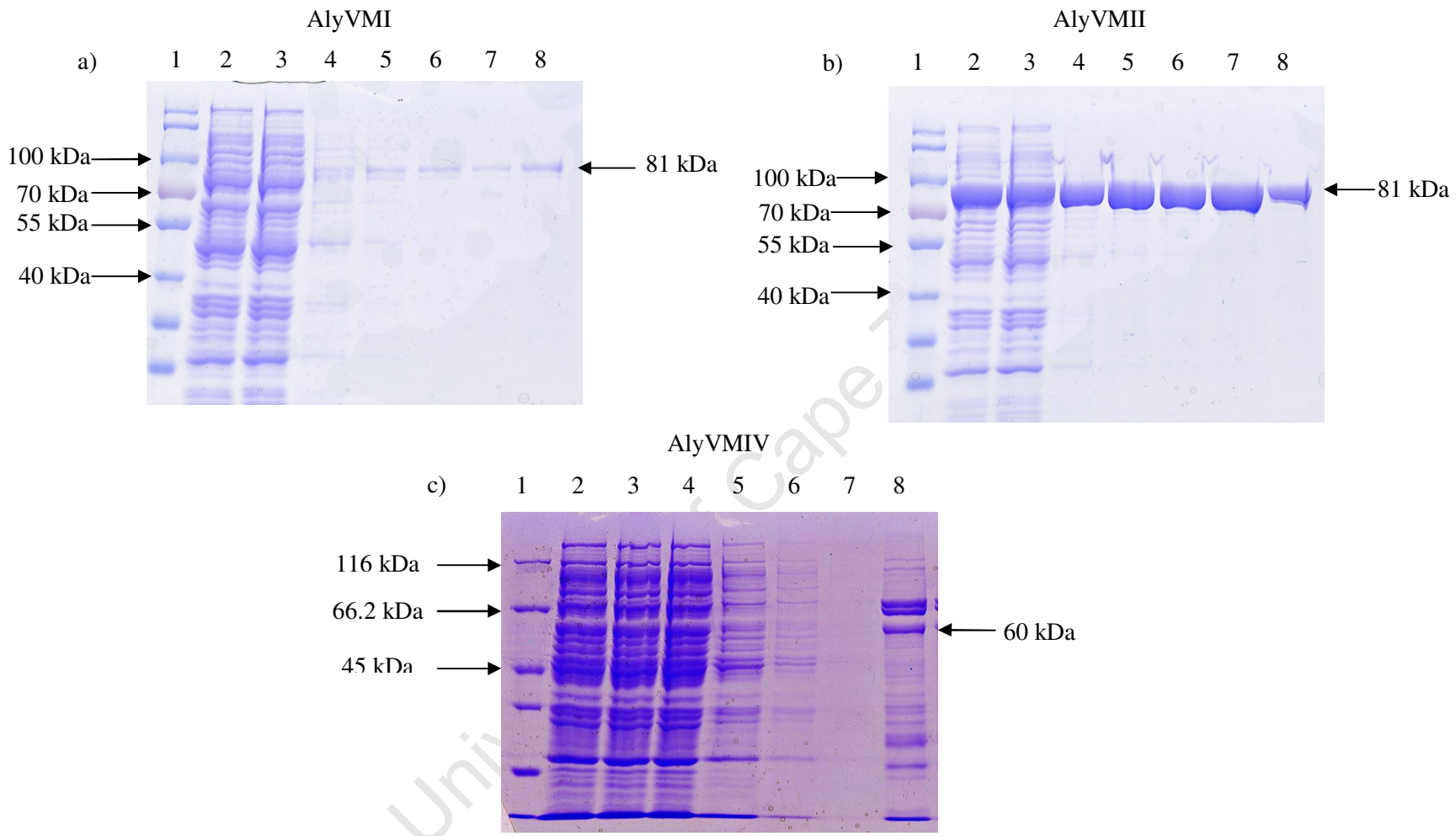



**Figure 3.3.** Large scale purification of AlyVMI (a), AlyVMII (b) and AlyVMIV (c) from cell lysates prepared from *E. coli* BL21 (DE3) pLysS transformed with pETalyVMI, pETalyVMII or pETalyVMIV, respectively, using His-Select® Nickel Affinity Gel. Lanes: 1) Fermentas molecular weight ladder, 2) *E. coli* cell lysate before application to the affinity column, 3) cell lysate after application to the affinity column, 4-7) wash fractions, 8) eluted fraction. Proteins were electrophoresed on an 8% SDS-PAGE gel and stained with Coomassie blue staining solution.

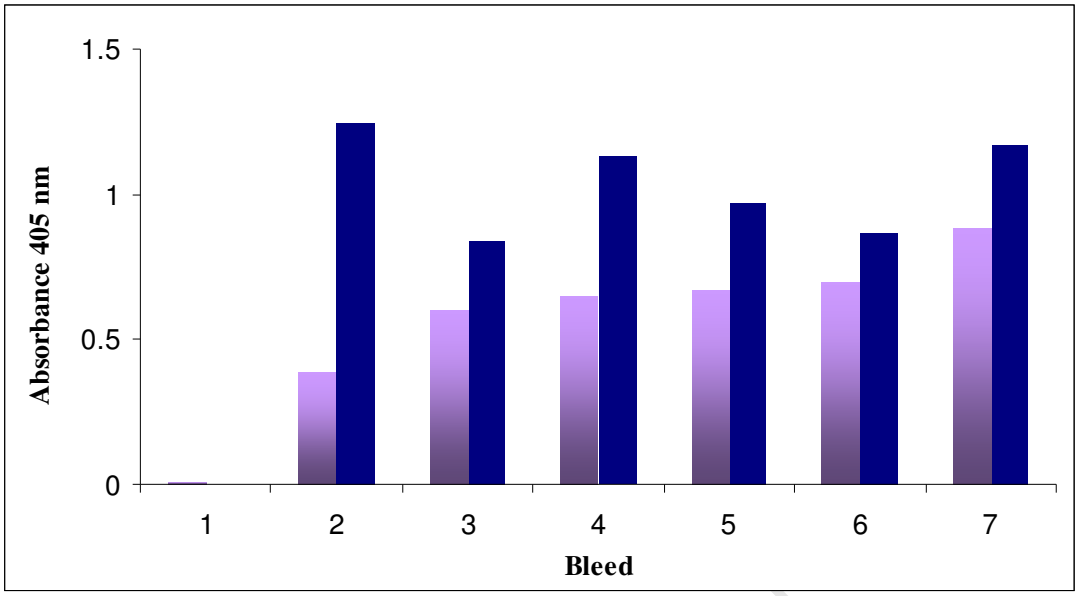



**Figure 3.4.** Titre determination of polyclonal antibodies raised in rabbits against purified recombinant AlyVMI (■) and AlyVMII (■) using indirect ELISA. A single experiment was performed. Bleed 1 represents the pre-immune serum and bleeds 2 to 7 represent anti-serum collected during the immunisation of rabbits with AlyVMI or AlyVMII.



### 3.4.5. Specificity of anti-AlyVMI and anti-AlyVMII antibodies

The anti-AlyVMI and anti-AlyVMII antibodies were tested for specificity to AlyVMI and AlyVMII, respectively, by conducting a western hybridisation analysis with cell lysates prepared from *E. coli* BL21 (DE3) pLysS transformed with either pET-29a, pETalyVMI, pETalyVMII or pETalyVMIV.

A negative result was obtained for all the cell lysates when tested with the anti-AlyVMI (Figure 3.5 a) or the anti-AlyVMII (Figure 3.6 a) pre-immune sera (bleed 1), respectively. Both the anti-AlyVMI (Figure 3.5 b) and anti-AlyVMII (Figure 3.6 b) antibodies recognized AlyVMI (~81 kDa), AlyVMII (~81 kDa) and AlyVMIV (~60 kDa) in the *E. coli* cell lysates.

Although both antibodies detected *E. coli* proteins in the cell lysates, the anti-AlyVMII antibodies cross-reacted to *E. coli* proteins in the cell lysates more noticeably than the anti-AlyVMI antibodies. The *E. coli* background was reduced by pre-absorbing the antibodies against cell lysates prepared from *E. coli* BL21 (DE3) pLysS cells transformed with pET-29a (Figures 3.5 c and 3.6 c). The anti-AlyVMII antibodies may not have been as pure as the anti-AlyVMI antibodies since more *E. coli* proteins were detected by the anti-AlyVMII antibodies than by the anti-AlyVMI antibodies after pre-absorbing against the *E. coli* cell lysates. There was also possibly some overflow from lane 3 into lanes 2, 4 and 5 when the gel was loaded, possibly due to an incompletely formed well, resulting in detection of AlyVMII in these lanes (Figure 3.6 c). Anti-AlyVMI and anti-AlyVMII antibodies no longer recognized AlyVMIV after pre-absorbing against cellular proteins of *E. coli* transformed with pET-29a (Figures 3.5 c and 3.6 c).

Pre-absorbing anti-AlyVMI or anti-AlyVMII antibodies against *E. coli* cell lysates containing AlyVMII or AlyVMI, respectively, reduced the *E. coli* background and rendered each antiserum specific for the protein it was raised against (Figure 3.7). Anti-AlyVMI and anti-AlyVMII antibodies also recognized native AlyVMI and AlyVMII, respectively, in *Vibrio midae* SY9 cell lysates (Figure 3.7 a and b, lane 4). No other *V. midae* SY9 proteins were recognized by the antibodies raised against recombinant AlyVMI or AlyVMII. Recombinant AlyVMI and AlyVMII were the same molecular weight, ~81 kDa, as native AlyVMI and AlyVMII detected in *V. midae* SY9 cell lysates.

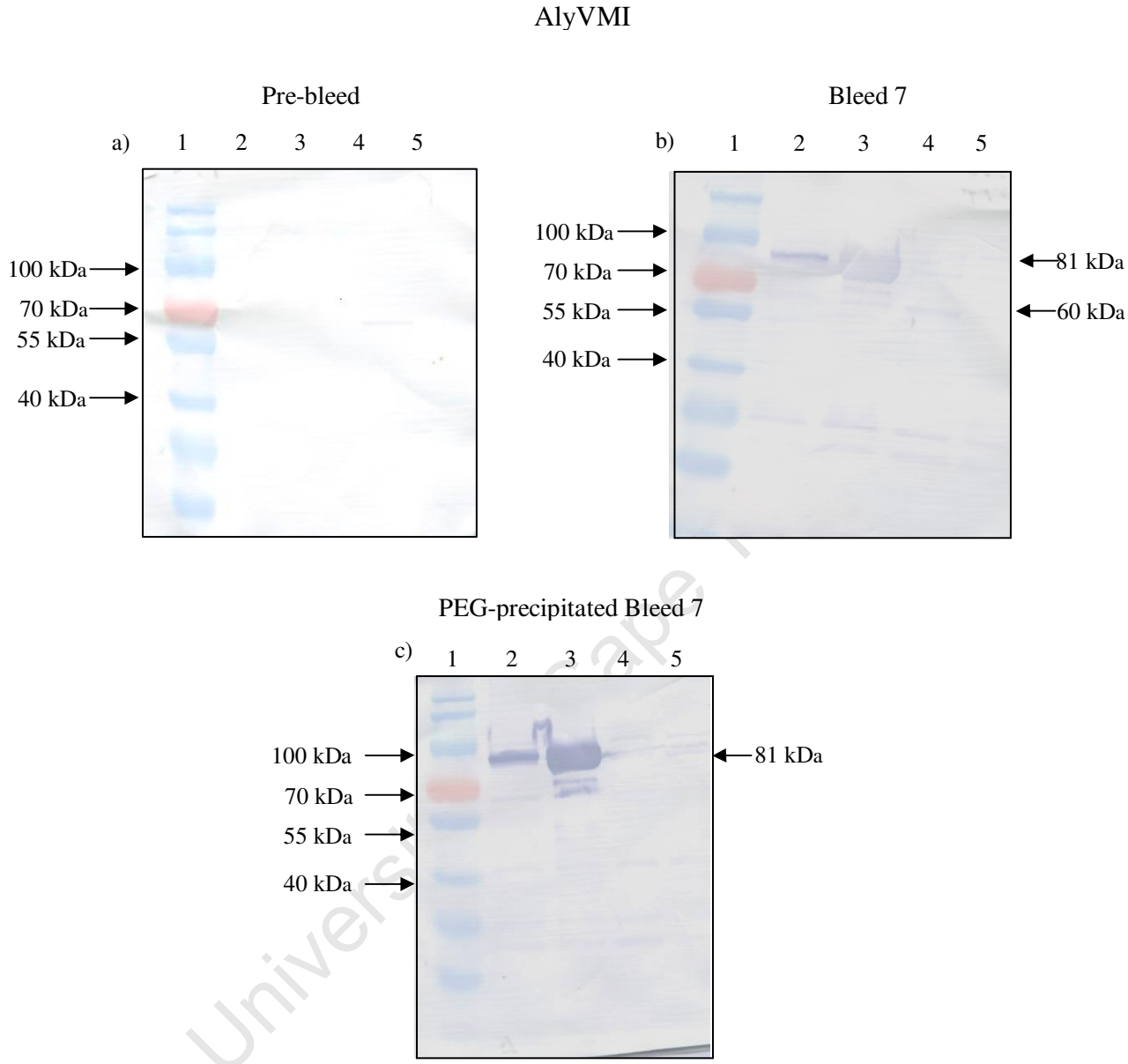



**Figure 3.5.** Western hybridisation analysis using anti-AlyVMI pre-immune serum (a), bleed 7 (b), and bleed 7 pre-absorbed against *E. coli* BL21 (DE3) pLysS cell lysates (c). Lanes: 1) Fermentas pre-stained molecular weight ladder, 2) *E. coli* cell lysate containing AlyVMI, 3) *E. coli* cell lysate containing AlyVMII, 4) *E. coli* cell lysate containing AlyVMIV, 5) *E. coli* cell lysate containing pET-29a. Proteins were electrophoresed on an 8% PAGE gel, transferred onto a nitrocellulose membrane and probed with anti-AlyVMI antibodies.

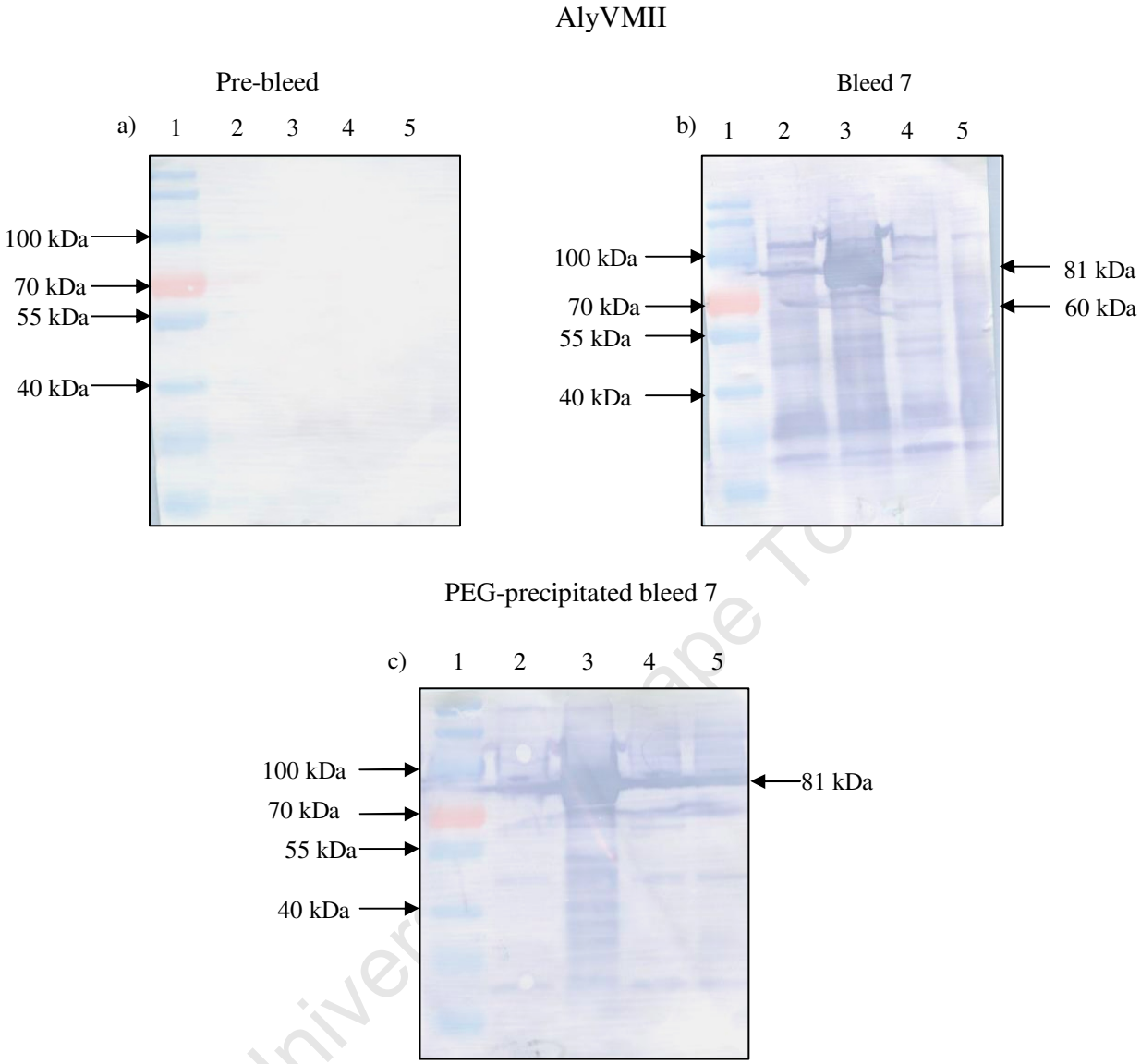



**Figure 3.6.** Western hybridisation analysis using anti-AlyVMII pre-immune serum (a), bleed 7 (b), and bleed 7 pre-absorbed against *E. coli* BL21 (DE3) pLysS cell lysates (c). Lanes: 1) Fermentas pre-stained molecular weight ladder, 2) *E. coli* cell lysate containing AlyVMI, 3) *E. coli* cell lysate containing AlyVMII, 4) *E. coli* cell lysate containing AlyVMIV, 5) cell lysate prepared from *E. coli* transformed with pET-29a. Proteins were electrophoresed on an 8% PAGE gel, transferred onto a nitrocellulose membrane and probed with anti-AlyVMII antibodies.

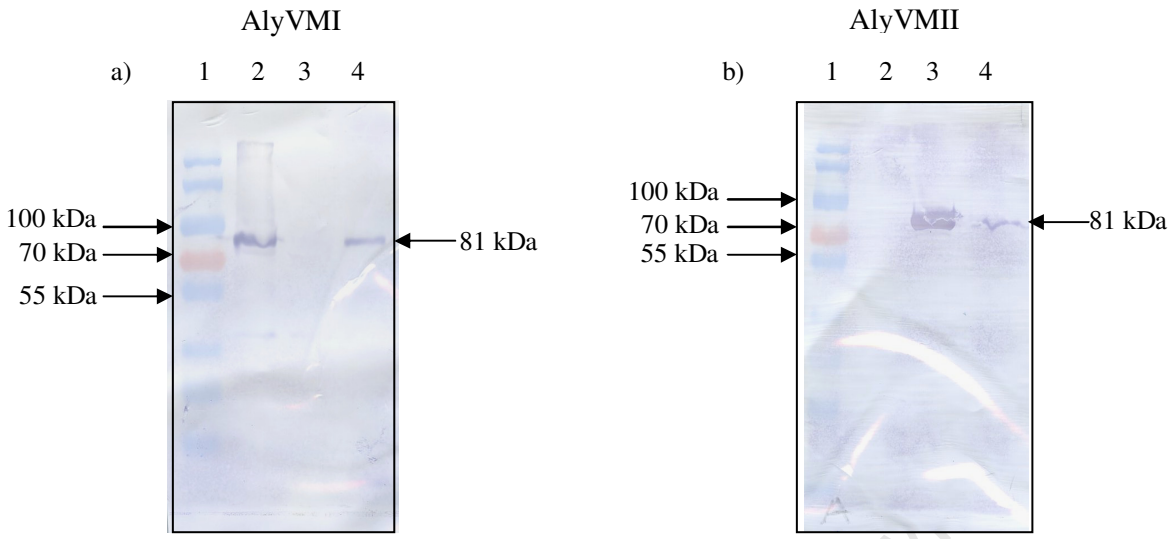



**Figure 3.7.** Specificity of anti-AlyVMI (a) and anti-AlyVMII antibodies (b). Western hybridisation analysis of *E. coli* cell lysates using anti-AlyVMI and anti-AlyVMII antibodies pre-absorbed against cell lysates prepared from *E. coli* BL21 (DE3) pLysS transformed with pETalyVMII or pETalyVMI, respectively. Lanes: 1) Fermentas pre-stained molecular weight ladder, 2) *alyVMI E. coli* cell lysate, 3) *alyVMII E. coli* cell lysate, 4) *Vibrio midae* SY9 cell lysate. Proteins were electrophoresed on an 8% PAGE gel, transferred onto a nitrocellulose membrane and probed with anti-AlyVMI or anti-AlyVMII antibodies.

## 3.5. DISCUSSION

The Novagen expression vector pET-29a was chosen to express the four alginate lyase genes of *Vibrio midae* SY9 since it carries a C-terminal 6x histidine tag, an N-terminal S tag and a kanamycin resistance selective marker. The histidine and S tag are both small, being just 6 and 15 amino acids in length, respectively, can be used for affinity purification as well as western hybridisation analysis and can be removed from the vector during cloning, if required, by digestion with certain restriction enzymes. The expression vector pET-29a is also a translation vector meaning it contains the ribosome binding site from the T7 phage major capsid protein and is used for the expression of target genes which do no have their own ribosomal binding site (pET System Manual, Novagen, EMD Biosciences, USA).

Alginate lyase genes *alyVMI*, *alyVMII*, *alyVMIII* and *alyVMIV* were amplified using the high fidelity DNA polymerase, *Pfu*, from *Pyrococcus furiosus*. *Pfu* exhibits 5' to 3' exonuclease activity which enables it to correct nucleotide incorporation errors (Lundberg *et al*. 1991). PCR products were cloned into pBluescript SK (+)$^{TM}$ and sub-cloned into pET-29a using the restriction sites included in the PCR amplification primers. Recombinant pET-29a DNA inserts were sequenced and found to align with the alginate lyase gene sequences determined in Chapter 2, possess a start codon, were in frame with the T7 promoter and the C-terminal histidine tag, and included the restriction sites used for sub-cloning.

*E. coli* DH5α was used as a cloning host, while *E. coli* BL21 (DE3) pLysS was used as a host for expression of the alginate lyase genes. *E. coli* BL21 strains are deficient in the *lon* protease and the *ompT* outer membrane protease which can degrade proteins during purification (Grodberg and Dunn, 1988). Thus, recombinant proteins may be more stable in *E. coli* BL21 strains than in strains which possess these proteases. Bacteriophage DE3 is a derivative of λ phage. It contains the immunity region of phage *21* and the *lacI* gene, the *lacUV5* promoter and the gene for T7 RNA polymerase on a single DNA fragment. This DE3 DNA fragment has been inserted into the *int* gene on the *E. coli* chromosome, thus preventing the phage from excising from the *E. coli* chromosome in the absence of a helper phage. These strains are lysogens of the bacteriophage DE3 and are designated *E. coli* (DE3) (Studier and Moffatt, 1986).

The *lacUV5* promoter of the lysogen drives transcription of the T7 RNA polymerase gene and is induced by the addition of IPTG to the culture medium. T7 RNA polymerase is highly selective for its own promoters which do not naturally occur in *E. coli* (Studier & Moffatt 1986) and directs transcription of the target gene present on the pET expression vector. Plasmid pLysS is a chloramphenicol resistant plasmid which is compatible with pET-29a and provides a small amount of T7 lysozyme. T7 lysozyme has a dual function in the cell. It binds to T7 RNA polymerase thereby inhibiting transcription (Huang *et al*. 1999, Zhang *et al*. 1997) and cleaves the peptidoglycan layer of the *E. coli* cell wall (Inouye *et al*. 1973). The presence of pLysS increases the tolerance of λDE3 lysogens for plasmids with toxic inserts by increasing the amount of control available over expression. It also makes the preparation of cell lysates easier by providing a small amount of lysozyme.

Western hybridisation analysis using rabbit anti-6x histidine antibodies was used to determine whether the recombinant proteins were being expressed in *E. coli* BL21 (DE3) pLysS cells and whether they were histidine tagged. The predicted molecular weight of AlyVMI and AlyVMII is ~81 kDa, AlyVMIII is ~54 kDa and AlyVMIV is ~57 kDa (Chapter 2, Section 2.4.3).

Detection of a protein of ~81 kDa in cell lysates prepared from *E. coli* BL21 (DE3) pLysS transformed with either pETalyVMI or pETalyVMII confirmed that *alyVMI* and *alyVMII* were expressed and that recombinant AlyVMI and AlyVMII were histidine tagged (Figure 3.2). A protein of ~54 kDa was not detected in the lane loaded with the *E. coli* cell lysate prepared from cells transformed with pETalyVMIII which confirmed that *alyVMIII* was not expressed. Detection of a ~60 kDa protein in the cell lysate prepared from *E. coli* transformed with pETalyVMIV confirmed that *alyVMIV* was expressed and that recombinant AlyVMIV was histidine tagged (Figure 3.2).

Alginate lyase gene *alyVMIII* was not expressed at all in *E. coli* BL21 (DE3) pLysS using pET-29a. Optimization of the growth temperature of *E. coli* BL21 (DE3) transformed with pETalyVMIII did not lead to the expression of *alyVMIII* in *E. coli*. The cloning of *alyVMIII* into the *Xba*I and *Xho*I restriction sites of pET-29a resulted in the removal of the S tag and ribosome

binding site from the vector, whereas the cloning of *alyVMI*, *alyVMII* and *alyVMIV* into the *Nde*I and *Xho*I restrictions sites of pET-29a resulted in the removal of the S tag only. Thus, *alyVMIII* may have been transcribed from pET-29a but the mRNA was not translated due to the lack of a ribosome binding site. Although *alyVMIII* could be cloned into another restriction site present in the multiple cloning region of pET-29a that does not also cleave the gene, the S tag and other additional amino acids would be included in the resulting recombinant plasmid. The expression of *alyVMIII* from such a plasmid would need to be confirmed experimentally and whether or not an additional tag and amino acid residues interfere with protein folding or enzyme activity. Alternatively, cloning *alyVMIII* into an expression vector with a different multiple cloning region to pET-29a may aid the cloning and expression of the gene.

AlyVMI and AlyVMII were purified by affinity chromatography (Figure 3.3 a and b). AlyVMIV was not purified to homogeneity since non-specific *E. coli* proteins co-purified with the enzyme (Figure 3.3 c). Optimization of the growth temperature of *E. coli* BL21 (DE3) pLysS transformed with pETalyVMIV, the concentration of IPTG used to induce expression, the concentration of imidazole in the affinity column wash buffers as well as purification under denaturing conditions did not improve AlyVMIV purity. Possible solutions to overcome this problem may include using a purer grade of imidazole, a larger nickel affinity column, and washing the column and eluting bound protein with an imidazole gradient rather than a set imidazole concentration (Ealand, 2010).

IPTG induced cultures of *E. coli* BL21 (DE3) pLysS cells transformed with pETalyVMIV grew slower than *E. coli* cells transformed with pETalyVMI, pETalyVMII, or pETalyVMIII as indicated by a ~50-60% reduction in absorbance at 600 nm. The slower growth rate of the IPTG induced cultures indicates that the protein is toxic to the *E. coli* cells (The QIAexpressionist Handbook, Qiagen, USA). The toxic effect of AlyVMIV may be attributed to the presence of a signal peptide (Chapter 2, Section 2.4.3.4) the hydrophobic regions of which most likely associate with the cell membrane (The QIAexpressionist Handbook, Qiagen, USA). Overexpression of exported heterologous proteins in *E. coli* can lead to a lethal overload of the protein export machinery and proteins which are not completely exported become 'jammed' in the cell membrane (Baneyx, 1999; Bieker and Silhavy, 1989; Bieker-Brady and Silhavy, 1992;

Makrides *et al*, 1996). This results in the normal processing and translocation of periplasmic and membrane proteins being inhibited (Ito *et al.* 1981). Since alginate lyase activity was found in the culture supernatant of *E. coli* cells transformed with pETalyVMIV, it is reasonable to conclude that *E. coli* recognizes the AlyVMIV signal peptide and attempts to export the protein. Cloning AlyVMIV without its signal peptide (first 24 amino acids) might alleviate this problem and improve purification of the protein. The xanthan lyase from *Bacillus* sp. strain GL1 was cloned and successfully purified without its signal peptide using pET-17b (Hashimoto *et al.* 2000a).

To determine whether recombinant proteins were active, alginate lyase activity of *E. coli* BL21 (DE3) pLysS cell lysates prepared with lysis buffer lacking imidazole was tested using the TBA assay. The alginate lyase activity of the purified proteins was not determined since the presence of imidazole in the eluted fractions interferes with the assay. AlyVMI and AlyVMII in *E. coli* BL21 (DE3) pLysS cell lysates were found to not be active, whereas AlyVMIV was active indicating that the latter enzyme is processed correctly in *E. coli* and that the histidine tag does not interfere with the folding or activity of the enzyme. It is possible that AlyVMI and AlyVMII are not correctly processed in *E. coli*, or that the histidine tag interferes with the folding or activity of the proteins. The assay was also performed at 20°C, however no activity was detected. It is may also be possible that a co-factor is required for the activity of AlyVMI and AlyVMII. It is also possible that since AlyVMI and AlyVMII contain transmembrane domains (Figure 2.4 and 2.5, page 57 and 61, respectively), a membrane component may be required for the activity of these enzymes. Alginate lyase activity detected in *Vibrio midae* SY9 cell lysates would be the combined activity of AlyVMI, AlyVMII and AlyVMIII which are present intracellularly and is indicative that the native enzymes are active after sonication in the lysis buffer used to prepare the *E. coli* cell lysates.

Purified AlyVMI and AlyVMII were used to raise antibodies in rabbits. Indirect ELISA confirmed that the pre-immune sera (bleed 1) did not react to AlyVMI and AlyVMII (Figure 3.4). Antibodies against AlyVMI and AlyVMII were present in bleeds 2 to 7. There was a greater immune response to purified AlyVMII than to AlyVMI since the anti-AlyVMII antibody titre was higher than that of anti-AlyVMI. This may be due to the fact that approximately 50 times

more AlyVMII than AlyVMI was used to immunise the rabbits. The recommended dosage of antigen for the immunisation of rabbits is 1 mg/ml divided over 4 injections followed by a booster immunisation. Purified AlyVMI and AlyVMII were not available in such great quantities, therefore, the amount of each protein that was purified was used to immunise the rabbits. Since AlyVMII was purified in larger amounts than AlyVMI, the rabbits were immunised with more AlyVMII than AlyVMI.

The specificity of anti-AlyVMI and anti-AlyVMII antibodies was tested by western hybridisation analysis. Recombinant AlyVMI, AlyVMII and AlyVMIV in *E. coli* BL21 (DE3) pLysS cell lysates were recognized by both anti-AlyVMI and anti-AlyVMII antibodies (Figures 3.5 b and 3.6 b). After pre-absorption of anti-AlyVMI and anti-AlyVMII antibodies against cell lysates of *E. coli* BL21 (DE3) pLysS cells transformed with pET-29a, recombinant AlyVMIV was no longer recognized by either antibody (Figures 3.5 c and 3.6 c). This could be due to the removal of anti-histidine antibodies which would have recognized all three recombinant histidine tagged proteins, suggesting that there are no epitopes in common between AlyVMI or AlyVMII and AlyVMIV.

Pre-absorbing anti-AlyVMI or anti-AlyVMII antibodies against *E. coli* BL21 (DE3) pLysS cell lysates containing AlyVMII or AlyVMI, respectively, rendered the antibodies specific to the recombinant protein against which each was raised (Figure 3.7). Therefore, there must be epitopes in common between the two proteins as well as epitopes specific to each protein, suggesting that there are structural differences between the two proteins even though they appear to be very similar with regard to amino acid sequence, predicted conserved domains and predicted secondary structure (Chapter 2, Section 2.4.3). In addition, AlyVMI and AlyVMII are expressed differently from the same expression vector in the same *E. coli* expression host. Recombinant AlyVMII was more soluble in *E. coli* BL21 (DE3) pLysS than AlyVMI under the same IPTG induction conditions (data not shown), resulting in a greater yield of recombinant AlyVMII than AlyVMI and supporting the suggestion that these two proteins are structurally different.

The predicted molecular weight of AlyVMI and AlyVMII is ~81 kDa, AlyVMIII is ~54 kDa and AlyVMIV is ~57 kDa (Chapter 2, Section 2.4.3). SDS-PAGE and western hybridisation analysis revealed AlyVMI and AlyVMII to be ~81 kDa and AlyVMIV to be ~60 kDa. An experimental molecular weight was not obtained for AlyVMIII since *alyVMIII* was not expressed in *E. coli* BL21 (DE3) pLysS. The predicted and experimental molecular weights of AlyVMI and AlyVMII are in agreement, while the experimental molecular weight of AlyVMIV was slightly larger than the predicted molecular weight.

The only difference between recombinant and native AlyVMIV is the presence of a histidine tag. The difference between the predicted and experimental molecular weight of AlyVMIV could be due to altered migration of AlyVMIV through SDS-polyacrylamide gels. AlyVMIV was predicted to contain a galactose-binding like domain (Chapter 2, Section 2.4.3.4). The function of this domain is to confer binding to ligands such as cell-surface attached carbohydrate residues or phospholipids. If the carbohydrate moiety remains attached to AlyVMIV, the protein will behave anomalously when electrophoresed through SDS-polyacrylamide gels. SDS will only bind to the protein and not to the carbohydrate, resulting in a reduced net charge due to reduced SDS binding and thus decreased mobility of the polypeptide through the polyacrylamide, resulting in an artificially high molecular weight (Hames, 1981). The difference between the predicted and experimental molecular weight of AlyVMIV may be due to the same phenomenon.

Western hybridisation analysis was also performed on *Vibrio midae* SY9 cell lysates using anti-AlyVMI or anti-AlyVMII antibodies. The molecular weight of native AlyVMI and AlyVMII was found to be ~81 kDa which is the same molecular weight as the recombinant proteins and is comparable to the predicted molecular weight. No other *Vibrio midae* SY9 proteins were recognized by the antibodies, confirming the specificity of anti-AlyVMI and anti-AlyVMII to AlyVMI and AlyVMII, respectively.

Alginate lyases can be classified according to their substrate specificity, molecular weight or amino acid sequence and structure similarity. As discussed in Chapter 2 (Section 2.5), AlyVMI and AlyVMII belong to polysaccharide lyase family 17 (PL-17), whereas AlyVMIII and AlyVMIV belong to PL-7. Although substrate specificity of the four alginate lyases remains

unknown, they can be grouped by molecular weight. Small alginate lyases range from 25-30 kDa, medium-sized lyases are ~40 kDa and large lyases are greater than 60 kDa (Osawa *et al*. 2005). AlyVMI, AlyVMII and AlyVMIV fall into the large alginate lyase group since their experimental molecular weights are 60 kDa or larger. AlyVMIII has a predicted molecular weight of 54 kDa and thus would also belong to the large molecular weight group.

The two alginate lyases of *Vibrio* sp. O2, AlyVOA and AlyVOB, are small lyases with predicted molecular weights of 28.4 kDa and 25.2 kDa, respectively (Kawamoto *et al*. 2006). AlyVGI and AlyVGII of *Vibrio halioticoli* fall into the ~40 kDa lyase group with predicted molecular weights of 39 kDa and 36 kDa, respectively, whereas AlyVGIII is a small lyase at 27 kDa (Sugimura *et al*. 2000). *Sphingomonas* sp. A1 has one small lyase (A1 II, 25 kDa), one 40 kDa lyase (A1 III), and two large lyases (A1 I, 63 kDa and A1, IV 86 kDa; Yoon *et al*. 2000, Hashimoto *et al* 2000b). The four alginate lyases of *Vibrio midae* SY9 are all large lyases. Therefore, alginate lyases vary greatly and bacteria can have multiple enzymes that belong to the same or different groups.

In conclusion *alyVMI*, *alyVMII*, *alyVMIII* and *alyVMIV* were successfully cloned into the expression vector pET-29a. Expression of recombinant *alyVMI*, *alyVMII* and *alyVMIV* was induced by IPTG in *E. coli* BL21 (DE3) pLysS and the presence of histidine tagged proteins confirmed by anti-histidine western blot analysis. Recombinant AlyVMI and AlyVMII were purified to homogeneity using nickel affinity chromatography. Polyclonal antibodies were raised against recombinant AlyVMI and AlyVMII in rabbits. Specificity of the antibodies was tested by western hybridisation analysis. Pre-absorbing anti-AlyVMI or anti-AlyVMII against *E. coli* cell lysates containing AlyVMII or AlyVMI, respectively, rendered the antibodies specific for the protein they were raised against. Anti-AlyVMI or anti-AlyVMII antibodies were also specific for native AlyVMI or AlyVMII in *Vibrio midae* SY9 cell lysates and the predicted, recombinant and native molecular weights of the proteins were found to be comparable.

# Chapter 4

## Expression Analysis of the Four Alginate Lyase Genes of *Vibrio midae* SY9

### CONTENTS

## 4.1. SUMMARY

Alginate lyases of *Saccharophagus degradans* 2-40 and *Sphingomonas* sp A1 have been shown to be induced in the presence of alginate and repressed in the presence of glucose. However, these studies only measured alginate lyase activity and did not investigate mRNA transcript or protein levels. The aim of this study was to determine whether the alginate lyase genes of *Vibrio midae* SY9 are regulated at the transcriptional, translational or post-translational level. *V. midae* SY9 was cultured in alginate media without and supplemented with glucose. Real-time RT PCR, a sensitive and accurate method for profiling gene expression, was used to monitor mRNA transcript levels of the four alginate lyase genes during growth. An indirect ELISA assay was used to monitor the levels of AlyVMI and AlyVMII in *V. midae* SY9 cells. Alginate lyase activity in culture supernatants and cell lysates was monitored using the thiobarbituric acid assay and the reducing sugar content of the culture supernatant was monitored using the dinitrosalicylic acid assay. The alginate lyase mRNA and protein levels did not differ significantly between the two treatments. However, alginate lyase activity was only detected in culture supernatants and cell lysates when the reducing sugar level in the culture supernatant was low. It was hypothesised that the four alginate lyase genes of *V. midae* SY9 are expressed when alginate is present in the growth media whether glucose is present or not and that they are regulated post-translationally, the mechanism of which remains to be determined.

## 4.2. INTRODUCTION

Microorganisms display a diverse metabolic ability and many bacteria are able to utilise a variety of carbon sources (Postma *et al*. 1993). For example, *Vibrio midae* SY9 is able to hydrolyse alginate, gelatin and starch (Macey, 2005) and *Saccharophagus degradans* 2-40 is able to utilise 10 complex carbohydrates as sole carbon sources (Ensor *et al*. 1999). This ability allows bacteria to adapt to a continuously changing environment and compete with other organisms for limited nutrients.

If provided with a variety of carbon sources, bacteria will preferentially take up and utilise the compound that allows the fastest growth (Stülke and Hillen, 1999). This phenomenon is known as carbon catabolite repression (CCR). The presence of a preferred carbon source will repress the expression of catabolic systems specific to an alternative carbon source(s) (Görke and Stülke, 2008). Glucose is often the preferred carbon source in many model organisms (Görke and Stülke, 2008). The classic example is the glucose-lactose diauxie in *Escherichia coli* (Monod, 1942; Inada *et al*. 1996). However, there are instances where glucose is not the preferred carbon source. Examples include *Streptococcus thermophilus* (van den Bogaard *et al*. 2000), *Bifidobacterium longum* (Parche *et al*. 2006) and *Pseudomonas aeruginosa* (Collier *et al*. 1996). An interesting case is the highly regulated co-fermentation of glucose with other carbon sources by *Corynebacterium glutamicum* (Frunzke *et al*. 2008, Wendisch *et al*. 2000). CCR occurs in nearly all bacteria since it is an important regulatory mechanism allowing bacteria to efficiently use available carbon sources. However, diverse mechanisms for repression exist as each group of bacteria has developed its own way of achieving CCR (Görke and Stülke, 2008).

The genome of an organism is an unchanging component; each cell contains a complete set of DNA encoded genes regardless of age, physiology or environment (Robertson, 2005). The transcriptome represents the mRNA complement of a cell and varies with physiology, infection or development (Bustin and Nolan, 2004). Gene expression studies analyse this varying component of a cell. The ability to quantify transcriptional levels and understand patterns of expressed genes could help to provide insight into regulatory pathways and signaling networks. This would lead to a new understanding of cellular processes and disease mechanisms which

would be useful in characterising the physiological state and stress response of organisms (Desroche *et al*. 2005; Schulze and Downward, 2001; Vandesompele *et al* 2002).

Two recently developed methods for measuring mRNA present in cells are microarray analysis and real-time reverse transcription PCR (real-time RT PCR). Microarrays allow the parallel analysis of thousands of genes in two differentially labeled RNA populations (Schena *et al.* 1995), whereas real-time RT PCR measures gene expression of a limited number of genes in many different samples concurrently (Heid *et al*. 1996; Higuchi *et al*. 1993). Real-time RT PCR has become the method of choice for quantifying mRNA transcripts in biological samples (Pfaffl, 2001). It is a highly sensitive technique allowing for the quantification of rare transcripts and detection of small changes in gene expression (Pfaffl, 2001; Huggett *et al.* 2005).

Real-time PCR combines amplification and detection into one step by the continuous collection of fluorescent data throughout the PCR (Wong and Medrano, 2005). Various fluorescent chemistries for detection are available and include TaqMan® probes, Molecular Beacons®, hybridisation probes and the DNA binding dye SYBR® Green I (Shipley, 2006). SYBR® Green I binds to the minor groove of double stranded DNA, is excited at 494 nm and emits at 521 nm (Shipley, 2006). The formation of a single product must be validated when using SYBR® Green I since the dye binds indiscriminately to double stranded DNA including primer dimers (Shipley, 2006).

Real-time RT PCR data must be normalised to account for the variability of RNA quantity and quality, reverse transcription and PCR efficiencies (Čikoš *et al*. 2007; Huggett *et al.* 2005). Current normalisation strategies include ensuring a similar sample size is used for RNA extractions; a similar amount of RNA is used for the reverse transcription reaction and normalisation to an internal reference gene is performed (Bustin, 2002; Huggett *et al.* 2005). These strategies can be used in combination and included at many stages of a real-time assay.

Two types of quantification are possible. Absolute quantification is based on an internal or external calibration curve (Pfaffl, 2001) and determines the initial number of transcripts present of the gene of interest (Livak and Schmittgen, 2001). Relative quantification describes the

change in expression of a gene of interest in different samples relative to each other (Čikoš *et al*. 2007). In certain contexts it is more meaningful to report a relative change in gene expression rather than an absolute transcript copy number.

Many relative quantification methods have been described and include the two standard curve method (Morrison *et al*. 1998), the delta-delta $C_T$ method (Livak and Schmittgen, 2001), the relative expression software tool (REST; Pfaffl, 2001; Pfaffl *et al*. 2002), comparative quantification (Rotorgene software, Qiagen), linear regression (Ramakers *et al*. 2003) and sigmoidal curve fitting (Liu and Saint, 2002). The delta-delta $C_T$ method and the REST method incorporate normalisation against an internal reference gene into the equation used for analysis. The REST method also includes the efficiency of the PCR reaction in the calculation.

This chapter describes the gene expression analysis of the four alginate lyases of *Vibrio midae* SY9. Real-time RT PCR and a quantitative indirect ELISA were used to determine the presence and relative quantity of the mRNA transcripts and proteins respectively at each stage of growth of cells cultured in alginate media without and supplemented with glucose. The TBA assay was used to determine alginate lyase activity in culture supernatants and cell lysates. The aim of this study was to determine whether the alginate lyases of *Vibrio midae* SY9 are regulated at the transcriptional, translational or post-translational level.

## 4.3. MATERIALS AND METHODS

All media and solutions used in this study are listed in Appendix A.

### 4.3.1. Bacterial strains and plasmids used

Bacterial strains and plasmids used in this study are listed in Table 4.1.

### 4.3.2. Culture conditions and media used

*Escherichia coli* BL21 (DE3) pLysS was cultured either in Luria broth (LB) or on Luria agar (LA) containing 20 µg/ml chloramphenicol at 37°C. *E. coli* BL21 (DE3) pLysS transformants harboring recombinant pET-29a plasmids were cultured either in LB or on LA containing 20 µg/ml chloramphenicol and 30 µg/ml kanamycin at 37°C. *Vibrio midae* SY9 was cultured either in marine broth (MB) or on marine agar (MA) at room temperature.

**Table 4.1.** Bacterial strains and plasmids

| Strain/plasmid | Genotype/relevant characteristic(s)[a] | Reference |
|---|---|---|
| **Strains** | | |
| *E. coli* BL21 (DE3) pLysS | F$^-$ *omp*T *gal dcm lon hsd*SB(rB- mB-) λ(DE3) pLysS(cm$^r$) | Moffatt and Studier (1987) |
| *Vibrio midae* SY9 | Putative probiont isolated from *Haliotis midae,* South Africa | Macey and Coyne (2005) |
| **Plasmids** | | |
| pETalyVMI | pET-29a carrying the 2163 bp *alyVMI* gene, Kan$^r$ | Chapter 3 |
| pETalyVMII | pET-29a carrying the 2163 bp *alyVMII* gene, Kan$^r$ | Chapter 3 |

[a] cm$^r$, chloramphenicol resistant; kan$^r$, kanamycin resistant.

### 4.3.3. *Vibrio midae* SY9 growth experiment

*Vibrio midae* SY9 was inoculated into 5 ml of alginate media and incubated at room temperature with shaking at 100 rpm on an orbital shaker for 8 h. This starter culture was used to inoculate 100 ml of fresh alginate media in a 1 L shake flask to a starting absorbance of 0.02 at 600 nm. The inoculated media was incubated at room temperature with shaking overnight. This overnight culture was used to inoculate 6x 500 ml of new alginate media in 5 L shake flasks to a starting absorbance of 0.02 at 600 nm. Glucose was added to the media of three of the inoculated cultures to a final concentration of 0.2%. The cultures were incubated at room temperature with shaking at 100 rpm. Absorbance of the cultures was monitored at 600 nm with 1 ml samples collected at 2, 4, 6, 8, 10, 12, 15 and 24 h after inoculation.

Samples of each culture were collected for monitoring alginate lyase activity, the amount of reducing sugar present and the presence and relative quantity of AlyVMI and AlyVMII in the cells. One milliliter samples at each time point were centrifuged at 8000 rpm. Culture supernatant was carefully removed and stored at -20°C until tested for alginate lyase activity and reducing sugar content. The pelleted cells were stored at -20°C until total protein was isolated. Finally, cell samples from each culture were collected from which total RNA was isolated to monitor gene expression using real-time RT PCR. Eight milliliters of culture were removed 2 hours after inoculation, 5 ml at 4 h, 3 ml at 6 h, 2 ml at 8 h and 1 ml at 10, 12, 15 and 24 h after inoculation. Samples were collected in RNAse free microcentrifuge tubes and centrifuged at 8000 rpm for 2 min. Culture supernatant was discarded and the cells stored at -20°C until total RNA was extracted.



### 4.3.4. Real-time RT PCR

Total RNA was isolated from frozen *Vibrio midae* SY9 cells collected at each time point of the growth experiment as described in Appendix B.12. Total RNA was treated with DNAse I (Promega) as described in Appendix B.12. Two micrograms of total RNA were separated on a 1.2% formaldehyde/MOPS agarose gel (Appendix B.13) to determine the integrity of the RNA. Total RNA was assessed for traces of genomic DNA by PCR amplification using primers

specific for the *Vibrio midae* SY9 16S rRNA gene (Table 4.2). Amplification conditions are described in Appendix C.5. First strand cDNA synthesis was performed on 5 µg of total RNA using random hexamers and the ImPromII$^{tm}$ Two Step Reverse Transcriptase Kit (Promega) in a total volume of 40 µl as described in Appendix B.14. The resulting cDNA was stored at -70°C until used as a template for real-time RT PCR.

Real-time RT PCR primers specific for *alyVMI*, *alyVMII*, *alyVMIII* or *alyVMIV* were designed using DNAMAN Version 4.13 (Lynnon Biosoft), FastPCR Version 5.3.5 (Ruslan Kalendar, Primer Digital LTD) and PerlPrimer Version 1.1.12 (Owen Marshall). Primer sequences and amplicon sizes are listed in Table 4.2. *V. midae* SY9 genomic DNA was isolated as described in Appendix B.8. The resulting DNA was subjected to PCR amplification using the primers described in Table 4.2. PCR conditions are described in Appendix C.8. PCR products were separated on a 2% TAE agarose gel (Appendix B.5) to verify product size and reaction specificity.



**Table 4.2.** Real-time RT PCR primer sequences and expected amplicon sizes.

| Gene | Primer | Primer sequence (5'-3') | Amplicon (bp)[a] | Ta[b] (°C) | Reference |
|---|---|---|---|---|---|
| *alyVMI* | RTF5 | CTTTCTCAAGCCTACGATAAAG | 267 | 55 | This study |
| | RTR5 | GTTTACCCAGCGACAGAAG | | | This study |
| *alyVMII* | RTF7 | CAACACCAGCCGTGACTC | 307 | 58 | This study |
| | RTR7 | GTCCAGTTTCAAGCCGTAAAG | | | This study |
| *alyVMIII* | RTF2 | CCACACCCTTTGTTACG | 279 | 53 | This study |
| | RTR2 | GTAGCTCAGGAGGTGCTG | | | This study |
| *alyVMIV* | RTF6 | CTGCCACCTTCGTCATAG | 288 | 55 | This study |
| | RTR6 | CAAAGATGATGAGCCAATTC | | | This study |
| 16S rRNA | 16SF | GAGTACGGTCGCAAGA | 246 | 53 | Huddy (2010) |
| | 16SR | CGCTGGCAAACAAGGA | | | Huddy (2010) |

[a] bp, base pairs; [b] Ta, annealing temperature

Real-time RT PCR was performed in triplicate using cDNA generated from *V. midae* SY9 sampled at each time point of the growth experiment as template. SYBR Green I was used as a fluorescent detection dye and reactions were performed in a Rotor-Gene 3000 (Qiagen). The reaction mixture contained 1 µl cDNA, 400 nM forward primer, 400 nM reverse primer, 0.25 µl 50x SYBR Green I (Quantace) and 6.25 µl 2x SensiMix (Quantace; contains reaction buffer, 3mM $MgCl_2$, dNTPs, Heat-Activated Taq DNA Polymerase and stabilizers) in a total of 12.5 µl. A separate master mix was used for each primer pair. Two microlitres of cDNA from each sample of the growth experiment were pooled and serially diluted (1:10 for the reference gene and 1:3 for the genes of interest) to prepare calibration curve standards to be used to determine PCR reaction efficiency. The calibration curve standards were amplified from two independent dilution series in each real-time run. No template controls (NTCs), where $dH_2O$ was substituted for cDNA template, were included in every real-time run.

PCR reaction conditions were as follows: 1 cycle of denaturation at 95°C for 10 min; 40 cycles of 95°C for 10 sec, annealing temperature as per Table 4.2 for 15 sec, 72°C for 20 sec; and 1 final elongation cycle at 72°C for 45 sec. Amplified products were detected by acquiring fluorescent data at the end of the 72°C elongation step of every cycle. Amplification product dissociation analysis was performed after the PCR reactions were completed: the temperature was increased by 1°C from 72°C to 95°C holding for 5 sec at each step. A dissociation curve was constructed using Rotor-Gene 600 software, Version 1.7.87 (Qiagen), where the first derivative of the fluorescence data was plotted against temperature.

Fluorescent threshold ($C_T$) values were determined for samples and standards using quantitative analysis on the Rotor-Gene 6000 software. Reaction efficiencies were calculated from the calibration curves according to the equation: efficiency = $10^{(-1/slope)}$ -1. An efficiency of 1 indicates a doubling of product in each PCR cycle. Quantitative data was analyzed using the two standard curve method (Morrison *et al*. 1998). The data was normalised to a reference gene, 16S rRNA, and to a calibrator sample, the 2 hour time course sample, to allow comparison between the four genes of interest. Error bars represent the standard error of the mean.

### 4.3.5. Total cellular protein analysis

The intracellular quantity of AlyVMI and AlyVMII in *Vibrio midae* SY9 cells collected at each time point of the growth experiment was determined using an indirect ELISA assay.

#### 4.3.5.1. Total protein isolation

Total protein was isolated from the frozen cell samples collected at various time points during the *Vibrio midae* SY9 growth experiment. Frozen cells were thawed by resuspending them in 1 ml lysis buffer containing 50 mM sodium phosphate pH 8.0, 300 mM NaCl and no imidazole. Lysozyme was added to a final concentration of 1 mg/ml and the cells incubated on ice for 30 min. The cells were sonicated (Misonix sonicator 3000 fitted with a mirotip) with twelve 15 sec bursts at power setting 4 (9 W) and a 15 sec cooling period between bursts. The samples were centrifuged at 15 000 rpm for 15 min at 4°C. The supernatant fractions, representing the cell lysates, were retained. The total protein concentration of each cell lysate was determined using the Bradford method (Appendix B.16.2).

#### 4.3.5.2. Indirect ELISA assays

AlyVMI and AlyVMII were purified from *E. coli* BL21 (DE3) pLysS cells transformed with either pETalyVMI or pETalyVMII, respectively, and quantitated as described in Chapter 3, Section 3.3.6. Anti-AlyVMI and anti-AlyVMII polyclonal antibodies were precipitated from bleed 8 immune sera using the polyethylene glycol (PEG) method as described in Chapter 3, Section 3.3.10. Anti-AlyVMI and anti-AlyVMII PEG-precipitated antibodies were pre-absorbed against *E. coli* cell lysates containing AlyVMII or AlyVMI respectively as described in Chapter 3, Section 3.3.10 except that 10 cm x 5 cm nitrocellulose membranes were incubated with 5 ml of *E. coli* cell lysates and that PEG precipitated anti-AlyVMI and anti-AlyVMII antibodies were diluted 1:100 in blocking solution.

The indirect ELISA assay described by Ausubel *et al*. (1989, unit 11.4) was modified and optimised to detect AlyVMI and AlyVMII in the *Vibrio midae* SY9 cell lysates. The wells of two 96-well flat-bottomed polysorp microtitre plates (Nunc) were coated with 5 µg of total protein from each time point (in triplicate) of the growth experiment in 1x PBS in a total of 100 µl. The microtitre plates were covered with plastic wrap and incubated at 4°C overnight. Excess antigen was removed and the wells washed three times with 1x PBS.

To prevent non-specific binding of the primary antibody to the microtitre plate, the wells were blocked with 200 µl 1% BSA in 1x PBS at 37°C for 1 h. Microtitre plates were covered with plastic wrap and placed in a humid chamber when incubated at 37°C to prevent evaporation. Excess blocking solution was removed and the wells washed three times with 1x PBS. One hundred microlitres of pre-absorbed anti-AlyVMI antibodies were added to the wells of one of the microtitre plates. Pre-absorbed anti-AlyVMII antibodies were diluted 1:4 in 1x PBS and 100 µl added to the wells of the second microtitre plate. The microtitre plates were incubated with primary antibody at 37°C for 1 h. Excess antibody was removed and the wells washed three times with 1x PBS. Alkaline phosphatase conjugated goat anti-rabbit secondary antibody (Sigma) was diluted 1:5000 in 1x PBS. One hundred microlitres of secondary antibody was added to each well of the microtitre plates and incubated at 37°C for 1 h.

Excess secondary antibody was removed and the wells were washed three times with 1x PBS. The wells were equilibrated with two 200 µl washes of equilibration buffer. One hundred microlitres of freshly prepared substrate, 1 mg/ml 4-nitrophenyl disodium-orthophospate (Merck) in equilibration buffer, was added to the wells. After 4 hours incubation at room temperature, absorbance of the microtitre plates was read at 405 nm on a Titretek Multiscan Plus spectrophotometer. A negative control accounting for non-specific interactions of the secondary antibody with sample proteins was prepared for each time point sample by adding 1x PBS to the well instead of primary antibody. Corrected absorbance values were obtained by subtracting the absorbance of the control well from the absorbance of the corresponding antigen containing wells.

A standard curve of purified AlyVMI or AlyVMII was prepared by performing doubling dilutions of purified protein in 1x PBS. The wells of a polysorp microtitre plate were coated with dilutions of either AlyVMI or AlyVMII in triplicate as well as a negative control for each dilution. The microtitre plate coated with AlyVMI was incubated with pre-absorbed anti-AlyVMI antibody. Similarly, the microtitre plate coated with AlyVMII was incubated with pre-absorbed anti-AlyVMII antibody. 1x PBS was added to the negative control wells instead of primary antibody. Corrected absorbance values were averaged and plotted against known concentrations of purified antigen to produce a calibration curve. Corrected absorbance values of the experimental samples were compared to the linear region of the calibration curve. The concentrations were normalised to the 2 hours post-inoculation sample in order to compare the results of the two proteins. Error bars represent the standard error of the mean.

### 4.3.6. Enzyme assays

Alginate lyase activity of the culture supernatant and cell lysates was determined using the TBA assay. Reducing sugar content of the culture supernatant was determined using the DNS reducing sugar assay.

#### 4.3.6.1. Determination of alginate lyase activity

The thiobarbituric acid (TBA) assay (Weissbach and Hurwitz, 1959) was performed on culture supernatants and cell lysates of each time point sample to determine the alginate lyase activity of *Vibrio midae* SY9 cells cultured without and supplemented with 0.2% glucose. The assay was performed in triplicate as described in Appendix B.6. One unit of alginate lyase activity is expressed as µg malonaldehyde equivalents released per 1 ml of culture supernatant in 30 minutes at 37˚C. Error bars represent the standard error of the mean.

#### 4.3.6.2. Determination of reducing sugar content

The dinitrosalicylic acid (DNS) assay (Miller, 1958; Sumner and Sisler, 1944) was performed on the culture supernatant of each sample to determine the amount of reducing sugar present at each time point of the *V. midae* SY9 growth experiment. Fifty microlitres of sample was added to 150 µl DNS reagent and mixed. Reactions were boiled for 5 min and rapidly cooled on ice. Eight hundred microlitres of $dH_2O$ was added to each reaction and mixed. Absorbencies were measured at 510 nm on a Beckman DU530 spectrophotometer. The assay was performed in triplicate for each sample. A negative control was prepared by adding 50 µl $dH_2O$ to 150 µl DNS reagent and performing the assay as described above. Corrected absorbance values were obtained by subtracting the absorbance of the negative control from the absorbance values of the samples. A calibration curve was prepared by plotting corrected absorbencies against known concentrations of glucose. Corrected absorbance values of experimental samples were compared to the calibration curve. Reducing sugar is expressed as mM glucose per 1 ml of culture supernatant. Standard errors represent the standard error of the mean.

### 4.3.7. Statistical analysis

All data is presented as means and standard errors. For comparisons of two means, an unpaired Student t-test was performed. For multiple comparisons, a two-way analysis of variance (ANOVA) was performed. When the result of the ANOVA was significant, the Tukey test was used as a post-hoc test to test for significant differences between sample means. When data were not normally distributed, a Mann-Whitney rank sum test or Kruskal-Wallis ANOVA on ranks test was performed. Spearman rank order correlations were performed to determine the strength of association between reducing sugar present in the culture medium, alginate lyase activity and absorbance of cultures at 600 nm. Prior to statistical analysis, the real time data and DNS reducing sugar assay data were $\log_{10}$ transformed, except for the correlation analysis. Significant differences were established at $P<0.05$.

## 4.4. RESULTS

### 4.4.1. *Vibrio midae* SY9 growth experiment

For the first 8 hours after inoculation, *Vibrio midae* SY9 grew similarly in alginate media without and supplemented with glucose (Figure 4.1). At 10, 12, 15 and 24 hours after inoculation, *V. midae* SY9 cultured in alginate media supplemented with glucose reached a significantly higher ($P$<0.05) absorbance at 600nm than *V. midae* SY9 cultured in alginate media without glucose. Twenty four hours after inoculation, *V. midae* SY9 reached an absorbance of 4.02 and 5.34 at 600 nm when cultured in alginate media without and supplemented with glucose, respectively, which is significantly different ($P$<0.05).

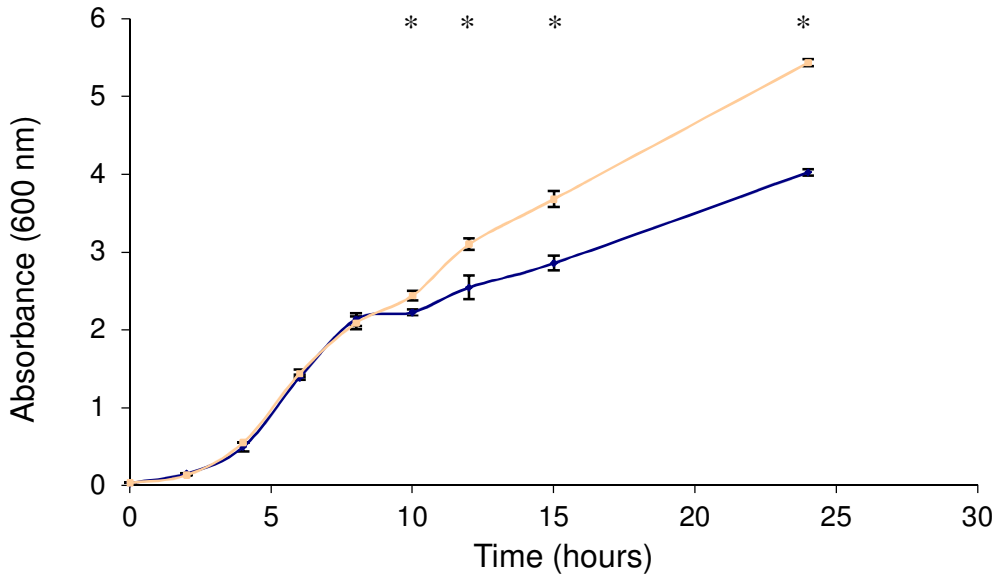



**Figure 4.1.** Growth profile of *Vibrio midae* SY9 cultured in alginate media without (■) and supplemented with (■) glucose. Absorbance of cultures was monitored at 600 nm for the duration of the growth experiment. Error bars represent the standard error of the means for experiments performed in triplicate. The absence of error bars indicates that the standard error is smaller than the symbol size. *($P$<0.05, Student t-test) represents a significant difference between means. There was a significant difference ($P$<0.05) between the absorbance at 600 nm of *V. midae* SY9 cultured in alginate media without and supplemented with glucose at 10, 12, 15 and 24 hours after inoculation as determined by a Student t-test.

### 4.4.2. Real-time RT PCR

Real-time RT PCR using SYBR Green I chemistry was used to quantify the presence of *alyVMI*, *alyVMII*, *alyVMIII* and *alyVMIV* mRNA transcripts in *Vibrio midae* SY9 cells at each time point of the growth experiment.

16S rRNA gene transcription was validated as a reference for quantitation of *V. midae* SY9 alginate lyase gene transcripts during growth of the bacterium in new alginate media without or supplemented with glucose by plotting $C_T$ values against time (Appendix D, Figure D.1.1). There is no significant difference ($P>0.05$) in $C_T$ values across time within each treatment. There was only a significant difference ($P<0.05$) in $C_T$ values between the two treatments at 8 hours post-inoculation.

Calibration curves for each gene of interest as well as the reference gene were generated using serial dilutions of pooled cDNA (Appendix D, Section D.2). The reaction efficiency and the correlation coefficient ($R^2$ value) of the calibration curves are listed in Table 4.3. A reaction efficiency of 1 indicates a doubling of amplification products in each PCR cycle. Dissociation curves were plotted for each real-time run (Appendix D, Section D.3). Each primer set yielded a single dissociation peak indicating a single product was amplified. The melt temperatures of the dissociation peaks are listed in Table 4.3. No products were formed in the no template controls (NTCs).



**Table 4.3.** Real-time RT PCR reaction efficiencies, calibration curve correlation coefficients and dissociation curve peaks for each gene amplified in the expression experiment.

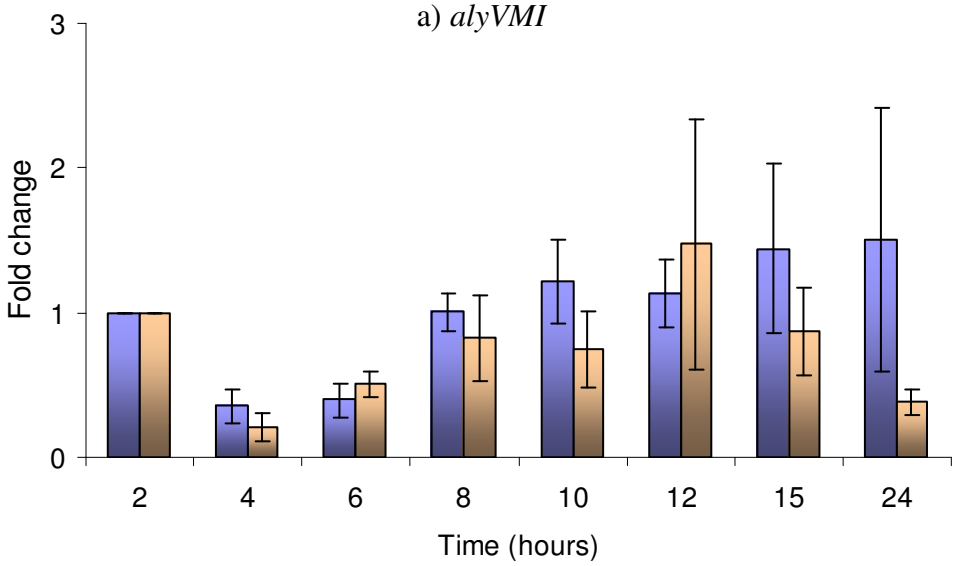
| Gene | Reaction Efficiency | Correlation coefficient ($R^2$) | Dissociation peak (°C) |
|---|---|---|---|
| *alyVMI* | 0.81 | 0.997 | 86.5 |
| *alyVMII* | 1.08 | 0.998 | 85.0 |
| *alyVMIII* | 0.93 | 0.985 | 82.5 |
| *alyVMIV* | 0.97 | 0.982 | 83.0 |
| 16S rRNA | 0.96 | 0.998 | 86.3 |

Real-time RT PCR data was analyzed using the two standard curve method (Morrison *et al*. 1998) and represented as a fold change relative to the two hours post-inoculation sample (Figure 4.2). There were no significant differences ($P$>0.05) in transcript levels at any time point between treatments for any of the four genes tested. The real-time RT PCR data was also analyzed using the Pfaffl method (Pfaffl, 2001; Pfaffl *et al*. 2002). This method of analysis (data not shown) also revealed that there were no significant differences ($P$>0.05) in transcript levels at any time point between treatments for any of the four genes tested. This suggests that there is no significant regulation of the alginate lyase gene transcripts when *Vibrio midae* SY9 is cultured in alginate media without or supplemented with glucose.

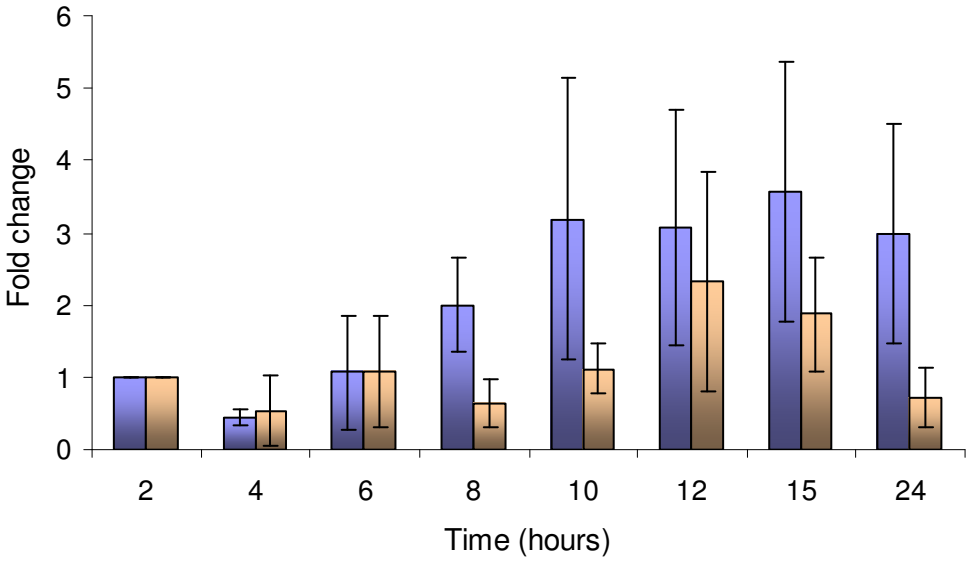
However, there were still small changes in mRNA transcript levels with time, the trend of which was similar for the four alginate lyase genes in each treatment but differed between the two treatments (Figure 4.2). In cells cultured without glucose added to the growth medium, there was a decrease in transcript levels from 2 to 4 hours post-inoculation followed by a steady increase to a maximum fold change at 24 hours post-inoculation (Figure 4.2). Alginate lyase gene *alyVMII* transcript levels differed by reaching a maximum fold change at 15 hours post-inoculation before decreasing from 15 to 24 hours post-inoculation. The maximum fold change of *alyVMI* was 1.50, of *alyVMII* was 3.55, of *alyVMIII* was 1.57 and of *alyVMIV* was 2.00.

In cells cultured with glucose added to the growth medium, there was a decrease in transcript levels from 2 to 4 hours post-inoculation followed by a steady increase to a maximum fold change at 12 hours post-inoculation after which transcript levels decreased to 24 hours post-inoculation. The maximum fold change of alyVMI was 1.47, of alyVMII was 2.32, of alyVMIII was 1.93 and of alyVMIV was 2.68. The large error bars indicate that the transcript levels were different between the three biological replicate cultures of each treatment.

a) *alyVMI*




b) *alyVMII*



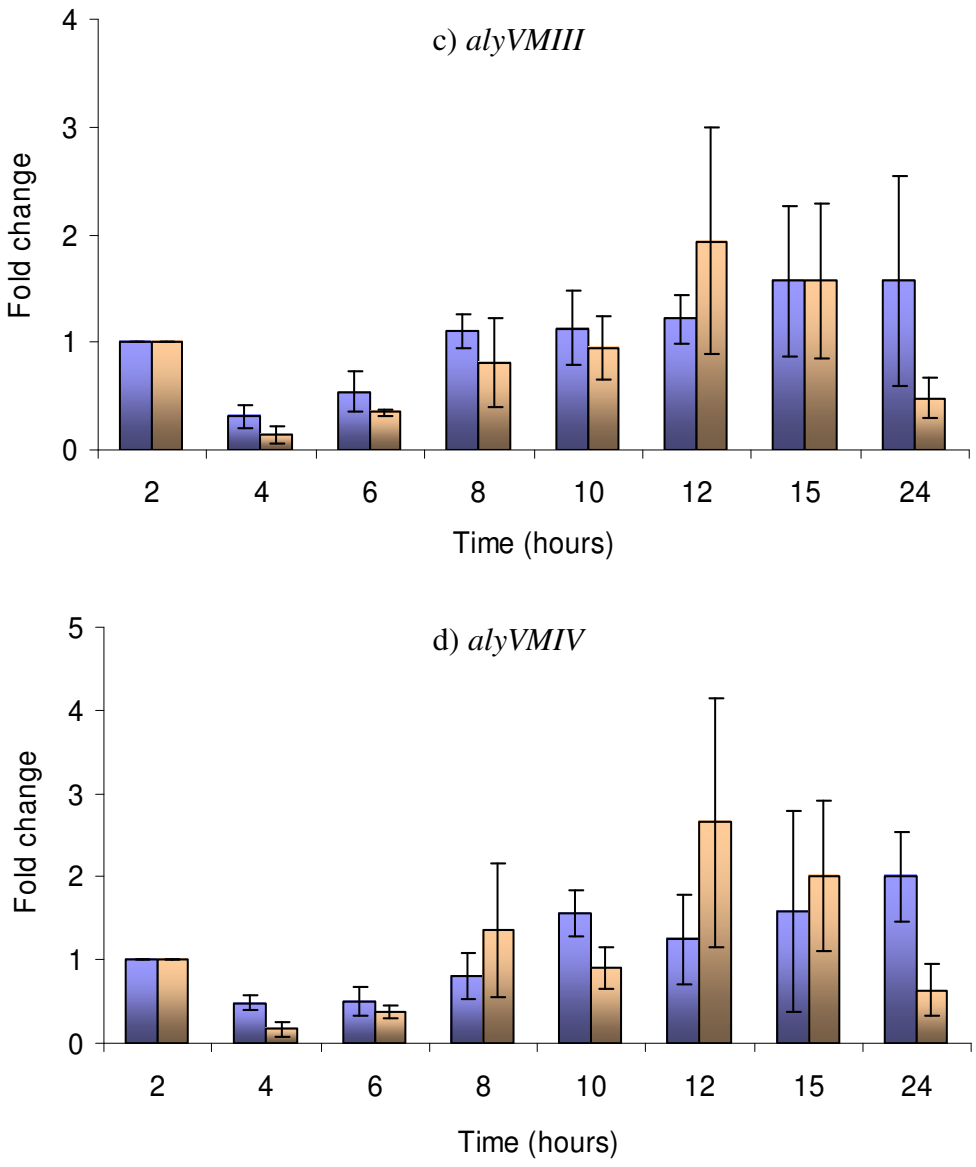



**Figure 4.2.** Real-time RT PCR analysis of *alyVMI* (a), *alyVMII* (b), *alyVMIII* (c) and *alyVMIV* (d) transcripts when *Vibrio midae* SY9 was cultured in alginate media without (■) and supplemented with (■) glucose. Real-time RT PCRs were performed in triplicate on a Rotor-Gene 3000 using SYBR Green I as the fluorescent detection chemistry. The Y-axis represents the fold change relative to the 2 hour post-inoculation sample. Histograms represent the mean of three experiments and error bars represent the standard error of the mean. There were no significant differences ($P$>0.05) at any time point between treatments as determined by a two-way ANOVA.

### 4.4.3. Quantitation of AlyVMI and AlyVMII present in *V. midae* SY9 cells

An indirect ELISA assay was used to quantitate the intracellular levels of AlyVMI and AlyVMII present in the *Vibrio midae* SY9 growth experiment samples. Results were normalised to the 2 hour post-inoculation samples. Except for 4, 6 and 24 hours post-inoculation where there was a greater amount ($P<0.05$) of AlyVMI in *V. midae* SY9 cells cultured without glucose compared to the cells cultured with glucose, there was no significant difference ($P>0.05$) in the amount of AlyVMI present in cells between treatments (Figure 4.3). There was no significant difference ($P>0.05$) in the amount of AlyVMII present in cells at any time point between treatments. The greatest fold change of AlyVMI (1.5) and AlyVMII (1.26) occurred in cells cultured without glucose at 24 and 8 hours post-inoculation, respectively. Therefore, the amount of AlyVMI and AlyVMII present in *V. midae* SY9 cells remained approximately constant under the experimental conditions tested.

### 4.4.4. Enzyme assays

Alginate lyase activity of the culture supernatants and cell lysates, as well as the reducing sugar content of the culture supernatants, were determined for each time point of the *Vibrio midae* SY9 growth experiment using the TBA alginate lyase and DNS reducing sugar assays, respectively.

#### 4.4.4.1. Alginate lyase activity of the culture supernatant and cell lysates

Two hours post-inoculation, alginate lyase activity was barely detected in the culture supernatant of *Vibrio midae* SY9 grown in alginate media without glucose as determined by the TBA assay (Figure 4.4 a). There was a steady increase of activity with time, except for a slight decrease at 15 hours post-inoculation, with maximal activity at 24 hours post-inoculation. There was no alginate lyase activity in the culture supernatant of *V. midae* SY9 cells grown in alginate media with glucose at 2 and 4 hours post-inoculation (Figure 4.4 a). Activity was first detected at 6 hours post-inoculation after which it increased steadily to a maximum activity at 24 hours post-inoculation. There was approximately twice as much alginate lyase activity in the culture supernatant of cells cultured without glucose at 24 hours post-inoculation than in the culture

supernatant of cells cultured in media supplemented with glucose. There was significantly ($P$<0.05) greater activity in the culture supernatant of cells cultured without glucose than in the culture supernatant of cells cultured with glucose from 4 to 24 hours post-inoculation (Figure 4.4a).

Alginate lyase activity of cell lysates of *V. midae* SY9 cells cultured without glucose was not detected 2 hours post-inoculation (Figure 4.4 b). Activity increased steadily from 4 hours to 8 hours post-inoculation, remained approximately constant from 8 to 12 hours post-inoculation and increased again from 12 to 24 hours post-inoculation. Maximum alginate lyase activity was at 24 hours post-inoculation in cells cultured without glucose. Alginate lyase activity of cell lysates of *V. midae* SY9 cells cultured with glucose added to the culture medium was not detected 2 hours after inoculation and barely detected 4 hours after inoculation (Figure 4.4 b). Activity was detected at 6 hours post–inoculation in cell lysates and increased steadily with time to a maximum activity at 24 hours post-inoculation. Alginate lyase activity was significantly ($P$<0.05) higher in cell lysates of *V. midae* SY9 cells cultured without glucose than cells cultured with glucose at 4, 6, 8 and 12 hours post-inoculation. However, cell lysates obtained from cells sampled from the two treatments had the same maximum activity 24 hours after inoculation (Figure 4.4 b).

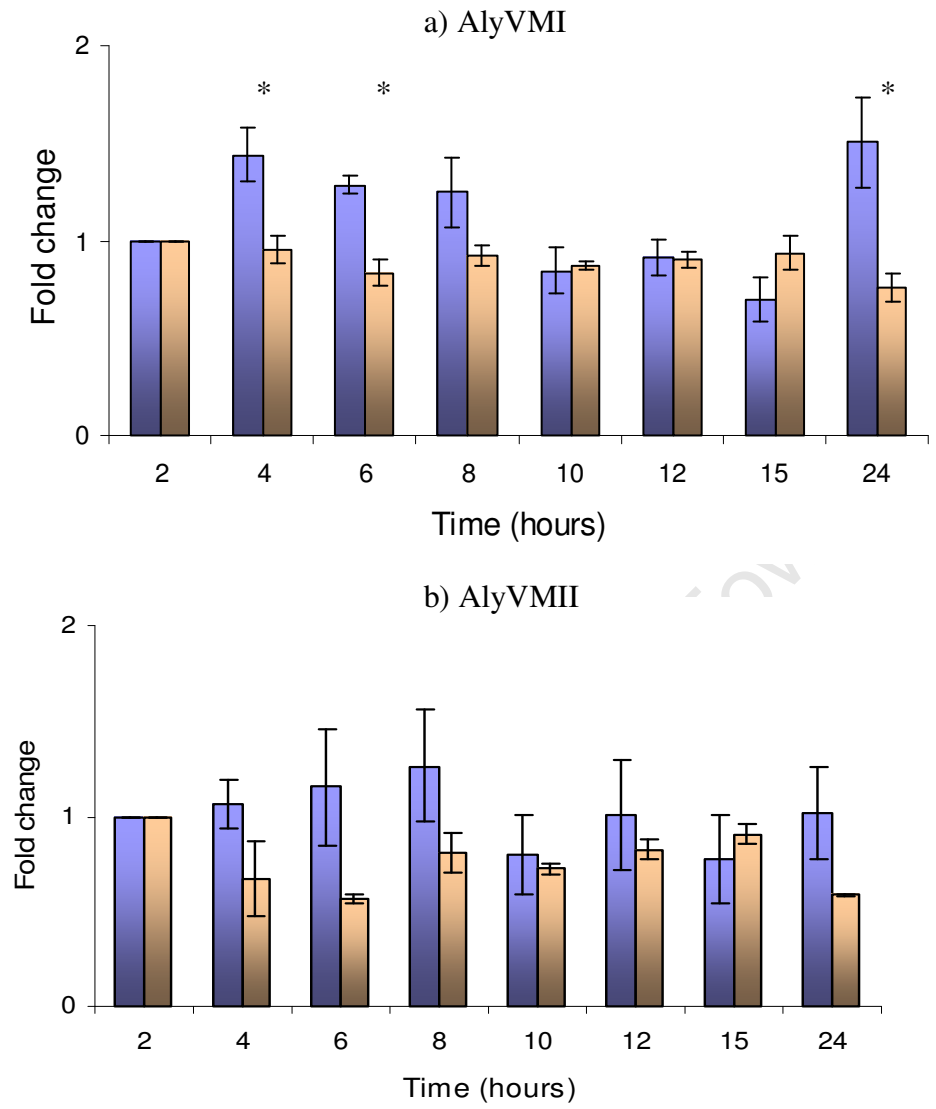



**Figure 4.3.** Quantitation of AlyVMI (a) and AlyVMII (b) present in *V. midae* SY9 cells during growth in alginate media without (■) and supplemented with (■) glucose. Indirect ELISA assays were performed in triplicate using anti-AlyVMI or anti-AlyVMII antibodies. Histograms represent the mean of three experiments and error bars represent the standard error of the mean. *($P$<0.05, Student t-test) represents a significant difference between means.

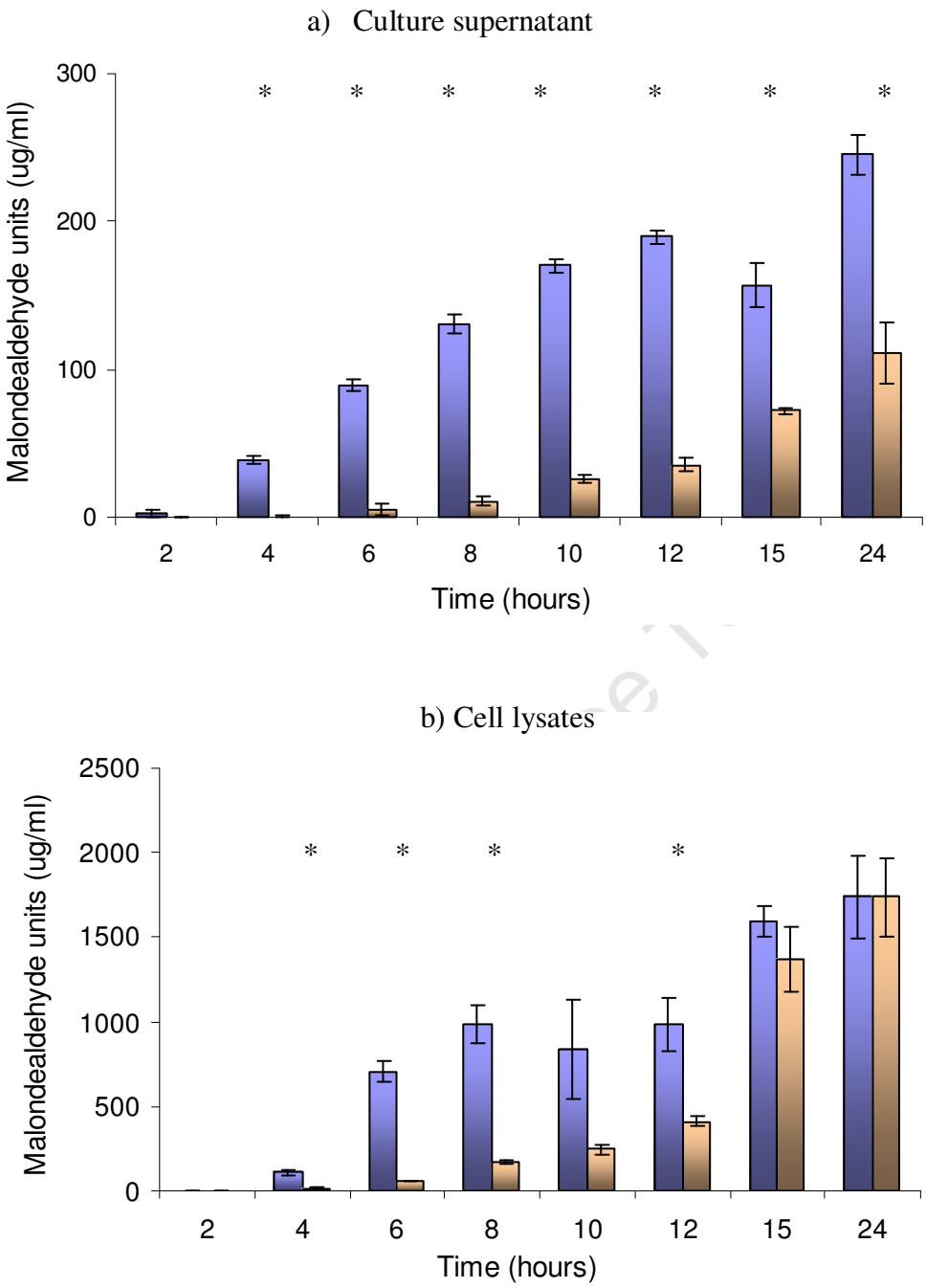



**Figure 4.4.** Alginate lyase activity in the culture supernatant (a) and cell lysates (b) of *V. midae* SY9 cells cultured in alginate media without (■) and supplemented with (■) glucose. Histograms represent the mean of three experiments and error bars represent the standard error of the mean. *($P$<0.05; Student t-test) represents a significant difference between the means.

#### 4.4.4.2. Reducing sugar content of the culture supernatant

Two hours after inoculation, the amount of reducing sugar present in the culture supernatant of *Vibrio midae* SY9 cultured in new alginate media without glucose was low (36.47 mmol/ml) as measured by the DNS reducing sugar assay (Figure 4.5). The amount of reducing sugar present in the culture supernatant increased steadily throughout the growth experiment. The maximum amount of reducing sugar (54.59 mmol/ml) was present 24 hours after inoculation and was ~1.5 times the amount present at 2 hours after inoculation.

Two hours after inoculation, the amount of reducing sugar present in the culture supernatant of *Vibrio midae* SY9 cultured in new alginate media supplemented with glucose was high (433.33 mmol/ml, Figure 4.5). The amount of reducing sugar present in the culture supernatant decreased from 2 to 8 hours post-inoculation and remained approximately constant from 8 to 24 hours post-inoculation. At 24 hours post-inoculation there was ~4.6 times less reducing sugar present in the culture supernatant than at 2 hours post-inoculation.

There was a significant difference ($P<0.05$) in the amount of reducing sugar present in the culture supernatant between the two treatments throughout the growth experiment (Figure 4.5). At two and 24 hours post-inoculation, the amount of reducing sugar present in the culture supernatant of cells cultured in the presence of glucose was ~11.9 and ~1.7 times more than that of cells cultured in the absence of glucose, respectively.

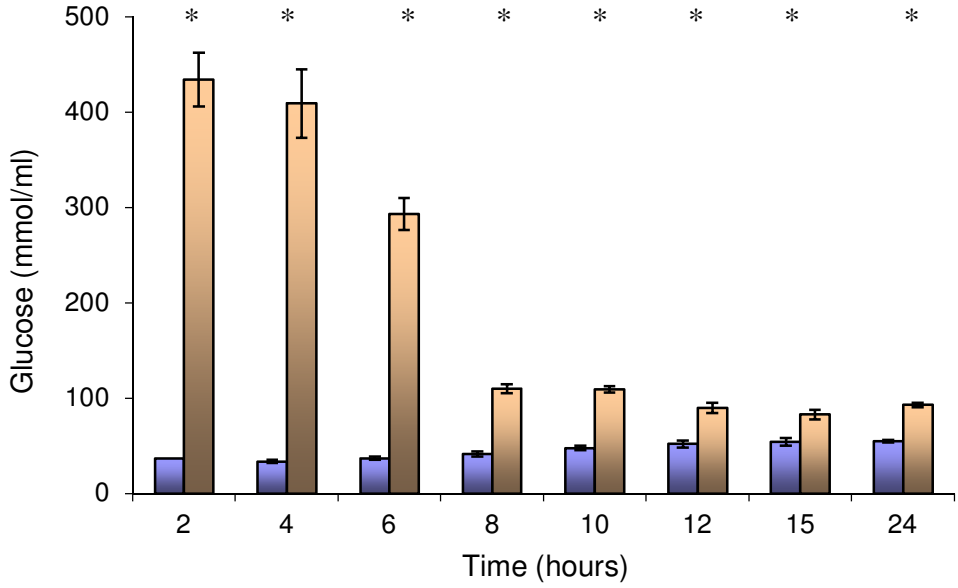



**Figure 4.5.** Reducing sugar present in the culture supernatant of *V. midae SY9* cells during growth in alginate media without (■) and supplemented with (■) glucose. Histograms represent the mean of three experiments and error bars represent the standard error of the mean. *($P$<0.05; Student t-test) represents a significant difference between the means.



#### 4.4.4.3. Correlation analysis

A correlation analysis was conducted to determine the strength of the association between the presence of glucose in the growth media, alginate lyase activity in the culture supernatant, alginate lyase activity in the cell lysates and the optical density of the culture over the course of the growth experiment. For cells cultured in alginate media supplemented with glucose, a Spearman rank order correlation revealed a significant negative correlation between the amount of reducing sugar present and alginate lyase activity in the culture supernatant ($r = -0.619$, $P<0.05$, $n = 24$), alginate lyase activity in the cell lysates ($r = -0.920$, $P<0.05$, $n = 24$) and absorbance of the culture at 600 nm ($r = -0.915$, $P<0.05$, $n = 24$). This indicates that as the amount of reducing sugar present in the media decreases, alginate lyase activity and absorbance of the culture at 600 nm (i.e. cell growth) increases. There was a significant positive correlation between the absorbance of the culture at 600 nm and alginate lyase activity in the culture

supernatant (r = 0.440, $P<0.05$, n = 24) and cell lysates (r = 0.972, $P<0.05$, n = 24). This indicates that as the absorbance of the culture at 600 nm increases, alginate lyase activity also increases. There was also a significant positive correlation between alginate lyase activity in the culture supernatant and the cell lysates (r = 0.494, $P<0.05$, n = 24).

For cells cultured in alginate media without glucose, a Spearman rank order correlation revealed a significant positive correlation between the amount of reducing sugar present and alginate lyase activity in the culture supernatant (r = 0.835, $P<0.05$, n = 24), alginate lyase activity in the cell lysates (r = 0.806, $P<0.05$, n = 24) and absorbance of the culture at 600 nm (r = 0.891, $P<0.05$, n = 24). This indicates that the amount of reducing sugar present in the media, alginate lyase activity and absorbance of the culture at 600 nm (i.e. cell growth) increase together with time. There was a significant positive correlation between the absorbance of the culture at 600 nm with alginate lyase activity in the culture supernatant (r = 0.912, $P<0.05$, n = 24) and cell lysates (r = 0.877, $P<0.05$, n = 24). There was also a significant positive correlation between alginate lyase activity of the culture supernatant and cell lysates (r = 0.800, $P<0.05$, n = 24).

## 4.5. DISCUSSION

Bacteria live in an ever changing environment and need to be able to cope with changes in temperature, nutrient and water availability as well as the presence of toxic substances (Ramos *et al*. 2001, 2005). Rapid and adaptive responses are essential for survival. Environmental signals are sensed on the outside of the cell by regulatory proteins and converted to a cellular response which may be in the form of gene transcription, protein synthesis, regulation of enzyme activity, changes in behaviour such as motility, or other processes (Postma *et al*. 1993; Ramos *et al*. 2001, 2005). Regulation at the transcriptional level is important in the response to environmental signals, but regulation at the translational and post-translational levels are just as important (Ramos *et al*. 2001).

Carbon catabolite repression (CCR) is an important regulatory mechanism that allows bacteria to efficiently use available substrates. Diverse mechanisms of CCR exist and each group of bacteria has developed its own way of achieving CCR (Görke and Stülke, 2008). The phospho*enol*pyruvate:sugar phosphotransferase (PTS) system is a multi-protein phosphorelay system that is an important generator of signals for regulatory events and is involved in the simultaneous phosphorylation and transport of carbohydrates across the cytoplasmic membrane, chemotaxis toward carbon sources and the regulation of other metabolic pathways (Görke and Stülke, 2008; Postma *et al*. 1993; Stülke and Hillen, 1999). Gram-positive and Gram-negative bacteria make use of the PTS system to convert signals to a response although different proteins are used as the effector molecules in each group of bacteria (Gunnewijk *et al*. 2001). In *Pseudomonas putida*, CCR occurs at the level of translation (Görke and Stülke, 2008). In the presence of a preferred substrate, a global regulatory protein binds to the 5' end of mRNA transcripts coding for activator proteins that activate gene expression for the utilisation of a less preferred substrate and in this way prevents their translation (Görke and Stülke, 2008).

Momma *et al*. (1999) found that the alginate lyases of *Sphingomonas* sp A1 are induced in the presence of alginate and induction is repressed in the presence of glucose. However, this was suggested from enzyme activity assays and experiments to determine mRNA transcript or protein levels were not conducted. Furthermore, cells were cultured in media containing either alginate

or glucose, not both carbon sources. *Saccharophagus degradans* 2-40 is able to utilise 10 complex carbohydrates as sole carbon sources (Ensor *et al.* 1999). When *S. degradans* 2-40 was cultured with glucose as a sole carbon source, agarase, alginate lyase, carboxymethylcellulase, carrageenanase, chitinases, laminarinase, pectinase, pullalanase and xylanase activities were not detected. Only amylase activity was detected (Ensor *et al*. 1999). The authors showed that the repression of the agarase system was relieved after the addition of cyclic adenosine monophosphate (cAMP) to the culture medium. Agarase activity was also detected in stationary phase cultures with glucose as a sole carbon source (Stosz, 1994; Whitehead *et al*. 2001). It was suggested that glucose did not fully repress agarase activity and that is was possible that some of the agarases of *S. degradans* 2-40 were repressed by glucose but not all of them. Again, mRNA transcript and protein levels were not investigated and cells were cultured with a single carbon source rather than a combination of carbohydrates.

In the present study, *Vibrio midae* SY9 was cultured in alginate media without or supplemented with glucose for 24 hours. Real-time RT PCR was performed on samples collected during the growth experiment to determine transcript levels of the four alginate lyase genes. Quantitative indirect ELISA assays were performed to determine the presence and relative quantity of intracellular AlyVMI and AlyVMII. Alginate lyase activity of the culture supernatant and cell lysates was determined using the thiobarbituric acid (TBA) assay and the dinitrosalicylic acid (DNS) assay was used to determine the amount of reducing sugar present in the culture supernatant. Antibodies were not raised against AlyVMIII or AlyVMIV (Chapter 3, Section 3.5). Therefore, it was not possible to determine either the intracellular levels of AlyVMIII or the intracellular and extracellular levels of AlyVMIV during the course of the growth experiment.

Analysis of the real-time data suggested that the mRNA transcripts of the four alginate lyase genes were not significantly ($P>0.05$) regulated at any time point between treatments (Figure 4.2). The indirect ELISA data showed that the intracellular levels of AlyVMI and AlyVMII also did not change significantly ($P>0.05$) with time between each treatment, except for AlyVMI which differed significantly ($P<0.05$) between treatments at 4, 6 and 24 hours post-inoculation (Figure 4.3). There was, however, a significant ($P<0.05$) change in alginate lyase activity in the culture supernatant and cell lysates between treatments (Figure 4.4). There was also a significant

difference ($P$<0.05) in the amount of reducing sugar present in the culture supernatant between the two treatments at each time point (Figure 4.5). A decrease in the amount of reducing sugar present in the culture supernatant of *V. midae* SY9 cultured in alginate media supplemented with glucose correlated with an increase in absorbance at 600 nm (i.e. cell growth) and with an increase in alginate lyase activity in the culture supernatant and in cell lysates.

Since the mRNA transcripts and protein levels were not regulated, but there was a change in alginate lyase activity in the culture supernatant and cell lysates in response to glucose in the culture medium, it is possible that the alginate lyase genes of *Vibrio midae* SY9 are regulated post-translationally rather than transcriptionally or translationally in response to glucose in the culture medium.

To the best of our knowledge, post-translational regulation of alginate lyase enzymes has not been reported before. Alginate lyases have been shown to be inducible in the presence of alginate as a sole carbon source (Ensor *et al*. 1999; Gacesa, 1992; Hansen and Nakamura, 1985; Lange *et al*. 1989; Momma *et al*. 1999) and repressed in the presence of glucose as a sole carbon source (Ensor *et al*. 1999; Momma *et al*. 1999). However, these studies did not determine whether the enzymes were induced in the presence of both alginate and glucose. It was also not determined at which level, transcriptional, translational or post-translational, these genes are regulated. The results of the present study show that the alginate lyase genes of *V. midae* SY9 were transcribed and translated when the bacterium was cultured in the presence of glucose and alginate. It is possible that the alginate lyases of *V. midae* SY9 are repressed when only a primary substrate, such as glucose, is present but are induced when alginate, a secondary substrate, is present even when glucose is present. Thus, CCR could still be an important regulatory mechanism in *V. midae* SY9. However, it is possible that *V. midae* SY9 utilises post-translation modification of enzymes as a means of utilising a preferred substrate before utilising a secondary substrate rather than full repression of gene transcription. Indeed, many biological processes are controlled by post-translational modifications of proteins which lead to the activation, inactivation or gain-of-function of proteins (Yamakura and Kawasaki, 2010). Post-translational modifications are reversible and thus able to provide the flexibility and adaptability that is so necessary for a rapid response to a changing environment (Chung *et al*. 2009). An example of a post-

translational modification which alters enzyme activity is the reversible mono-ADP-ribosylation of the nitrogenase complex of the cyanobacterium *Rhodospirillum rubrum*.

The nitrogenase complex of *Rhodospirillum rubrum*; a purple nonsulphur, photosynthetic, nitrogen-fixing bacterium; consists of two proteins: a dinitrogenase which contains the nitrogenase active site and a dinitrogenase reductase which provides reducing power to the dinitrogenase (Liang *et al*. 1991). Activity of the dinitrogenase reductase is post-translationally regulated by reversible mono-ADP-ribosylation in response to the level of light and concentration of nitrogen (Zhang *et al*. 1995, 2003, 2006). Dinitrogenase reductase ADP-ribosyltransferase (DRAT) inactivates dinitrogenase reductase by preventing the flow of electrons within the nitrogenase complex by ADP-ribosylation and dinitrogenase reductase-activating glycohydrolase (DRAG) restores activity by removing the ADP-ribose. Under conditions of excess ammonium ($NH_4^+$) or when cells are shifted from light to dark (energy limitation), dinitrogenase reductase is ribosylated and inactivated by DRAT. When the ammonium source is depleted or the cells are returned to light, the ribose group is removed by DRAG and nitrogenase activity is restored. Both DRAT and DRAG are also subject to post-translational control. The nitrogen regulatory protein, $P_{II}$ and its homologues, regulate the reversible ribosylation of dinitrogenase reductase to control nitrogenase activity (Zhang *et al*. 2003, 2006).

New findings indicate that transcription in *Plasmodium falciparum* is rigid and deviates from the classical models of transcriptional gene regulation (Chung *et al*. 2009). This suggests that post-transcriptional and post-translational mechanisms are important in regulating the life-cycle of the parasite. In humans, the adenosine-uridine binding factor (AUBF) binds to 3' AUUUA motifs of lymphokine and cytokine mRNAs and prevents their degradation by forming stable complexes (Malter and Hong, 1991). AUBF activity was shown to be independent of mRNA transcription and protein synthesis suggesting that pre-existing AUBF is post-translationally modified to an active form. Indeed, AUBF was found to be subject to two levels of post-translational regulation, redox switch and phosphorylation.

In *V. midae* SY9, the enzymes required to utilise a secondary substrate would be inactive while the preferred substrate is assimilated and then activated when required. The advantage of this type of regulation is that there would be no delay in utilising a secondary substrate since the enzymes would already be synthesized. Since *V. midae* SY9 may occur as a free living strain as well as in the digestive tract of *Haliotis midae*, this advantage could be important in the marine environment where nutrients are easily diffused and the bacterium could be unintentionally moved away from the substrate. The disadvantage of this regulation is that it would be energetically expensive to the cell to produce enzymes that are not used immediately and might not be used at all if the substrate diffuses away.

Post-translational modifications may alter enzyme activity, binding affinity, hydrophobicity, protein-protein interactions and subcellular localisations (Chung *et al*. 2009; Yamakura and Kawasaki, 2010). Post-translational modifications such as phosphorylation, methylation, acetylation or glycosylation of a specific peptide or of the active site would cause the alginate lyase enzymes to be inactive. Once de-phosphorylated, the enzymes would be active again. It is also possible that one of these modifications could change the conformation of the protein or active site thereby rendering it inactive. There may be a change in redox potential of the enzyme or active site. There could be an unknown inhibitor of these enzymes that binds to the active site. Such an inhibitor could also cause an allosteric change of the protein. It could even be the removal of a co-factor necessary for activity. Whatever the mechanism causing inactivity, it would need to be temporary and easily reversed when required. Alternatively, the enzymes may require a post-translational modification to be activated. An example is human dermatan sulphate epimerase 1 which requires *N*-glycosylation at four sites to be active (Pacheco *et al*. 2009) and *Saccharomyces cerevisiae* superoxide dismutase 2 which requires the addition of an essential manganese co-factor for activation (Luk *et al*. 2003).

It is important to take into consideration that these experiments do not reflect the natural environment of *Vibrio midae* SY9. The marine environment is nutrient limiting whereas laboratory media is rich in nutrients. It is possible that *V. midae* SY9 would behave differently in its natural environment. However, *V. midae* SY9 could be in a more nutrient rich environment in

the digestive tract of *Haliotis midae* than it would as a pelagic bacterium in the marine environment.

Regulation of the alginate lyase genes of *V. midae* SY9 should be investigated further to validate the hypothesis of post-translational regulation. Carbon catabolite repression by cAMP as seen in *E. coli* should be conclusively excluded. This can be achieved by monitoring alginate lyase mRNA transcript levels, protein levels and activity after the addition of cAMP to cultures grown in alginate media containing glucose. The present gene expression study could be repeated with different substrates to determine whether similar results are obtained. Antibodies against AlyVMIII and AlyVMIV should be raised and used to determine whether the levels of these proteins also remain constant during the expression experiments. Subsequent to this, the mechanism of post-translational modification of the enzymes should be elucidated. Techniques that have been used, singularly and in combination, for the detection of post-translational modifications and modified sites include: one- and two-dimensional PAGE, antibodies specific for the modification or modification site, matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS), tandem mass spectrometry (MS/MS), liquid chromatography (LC)-MS/MS, nanoelectrospray MS/MS, Fourier transform ion cyclotron resonance (FT-ICR) MS, and electron capture dissociation (ECD) FT-ICR MS (Jensen, 2004; Yamakura and Kawasaki, 2010). Enzyme activity studies could also be performed to establish whether a co-factor or an inhibitor exists that could play a role in the activity of these enzymes.

Post-translational regulation of the nitrogenase complex of *R. rubrum* is complex, as described above, and involves a number of proteins and signals. Depending on the modification, the regulatory pathway of *Vibrio midae* SY9 alginate lyases could also be complex and involve intermediate proteins for the addition and removal of the modification. Intermediate proteins and their function could be elucidated and whether they are also post-translationally regulated determined.

Although there was no significant ($P>0.05$) regulation of the four alginate lyase genes, there were still small changes in mRNA transcript levels with time, the trend of which was similar for the four genes in each treatment but differed between the two treatments (Figure 4.2). A similar

trend for *alyVMI* and *alyVMII* expression is not unexpected since the two genes were shown to be co-transcribed (Chapter 2, Section 2.4.5). The expression of *alyVMIII* and *alyVMIV* had a similar trend to the expression of *alyVMI* and *alyVMII*. It is unknown whether these genes are also co-transcribed. Alternatively, it is possible that the actions of the four alginate lyases are required together for the depolymerization of alginate and are therefore transcribed at the same time but not necessarily on the same transcript.

There was approximately twice the amount of alginate lyase activity in the culture supernatant of *Vibrio midae* SY9 cells cultured in alginate media without glucose than in the culture supernatant of cells cultured in alginate media supplemented with glucose at 24 hours post-inoculation (Figure 4.4 a). However, enzyme activity in the cell lysates reached the same level in both media 24 hours after inoculation (Figure 4.4 b). Since AlyVMIV is the only known alginate lyase of *V. midae* SY9 to possess a signal peptide (Chapter 2) it is reasonable to believe that the activity seen in the culture supernatant is attributable to at least AlyVMIV. It is possible that other unknown extracellular alginate lyases of *V. midae* SY9 are also present and contribute to the activity as well. The alginate lyase activity detected in the cell lysates may be a combination of the activity of AlyVMI, AlyVMII and AlyVMIII since they are not exported and remain in the cell (Chapter 2). There may also be other unknown extracellular alginate lyases of *V. midae* SY9 that contribute to the activity seen in the culture supernatant. The reducing sugar content in the media of *V. midae* SY9 cultured in alginate media without and supplemented with glucose increased and decreased with time, respectively (Figure 4.5). An increase in the amount of reducing sugar present in the culture media of cells cultured without glucose may be attributed to reducing sugars produced from the degradation of alginate which had not yet been taken up by the cells.

AlyVMIV contains a signal peptide and was predicted to be located extracellularly (Chapter 2). The proteolytic removal of a signal peptide during the translocation of a protein is considered to be a post-translational modification. Examples of extracellular alginate lyases which contain signal peptides include AlyVOA and AlyVOB from *Vibrio* sp. O2 (Kawamoto *et al*. 2006), AlyVI from *Vibrio* sp. QY101 (Han *et al*. 2004) and AlyVG1, AlyVG2 and AlyVG3 from *Vibrio halioticoli* (Sawabe *et al*. 2000). Once AlyVMIV has been exported from the cell, it cannot be

modified further or have a modification reversed. The control mechanism of AlyVMIV could include the enzyme remaining inside the cell until required extracellularly. Alternatively, there could be a different mechanism. For instance, AlyVMIV contains a galactose binding-like and a coagulation factor 5/8-type C-terminal or FA58C domain which could function in anchoring the enzyme to the cell via binding to a cell-surface attached carbohydrate (Chapter 2). Agarase AgaE of *Saccharophagus degradans* 2-40 was shown to be attached to the cell surface during growth in agarose containing media (Ekborg, 2005). Immunolabeling of whole cells and ultra-thin sections revealed that AgaE was initially bound to the cell surface during logarithmic growth and later released from the cell in aggregates during stationary phase. A similar mechanism for AlyVMIV could exist whereby the enzyme is attached to the cell surface when glucose is present in the media and released when reducing sugar levels in the media are low. Either of these mechanisms could account for the difference in alginate lyase activity detected in the supernatant of the two cultures following growth in media either lacking or supplemented with glucose for 24 hours.

In order to determine the mechanism employed for controlling the activity of AlyVMIV, antibodies specific to AlyVMIV should be raised and used in immunolocalisation studies to determine the location of the enzyme when *Vibrio midae* SY9 is cultured in alginate media without and supplemented with glucose. Indirect ELISA assays or western hybridisation analysis could also be used to determine the relative amounts of AlyVMIV in the different cellular fractions during growth. First, AlyVMIV would need to be purified.

In conclusion, gene expression studies were conducted to determine whether the four alginate lyase genes of *Vibrio midae* SY9 are regulated at the transcriptional, translational or post-translational level when cultured in alginate media without and supplemented with glucose. Real-time RT PCR was used to monitor mRNA transcript levels and an indirect ELISA assay was used to monitor the intracellular levels of AlyVMI and AlyVMII. Enzyme activity assays were used to determine alginate lyase activity present in the culture supernatants and cell lysates. The amount of reducing sugar present in the culture supernatant was also determined. The mRNA transcripts of the four genes were found not to be significantly regulated by either treatment nor were the enzyme levels inside the cell. However, enzyme activity was only

detected in the culture supernatant and cell lysates when reducing sugar levels in the culture supernatant were low. Thus, it is suggested that the alginate lyase genes of *V. midae* SY9 are regulated post-translationally under the experimental conditions tested, the mechanism of which remains to be determined.

# Chapter 5

# Immunolocalisation of AlyVMI and AlyVMII in *Vibrio midae* SY9

## CONTENTS

## 5.1. SUMMARY

*Vibrio midae* SY9 was negatively stained using methylamine tungstate and shown to be a rod-shaped bacterium with a single polar flagellum. Fixative conditions for immunolabeling were optimised using western hybridisation analysis. Recognition of AlyVMII by anti-AlyVMII antibodies was sensitive to glutaraldehyde fixation. Optimal fixation conditions for immunolabeling *V. midae* SY9 was found to be a solution containing 4% formaldehyde and 0.2% glutaraldehyde. *V. midae* SY9 was cultured for 10 h in alginate media, fixed in 4% formaldehyde and 0.2% glutaraldehyde and embedded in LR White acrylic resin or cryo-fixed according to the method of Tokuyasu (1973). Ultra-thin sections of resin embedded or cryo-fixed cells were immunolabeled with either pre-immune sera, anti-AlyVMI or anti-AlyVMII antibodies using a 10 nm gold conjugated secondary antibody. A clear double cell membrane was observed in the cryo-sections suggesting that *V. midae* SY9 cells were better preserved when cryo-fixed than when embedded in resin. No gold particles were observed when sections were labeled with pre-immune sera which acted as a negative control. Low levels of labeling were observed with the anti-AlyVMI and anti-AlyVMII antibodies in both the resin-embedded and cryo-fixed *V. midae* SY9 sections. Gold particles were observed in the cytoplasm and periplasm of *V. midae* SY9 cells when sections were labeled with anti-AlyVMI or anti-AlyVMII antibodies. It remains unknown whether it is AlyVMI or AlyVMII or both alginate lyases which are present in the cytoplasm and periplasm.

## 5.2. INTRODUCTION

The cell envelope of Gram-negative bacteria consists of an inner and an outer membrane separated by a periplasmic space (Tamm *et al*. 2001, 2004). The inner membrane is a lipid bilayer consisting of phosphatidylethanolamine, phosphatidylglycerol and cardiolipin and is the major permeability barrier between the interior and exterior of the cell. Most membrane associated metabolic functions are carried out by the inner membrane. The outer membrane is a highly asymmetric lipid bilayer consisting of an outer leaflet of lipopolysaccharides (LPS) and an inner leaflet of the same classes of phospholipids as the inner membrane. The outer membrane provides for the exchange of solutes with the environment by the presence of specific and non-specific pores (porins) which act as molecular sieves. The periplasmic space separating the inner and outer membranes consists of soluble proteins and a cross-linked peptidoglycan layer. Inner membrane proteins are α-helical whereas outer membrane proteins are membrane spanning β-barrels. The inner and outer membranes of Gram-negative bacteria appear as a 'double-track' in electron micrographs after fixation due to the denaturation of each lipid bilayer (Costerton *et al*. 1974).

Complex polysaccharides are abundant in nature and include agar, alginate, carrageenan, cellulose, chitin, glucan, laminarin, pectin, pullulan, starch and xylan. Bacteria have developed different methods of complex polysaccharide degradation which are characterised by two mechanisms. The first mechanism involves uptake of the macromolecule into the cell where specific intracellular enzymes degrade the polymer. *Sphingomonas* sp. A1 is unique in that it forms a pit-like structure on the cell surface through which alginate is imported into the cell (Hisano *et al.* 1995, 1996). Alginate lyases A1-I, A1-II and A1-III are formed from a common precursor and act endolytically to depolymerize the alginate molecule into oligosaccharides (Momma *et al.* 1999). Alginate lyase A1-IV acts endolytically to convert the oligosaccharide products of A1-I, A1-II and A1-III digestion into monomers which can enter cell metabolism (Hashimoto *et al.* 2000b; Miyake *et al*. 2003).

The second mechanism involves export of enzymes from the cell where they come into contact with the environment. The advantage of this approach is that the enzymes may reach distant substrates. The disadvantage is the potential loss of degradation products due to diffusion especially in the marine environment. A strategy to overcome this is to anchor the enzyme to the cell surface. Although distant substrates cannot be accessed, products of enzyme activity would be in close proximity to the cell.

One mechanism of attaching enzymes to cell surfaces is the formation of lipoproteins whereby a cysteine residue is post-translationally modified with a diacylglycerol group after cleavage of a signal sequence and subsequently embedded into the outermembrane, exposing the protein to the extracellular environment (Pugsley, 1993). An example of a carbohydrate degrading lipoprotein is the extracellular pullalanase from *Klebsiella pneumoniae* (Pugsley *et al.* 1986). Complex polysaccharide degradation of *Saccharophagus degradans* 2-40 has been extensively studied and several lipoproteins characterised in the agarase (Ekborg *et al.* 2006), cellulase (Taylor *et al.* 2006) and chitinase (Howard *et al.* 2003, 2004) systems.

A less characterised mechanism is covalent linking of proteins to a glycosyl phosphatidylinositol moiety which is embedded in the outer membrane. An example is the ice-nucleation protein of *Pseudomonas syringae* (Wolber *et al*. 1986). This strategy has been used in recombinant studies for the display of fusion proteins (Jung *et al.* 1998a, 1998b). Another strategy is the use of cellulosomes by cellulose degrading *Clostridia* (Bayer *et al*. 1998, 2004). Cellulosomes are highly organized complexes of non-catalytic scaffolding proteins (scaffoldin) to which cellulases are attached through interactions with dockerin and cohesion proteins. Cellulosomes are exposed to the environment and allow the cell to bind to and degrade cellulose, resulting in released soluble sugars being kept in close proximity to the cell.

Marine bacteria which produce both extracellular and intracellular alginate lyases have been described. An example is *Alteromonas* sp. strain H-4 which produces one intracellular and two extracellular alginate lyases (Sawabe *et al.* 1992, 1997, 1998a). Although anchoring of extracellular alginate lyases to the cell surface has not been described, alginate lyases present in the periplasmic space have been reported. Alginate lyase AlgL of the alginate producing

bacterium *Pseudomonas aeruginosa* has a dual role in alginate biosynthesis. It is present in the periplasm where it is part of a scaffold of proteins essential for alginate transport out of the cell and where it degrades any alginate remaining in the periplasm after disassembly of the scaffold (Jain and Ohman, 2005).

Although antibodies have been raised against some enzymes, immunolabeling bacterial alginate lyases has not been described. Antibodies against alginate lyases A1-II' and A1-IV' from *Sphingomonas* sp. A1 (Hashimoto *et al*. 2005; Miyake *et al*. 2004) and HdAly from *Haliotis discus hannai* (Hata *et al*. 2009) were raised in rabbits and used in western hybridisation analysis.

*Vibrio midae* SY9 produces four alginate lyases expressed by genes adjacent to each other on the chromosome (Chapter 2). Sequence analysis of the four genes revealed that AlyVMIV contains a signal sequence and is thus probably exported from the cell. AlyVMIV was also predicted to contain a galactose-binding like domain. Secondary structure analysis predicted the N-terminal of AlyVMI and AlyVMII to consist of α-helices and the C-terminal to consist of β-sheets (Chapter 2). Transmembrane domains were also predicted in the N-terminal α-helices of AlyVMI and AlyVMII.

AlyVMI and AlyVMII were purified by affinity chromatography and antibodies were raised in rabbits (Chapter 3). This chapter describes the immunolocalisation of AlyVMI and AlyVMII in *Vibrio midae* SY9 using anti-AlyVMI and anti-AlyVMII antibodies. Two methods of sample preparation were used: embedding cells in acrylic resin and cryo-fixation of cells. *V. midae* SY9 cells were also negatively stained and viewed by transmission electron microscopy.

## 5.3. MATERIALS AND METHODS

All media and solutions used in this study are listed in Appendix A.

### 5.3.1. Bacterial strains and plasmids used

Bacterial strains and plasmids used in this study are listed in Table 5.1.

### 5.3.2. Culture conditions and media used

*Escherichia coli* BL21 (DE3) pLysS was cultured either in Luria broth (LB) or on Luria agar (LA) containing 20 µg/ml chloramphenicol at 37°C. *E. coli* BL21 (DE3) pLysS transformants harboring recombinant pET-29a plasmids were cultured in LB or on LA containing 20 µg/ml chloramphenicol and 30 µg/ml kanamycin at 37°C. *Vibrio midae* SY9 was cultured either in marine broth (MB) or on marine agar (MA) at room temperature.



**Table 5.1.** Bacterial strains and plasmids

| Strain/plasmid | Genotype/relevant characteristic(s)[a] | Reference |
|---|---|---|
| **Strains** | | |
| *E. coli* BL21 (DE3) pLysS | F⁻ *omp*T *gal dcm lon hsd*SB(rB- mB-) λ(DE3) pLysS($cm^r$) | Moffatt and Studier (1987) |
| *Vibrio midae* SY9 | Putative probiont isolated from *Haliotis midae,* South Africa | Macey and Coyne (2005) |
| **Plasmids** | | |
| pETalyVMI | pET-29a carrying the 2163 bp *alyVMI* gene, $Kan^r$ | Chapter 3 |
| pETalyVMII | pET-29a carrying the 2163 bp *alyVMII* gene, $Kan^r$ | Chapter 3 |

[a] $cm^r$, chloramphenicol resistant; $kan^r$, kanamycin resistant.

### 5.3.3. Negative staining of *Vibrio midae* SY9

*Vibrio midae* SY9 was cultured on marine agar (MA) for 3 days at room temperature. Cells were transferred to 1x PBS using a sterile toothpick and allowed to stand for 10 min. A carbon coated copper grid (Agar Scientific) was held in a pair of tweezers while 3 µl of cells in 1x PBS were placed onto the carbon side of the grid. The cells were allowed to bind to the positively charged grid for 1 min. Thereafter cells were stained with 2% methylamine tungstate by dripping 50 µl of stain followed by 50 µl of $dH_2O$ over the grid three times. The grid was allowed to air dry. Grids were stored in a grid box at room temperature until viewed on a LEO 912 transmission electron microscope (TEM) and photographed with a ProScan CCD Camera and EsiVision Pro Software Version 3.2 (Soft Imaging Software, Germany).

### 5.3.4. Immunolabeling of *Vibrio midae* SY9 cells embedded in resin

#### 5.3.4.1. Fixation and resin embedding of *Vibrio midae* SY9 cells

Western hybridisation analysis was used to determine the fixative conditions under which anti-AlyVMI and anti-AlyVMII antibodies would still recognize and bind to their respective antigens. Recognition of AlyVMII by anti-AlyVMII was sensitive to the presence of glutaraldehyde (0.5% to 2.5%) and thus the milder fixative formaldehyde was tested. Optimum conditions were determined to be 4% formaldehyde (Merck) containing 0.2% EM grade glutaraldehyde (Agar Scientific) in 1x PBS.

*Vibrio midae* SY9 was cultured in alginate media and incubated at room temperature as described in Chapter 2, Section 2.3.9. Ten hours after inoculation, the culture was centrifuged at 10 000 rpm for 2 min. The culture supernatant was discarded and cells washed three times with 1x PBS. Two hundred microlitres of pelleted cells were resuspended in 1 ml of 1x PBS containing 4% formaldehyde and 0.2% glutaraldehyde. The cells were fixed overnight at 4°C and then washed twice in 1x PBS for 5 min followed by two 5 min $dH_2O$ washes. The cells were dehydrated in a series of 5 min ethanol washes at 30%, 50%, 70%, 80%, 90% and 95% at room temperature and subsequently twice in 100% ethanol for 15 min each wash. The cells were

centrifuged at 3000 rpm for 2 min, the supernatant discarded and the cells resuspended in the next solution for each wash and dehydration step.

After the final dehydration step, the cells were centrifuged at 3000 rpm for 2 min, the supernatant discarded and the cells resuspended in LR White acrylic embedding medium (London Resin Company) and 100% ethanol at a ratio of 1:1 (resin:ethanol). The cells were incubated in the resin/ethanol mixture overnight at room temperature with gentle agitation. The cells were centrifuged at 3000 rpm for 2 min and the resin/ethanol mixture discarded. The cells were resuspended in LR White resin and 100% ethanol at a ratio of 3:1 (resin:ethanol) and incubated at room temperature for 8 h with gentle agitation. The cells were centrifuged at 3000 rpm for 2 min and the resin/ethanol mixture discarded. The cells were resuspended in 100% LR White resin and incubated overnight at room temperature with gentle agitation. The cells were centrifuged at 3000 rpm for 2 min and the resin discarded. The cells were resuspended in 100% LR White resin and transferred to a Beem capsule. The Beem capsule was filled with 100% LR White resin taking care not to introduce air bubbles. Samples were incubated at 60°C for 24 h to polymerise the resin. The resin blocks were removed from the Beem capsules and stored at room temperature until sectioned.

#### 5.3.4.2. Sectioning and immunolabeling of *Vibrio midae* SY9 cells embedded in resin

*E. coli* BL21 (DE3) pLysS transformed with pETalyVMI or pETalyVMII were cultured and induced with IPTG as described in Chapter 3, Section 3.3.4. Cell lysates were prepared from the IPTG induced *E. coli* cells in lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 0 mM imidazole) as described in Chapter 3, Section 3.3.5. Anti-AlyVMI and anti-AlyVMII PEG-precipitated antibodies (bleed 8) were pre-absorbed against cell lysates prepared from E. coli transformed with pETalyVMII or pETalyVMI, respectively, as described in Chapter 3, Section 3.3.10, except that the antibodies were diluted and pre-absorbed in 1x PBS containing 0.5% BSA, 0.1% FSG and 0.05% Tween 20.

Ultra-thin (100 nm to 120 nm) sections of resin embedded *Vibrio midae* SY9 cells were prepared using a Reichert Ultracut S Ultramicrotome (Leica). Sections were collected on formvar coated nickel grids which were stored in a grid box at room temperature until labeled. Sections were immunolabeled according to Geuze and Slot (1980), Griffiths and Jockusch (1980) and Griffiths *et al*. (1983) with the following modifications. Immunolabeling was performed at room temperature unless otherwise stated. The sections were not allowed to dry at any time during the procedure and the back of the grid was prevented from becoming wet.

The sections were hydrated by floating grids section side down on 20 µl droplets of 1x PBS for 5 min at room temperature and then transferred to 20 µl droplets of 1x PBS containing 150 mM glycine for 5 min. The sections were blocked for 30 min on 20 µl droplets of 1x PBS containing 0.5% BSA, 0.5% fish skin gelatin (FSG, Sigma) and 0.05% Tween 20. The sections were transferred to 10 µl droplets of anti-AlyVMI or anti-AlyVMII antibodies pre-absorbed against *E. coli* cell lysates or 10 µl droplets of anti-AlyVMI or anti-AlyVMII PEG-precipitated antibodies (bleed 8) diluted 1:10 in 1x PBS containing 0.5% BSA, 0.5% FSG and 0.05% Tween 20.

The sections were labeled with primary antibody overnight and subsequently washed 5x with 20 µl droplets of 1x PBS containing 0.1% Tween 20 for 5 min each. Goat anti-rabbit 10 nm gold conjugated IgG secondary antibody (Sigma) was diluted 1:50 in 1x PBS containing 0.1% BSA, 5% fetal calf serum and 0.05% Tween 20. The sections were floated on 10 µl droplets of diluted secondary antibody for 1 h and then washed 5x with 20 µl droplets of 1x PBS containing 0.1% Tween 20 for 5 min each. The sections were fixed with 20 µl 1% glutaraldehyde in 1x PBS for 10 min to cross-link bound antibodies and then washed 5x with 20 µl droplets of $dH_2O$ for 5 min each.

Staining of the sections with uranyl acetate and Reynold's lead citrate (Reynolds, 1963) was optimised. Sections were stained with 2% uranyl acetate for 5 min and washed 5x with $dH_2O$ for 1 min each. Sections were then stained with Reynold's lead citrate for 2 min and washed in a stream of $dH_2O$ (Reynolds, 1963). Both the uranyl acetate and Reynold's lead citrate solutions

were centrifuged for 2 min before use. Grids were stored in a grid box at room temperature until viewed on a LEO 912 transmission electron microscope (TEM) and photographed with a ProScan CCD Camera and EsiVision Pro Software V3.2 (Soft Imaging Software, Germany).

Additional sections were prepared as described above except they were incubated with pre-immune serum (bleed 1) instead of primary antibody as a negative control. Following blocking with 0.5% BSA, 0.5% FSG and 0.05% Tween 20 for 30 min, sections used as negative controls were transferred to 10 µl droplets of pre-immune serum (bleed 1) of anti-AlyVMI or anti-AlyVMII diluted 1:10 in 1x PBS containing 0.5% BSA, 0.5% FSG and 0.05% Tween 20 and incubated overnight. Sections used as negative controls were washed, incubated with secondary antibody, stained and viewed as described above for the experimental sections.



### 5.3.5. Cryo-fixation and immunolabeling *Vibrio midae* SY9

The Tokuyasu (1973) method of cryo-fixation was followed. *Vibrio midae* SY9 was cultured in alginate media as described in Chapter 2, Section 2.3.9. Ten hours after inoculation, the culture was centrifuged at 10 000 rpm for 2 min. The culture supernatant was discarded and the cells washed three times with 1x PBS containing 0.4 M NaCl. Two hundred microlitres of pelleted cells were fixed in 1 ml of 4% formaldehyde containing 0.2% glutaraldehyde in 1x PBS overnight at 4°C. The cells were washed twice in 1x PBS containing 0.4 M NaCl for 5 min each. An equal volume of molten 2% agarose was added to the fixed and washed cells and mixed. Once the agarose had set, the agarose-cells mixture was cut into small blocks which were incubated in 1 ml of 2.3 M sucrose overnight at 4°C. The agarose blocks were trimmed to fit onto a cryo-pin and fixed to cryo-pins using 2.3 M sucrose. Samples were frozen by plunging the cryo-pins into liquid ethane (-165°C) using a KF80 Leica plunge-freezer. Frozen samples were stored in cryo-vials under liquid nitrogen at -196°C.

Ultra-thin cryo-sections were prepared using the Reichert Ultracut S Ultramicrotome under liquid nitrogen vapours at -100°C. Sections were lifted onto formvar-coated nickel grids using 2.3 M sucrose and then floated section side down on drops of 1x PBS containing 0.4 M NaCl. The sections were immunolabeled as described in Section 5.3.4.2. Sections were labeled with

10 µl droplets of anti-AlyVMI or anti-AlyVMII PEG-precipitated antibodies (bleed 8) diluted 1:10 in 1x PBS containing 0.5% BSA, 0.5% FSG and 0.05% Tween 20 or with a 1:10 dilution of pre-immune serum (bleed 1) of anti-AlyVMI or anti-AlyVMII in 1x PBS containing 0.5% BSA, 0.5% FSG and 0.05% Tween 20 as a negative control.

Cryo-sections were stained with uranyl acetate and methyl cellulose (Griffiths *et al.* 1982). One hundred microlitres of uranyl acetate was mixed with 100 µl methyl cellulose. Grids were incubated, section side down, on a 20 µl drop of stain mixture twice for 5 min each on ice. Grids were picked up with a loop and excess stain removed by touching the surface of the grid to filter paper. Grids were air-dried overnight, removed from the loops and stored in a grid box until viewed on a LEO 912 TEM and photographed with a ProScan CCD Camera and EsiVision Pro Software Version 3.2 (Soft Imaging Software, Germany).

## 5.4. RESULTS

### 5.4.1. Negative staining of *Vibrio midae* SY9

*Vibrio midae* SY9 was cultured on marine agar for 3 days and then negatively stained. Initially, cells were stained with uranyl acetate which made them appear very dark and obscured cellular features. Consequently, *V. midae* SY9 cells were then stained with methylamine tungstate. The cells were lighter and cell membranes could be observed (Figure 5.1). *V. midae* SY9 cells are rod-shaped with a single polar flagellum. The cells are ~0.7 µm in diameter and ~2 µm in length.

### 5.4.2. Immunolabeling of resin embedded *Vibrio midae* SY9 sections

No distinct organelles could be distinguished in *Vibrio midae* SY9 cells that had been fixed, dehydrated in ethanol and embedded in LR White resin (Figure 5.2). The edge of the cell appeared to be wavy and the cell membrane was not clearly defined.

No antigens were labeled on negative control resin sections which were treated with pre-immune serum (bleed 1) (Figure 5.2 a and b). Antigens in experimental sections treated with anti-AlyVMI or anti-AlyVMII antibodies that had been pre-absorbed against *E. coli* cell lysates containing AlyVMII or AlyVMI, respectively, were not labeled (results not shown). It was possible that the antibodies were too dilute after pre-absorption and consequently, antibodies that were not pre-absorbed were used for further labeling experiments.

Gold particles marking AlyVMI and AlyVMII were observed when sections were treated with anti-AlyVMI (Figure 5.2 c) or anti-AlyVMII (Figure 5.2 d) antibodies that had not been pre-absorbed against *E. coli* cell lysates containing AlyVMII and AlyVMI, respectively. However, the level of antigen labeling of each cell was low and not every cell of each section contained antigens that were labeled. Generally, there was greater labeling with the anti-AlyVMI antibody than with the anti-AlyVMII antibody. Gold particles were observed in the cytoplasm and on the edges of the cell.

Anti-AlyVMI and anti-AlyVMII antibodies recognize both AlyVMI and AlyVMII (Chapter 3). Therefore, it is likely that both AlyVMI and AlyVMII were labeled in sections treated with anti-AlyVMI and anti-AlyVMII antibodies not pre-absorbed against *E. coli* cell lysates containing AlyVMII and AlyVMI, respectively.

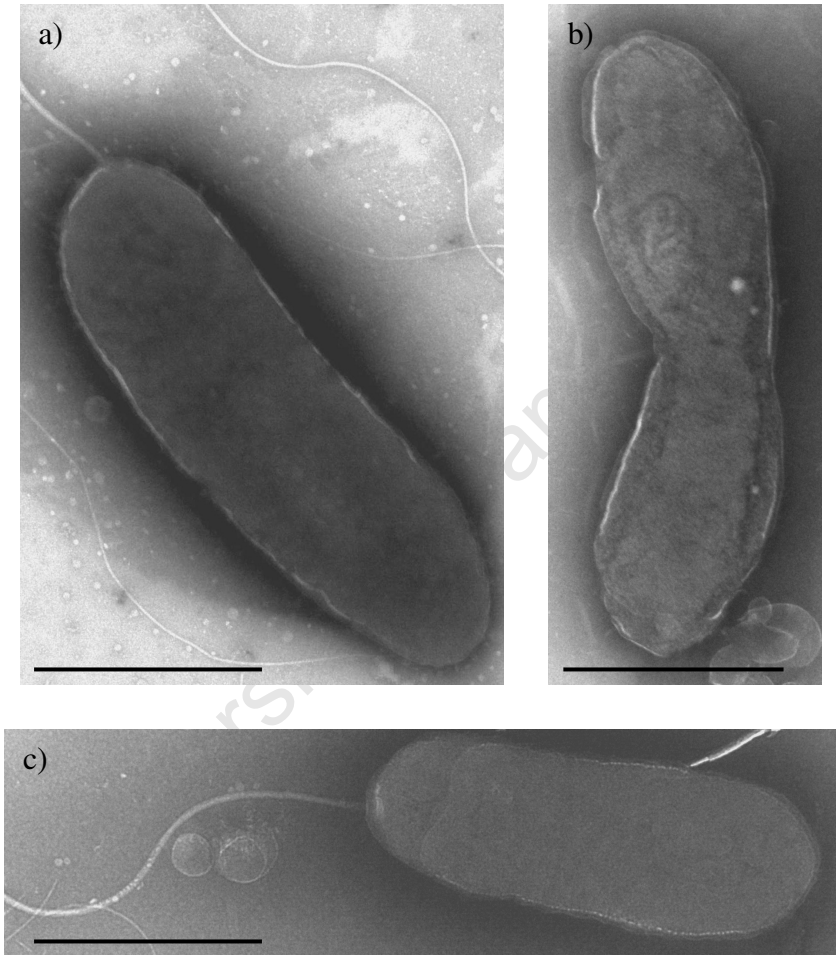



**Figure 5.1.** Electron micrographs of *Vibrio midae* SY9 negatively stained with methylamine tungstate. Rod-shaped cells with a single, polar flagellum were observed (a, c) and dividing cells in which the cell membrane is clearly visible (b). Scale bar: 1 µm.

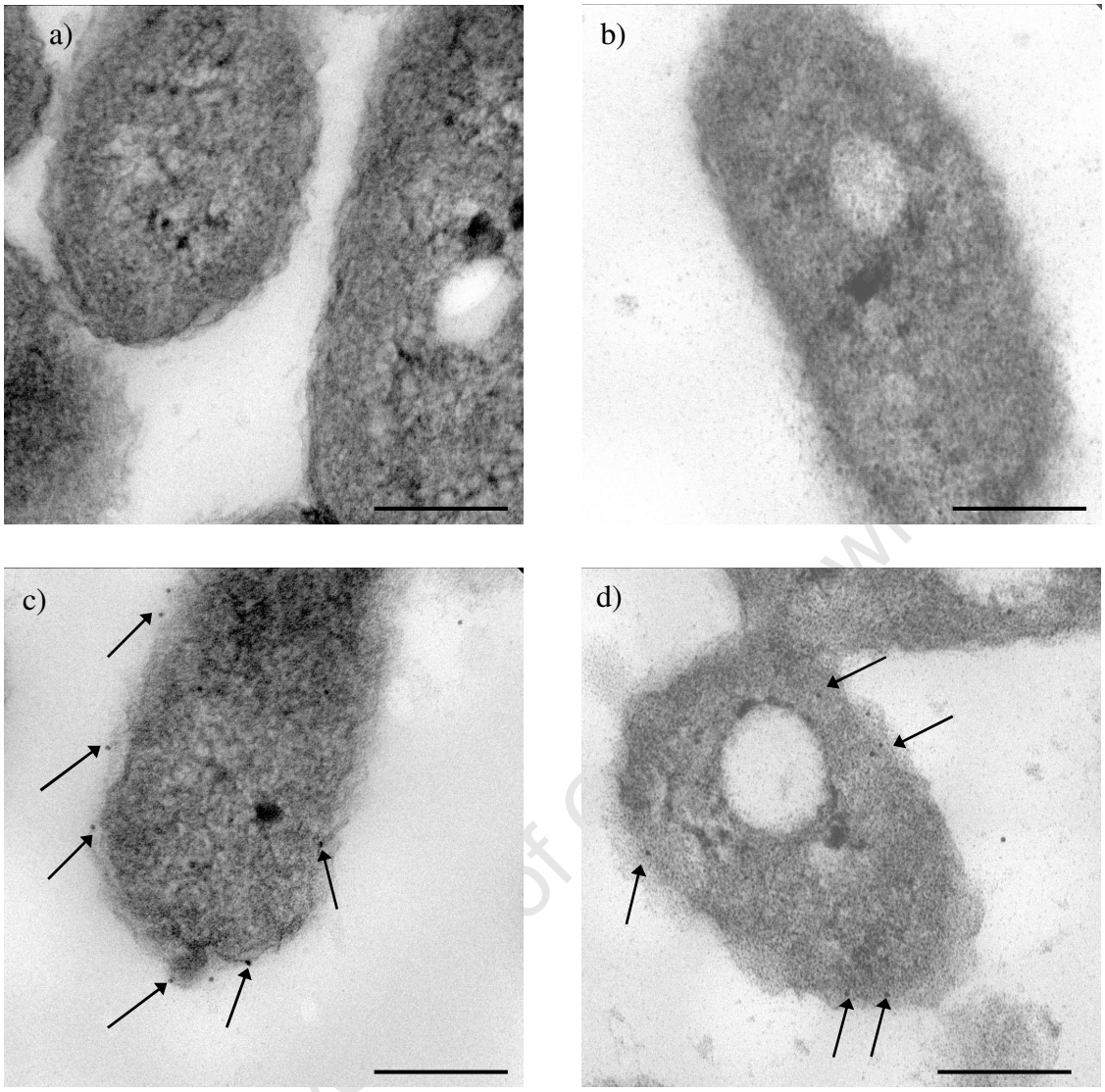



**Figure 5.2.** Electron micrographs of cross-sections of *Vibrio midae* SY9 cells embedded in LR White resin. Sections were labeled with pre-immune serum (bleed 1) obtained from rabbits prior to immunisation with purified AlyVMI (a), pre-immune serum (bleed 1) obtained from rabbits prior to immunisation with purified AlyVMII (b), anti-AlyVMI antibodies (c), and anti-AlyVMII antibodies (d). Arrows indicate 10 nm gold particles. Scale bar: 0.2 µm.

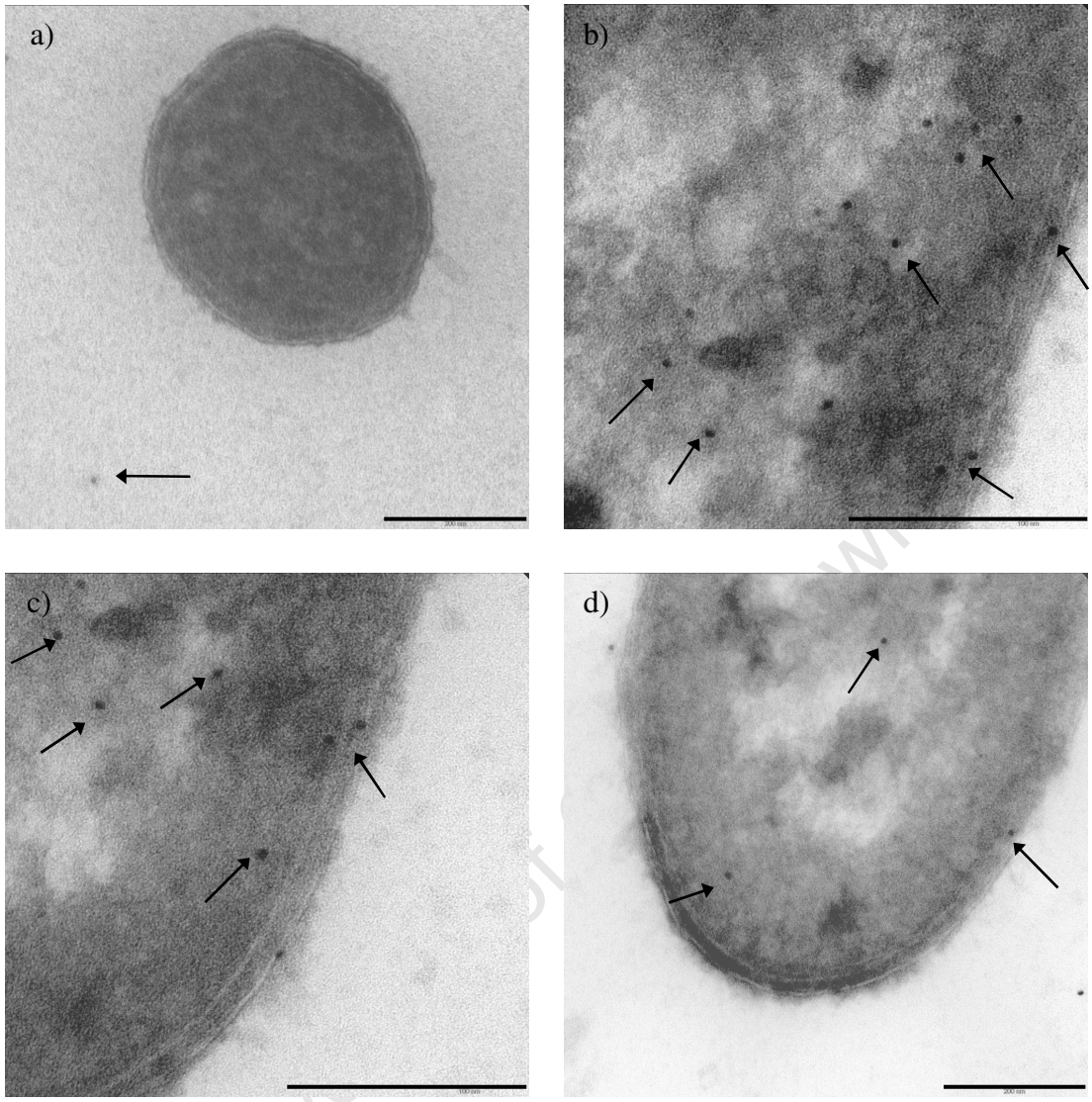



**Figure 5.3.** Electron micrographs of *Vibrio midae* SY9 cryo-sections immunolabeled with pre-immune serum (bleed 1) obtained from rabbits prior to immunisation with purified AlyVMI (a) or anti-AlyVMI antibodies (b, c and d). Arrows indicate 10 nm gold particles. Scale bar: 0.2 µm.

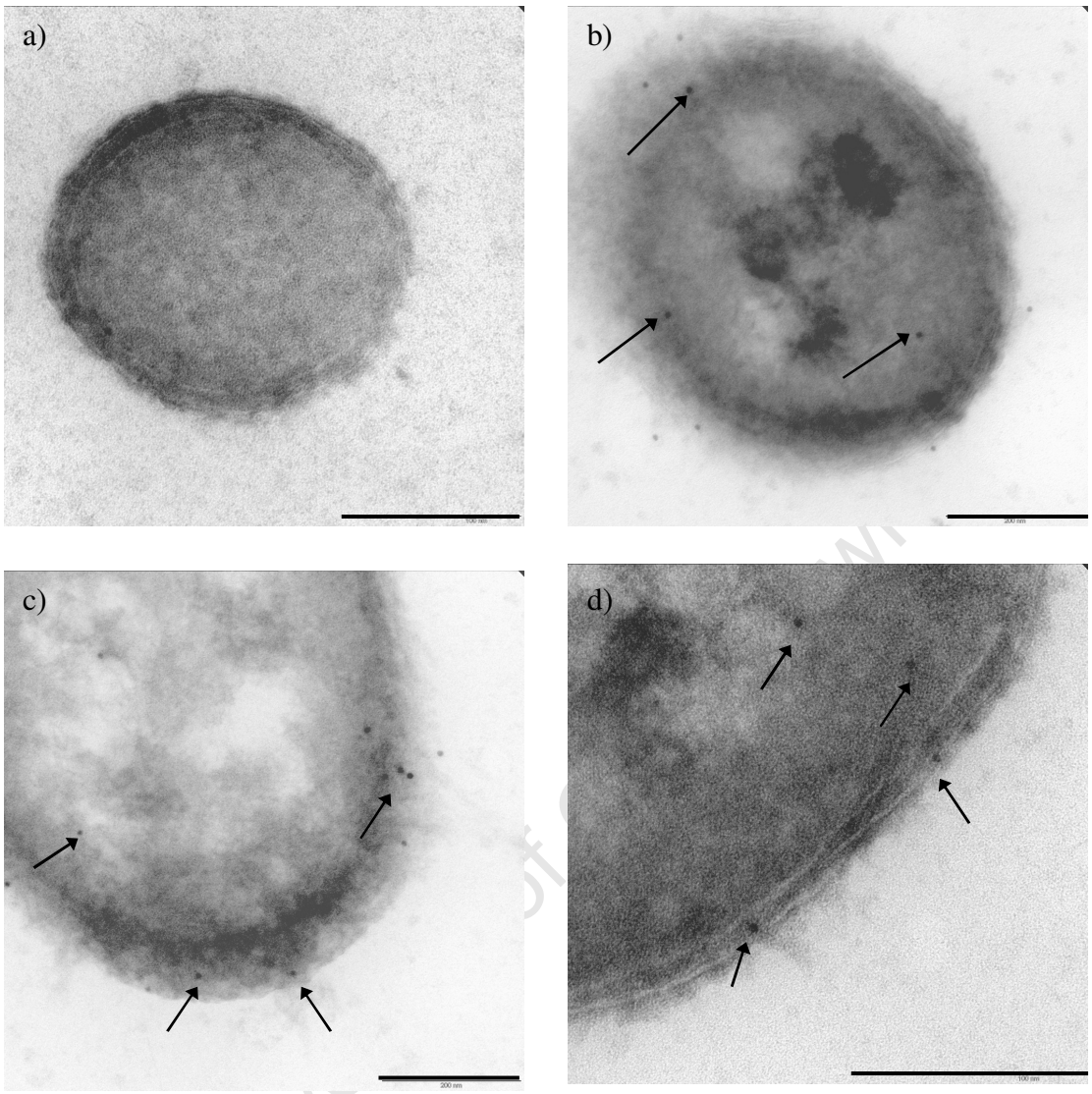



**Figure 5.4.** Electron micrographs of *Vibrio midae* SY9 cryo-sections labeled with pre-immune serum (bleed 1) obtained from rabbits prior to immunisation with purified AlyVMII (a) or anti-AlyVMII antibodies (b, c and d). Arrows indicate 10nm gold particles. Scale bar: 0.2 µm.

### 5.4.3. Immunolabeling of *Vibrio midae* SY9 cryo-sections

*V. midae* SY9 cells were more distinct in the cryo-sections than in resin sections (Figure 5.3). A double cell membrane was clearly observed, the cells were not as darkly stained and gold particles were easier to distinguish in the cryo-sections than in the resin sections.

No antigens were detected in the cells of cryo-sections treated with pre-immune sera (bleed 1) which acted as a negative control (Figures 5.3 and 5.4 a). AlyVMI and AlyVMII were labeled in the cells of cryo-sections treated with anti-AlyVMI or anti-AlyVMII antibodies that were not pre-absorbed against *E. coli* cell lysates containing AlyVMII or AlyVMI, respectively (Figures 5.3 and 5.4 b, c and d). Gold particles were observed in the cytoplasm and periplasm. It was not established whether the gold-labeled antigen was attached to the cell membrane or just present in the periplasmic space. There was an equivalent number of antigens labeled per cell in the cryo-sections compared to the resin sections, therefore approximately the same level of antigen labeling was achieved in the cryo- and resin sections. As observed in the resin sections, there was generally a greater degree of labeling with the anti-AlyVMI antibody than with the anti-AlyVMII antibody.

## 5.5. DISCUSSION

*Vibrio midae* SY9 was cultured on marine agar for 3 days and then negatively stained. Cells initially stained with uranyl acetate appeared very dark and cellular features could not be distinguished. Methylamine tungstate was then used as a negative stain and staining times were optimised. Cells had more contrast when stained with methylamine tungstate and cell membranes were evident. *V. midae* SY9 has been reported to be a Gram-negative rod of ~0.7 µm in diameter and ~2 µm in length with between one and three polar flagella (Macey, 2005). This study also found *V. midae* SY9 cells to be rods ~0.7 µm in diameter and ~2 µm in length. However, only cells with a single polar flagellum were observed. This could be due to flagella becoming detached from cells during the negative staining procedure.

Western hybridisation analysis was used to determine optimum fixative conditions for immunolabeling *Vibrio midae* SY9 cells. Recognition of AlyVMII by anti-AlyVMII was sensitive to the presence of glutaraldehyde whereas recognition of AlyVMI by anti-AlyVMI was not affected. Optimum fixation conditions were obtained using a mixture of 4% formaldehyde and 0.2% glutaraldehyde.

It is unknown why anti-AlyVMII antibodies did not recognize AlyVMII after incubation in glutaraldehyde. Aldehydes form cross-links within and between proteins; i.e. intra- and intermolecular cross-links. This results in the formation of polymers between soluble and structural proteins thus forming an insoluble network (Hopwood, 1969). Glutaraldehyde reacts with the amine, thiol, phenol and imidazole functional groups of amino acid residues such as lysine, tyrosine, tryptophan, histidine, phenylalanine, cysteine, proline, serine, glycine and arginine through aldol condensations, Michael-type additions or through the formation of Schiff bases (Migneault *et al*. 2004). The most reactive moiety of an amino acid residue is the ε-amino group, the reason why lysine residues are so reactive with glutaraldehyde, followed by the α-amino, guanidinyl, secondary amino and hydroxyl groups (Migneault *et al*. 2004). Extensive cross-linking caused by glutaraldehyde can result in the distortion or conformational change of enzyme structure, particularly the active site, rendering the enzyme inactive (Chui and Wan, 1997). Lenard and Singer (1968) also showed that glutaraldehyde causes significant structural

changes in proteins as determined by circular dichroism. It is also known that glutaraldehyde can affect the conformational stability of membrane proteins (Lenard and Singer, 1968; Nicolson, 1973). Thus, it is possible that the conformation of AlyVMII changed after incubation in glutaraldehyde rendering anti-AlyVMII antibodies unable to recognize it. Although AlyVMI and AlyVMII share ~50% amino acid identity, it may be the arrangement of the above mentioned amino acids in the secondary or tertiary structure that differs, allowing glutaraldehyde to have a greater effect on AlyVMII than AlyVMI. Fixation with glutaraldehyde is irreversible whereas fixation with formaldehyde is reversible upon washing of samples (Baschong *et al*, 1983), possibly explaining why fixation of AlyVMII in formaldehyde did not affect the binding of antibodies.

AlyVMI and AlyVMII appear to be very similar in amino acid sequence, predicted conserved domains and predicted secondary structure (Chapter 2, Section 2.4.3 and 2.5). However, the two alginate lyases are expressed differently from the same expression vector and *E. coli* expression host (Chapter 3, Section 3.5). Recombinant AlyVMII is more soluble and obtained in greater amounts than recombinant AlyVMI under the same expression conditions. Furthermore, pre-absorption against *E. coli* cell lysates containing AlyVMII or AlyVMI improved the specificity of anti-AlyVMI and anti-AlyVMII, respectively, to the protein against which each antibody was raised. This suggests that there are common epitopes and others that are specific to each protein reflecting structural differences between AlyVMI and AlyVMII (Chapter 3, Section 3.5). In addition, the observation that AlyVMII was affected by incubation in glutaraldehyde rendering it unrecognizable to anti-AlyVMII antibodies, suggests that a structural change occurred in AlyVMII but not in AlyVMI, or not to the same extent, further supporting the suggestion of structural differences between the two alginate lyases.

*Vibrio midae* SY9 cells cultured in alginate media for ten hours were either fixed in 1x PBS containing 4% formaldehyde and 0.2% glutaraldehyde, dehydrated and embedded in LR White resin or fixed in formaldehyde and glutaraldehyde and cryo-fixed using sucrose as a cryo-protectant. One hundred nanometer sections were prepared from the resin embedded or cryo-fixed cells. *V. midae* SY9 cells were more distinct in the cryo-sections than the resin embedded sections in that a clear double membrane could be observed in the cryo-sections (Figures 5.3 and

5.4). Costerton *et al.* (1974) described the cell membrane of Gram-negative bacteria in electron micrographs as a 'double-track' due to denaturation of the lipid bilayer during fixation. The protocol for resin embedding involves fixing the cells, ethanol dehydration and multiple washing steps. All of these steps influence the structure of the cells as well as the efficiency of immunolabeling. Fewer chemicals and less washing steps are used in cryo-fixation, resulting in samples being handled less. The only step in cryo-fixation that could denature proteins is the fixation step. Consequently, cells approach a more natural state when cryo-fixed than when resin embedded.

No labeling of AlyVMI and AlyVMII was observed in *V. midae* SY9 cells when resin sections were treated with anti-AlyVMI and anti-alyVMII antibodies pre-absorbed against *E. coli* cell lysates containing AlyVMII or AlyVMI, respectively. This could be due to the antibodies being too dilute in the labeling solution after pre-absorption to detect the recombinant alginate lyase they are specific to in the small volumes used in the immunolabeling procedure. As a result, antibodies not pre-absorbed against *E. coli* cell lysates were used for further labeling experiments. AlyVMI and AlyVMII were labeled in cells when resin and cryo-sections were treated with anti-AlyVMI and anti-alyVMII antibodies that had not been pre-absorbed against *E. coli* cell lysates containing AlyVMII or AlyVMI, respectively.

The labeling efficiency of AlyVMI and AlyVMII in *V. midae* SY9 cells of resin and cryo-sections was low. AlyVMI and AlyVMII were not labeled in every cell present in a section and each cell had a different degree of AlyVMI and AlyVMII labeling; some cells contained many gold particles and others had few. When a section is labeled and viewed under the electron microscope, only a small part of the cell is viewed, not the entire cell. Thus, there may be additional proteins of interest in a given cell that were not present on the labeled section. Since AlyVMI and AlyVMII were predicted to contain transmembrane domains and at least one of the enzymes was localised to the periplasm, it is possible that the protein may be localized to a specific area within the periplasm. The protein may accumulate in an area where a specific receptor is located. However this remains unknown. These may be the reasons why all the cells of a given section were not uniformly labeled.

Gold particles, and thus AlyVMI and AlyVMII, were observed in the cytoplasm and periplasm of cryo-sectioned cells treated with anti-AlyVMI or anti-AlyVMII antibodies that were not pre-absorbed against *E. coli* cell lysates containing AlyVMII or AlyVMI, respectively. It was not determined whether the gold-particles, and thus the protein, were attached to the cell membrane or just present in the periplasmic space. It could be that one of the enzymes is present in the cytoplasm of *V. midae* SY9 cells and the other in the periplasm or it could be that both enzymes are present in the cytoplasm and periplasm.

The literature cites antibodies raised against only three alginate lyases, A1-II' and A1-IV' from *Sphingomonas* sp. A1 (Hashimoto *et al* 2005; Miyake *et al*. 2004) and HdAly from *Haliotis discus hannai* (Hata *et al*. 2009), which were not used in immunolocalisation studies. Periplasmic alginate lyases have been described from *Azotobacter chroococcum* (Kennedy *et al* 1992), *A. vinelandii* (Kennedy *et al* 1992), the marine *Photobacterium* sp. ATCC 433367 (Malissard *et al*. 1993), *Pseudomonas aeruginosa* (Jain and Ohman, 2005) and *P. syringae* (Preston *et al*. 2000). An intracellular alginate lyase was also purified from *A. vinelandii* (Davidson *et al*. 1977a). Periplasmic alginate lyases are not normally expected to be in contact with their substrate alginate (Kennedy *et al* 1992). The periplasmic alginate lyase of *A. vinelandii* was proposed to prime the biosynthesis of alginate molecules, determine the length of the polymers and degrade the cyst coat during germination (Ertesvåg *et al*. 1998). The *P. aeruginosa* alginate lyase forms part of a scaffold of proteins in the periplasm which directs alginate polymers through the periplasm for secretion across the outer membrane (Jain and Ohman, 2005). The role of a periplasmic alginate lyase in *Vibrio midae* SY9 which does not produce alginate or form cysts is unknown.

In order to determine whether it is AlyVMI, AlyVMII or both present in the cytoplasm or periplasmic space, antibodies specific to each of these enzymes would be required to perform immunolocalisation experiments. Pre-absorbing anti-AlyVMI and anti-AlyVMII antibodies against *E. coli* cell lysates containing AlyVMII or AlyVMI, respectively, rendered the antibodies specific for the protein they were raised against (Chapter 3, Section 3.5). However, pre-absorbed antibodies proved to be too dilute for immunolabeling experiments. Optimization of the labeling protocol or possibly concentrating the antibodies might improve labeling with the pre-absorbed

antibodies. Alternatively, if these two alginate lyases were found to have different substrate specificities, enzyme activity studies of the periplasmic and cytoplasmic fractions of *Vibrio midae* SY9 cells could be performed.

In conclusion, *Vibrio midae* SY9 was negatively stained with methylamine tungstate and found to be rod-shaped with a single polar flagellum. Gold particles, and thus AlyVMI and AlyVMII, were observed in the cytoplasm and periplasm of cryo-sectioned cells treated with anti- AlyVMI or anti-AlyVMII antibodies. It remains unknown whether AlyVMI, AlyVMII or both enzymes are present in the cytoplasm or periplasm.

# Chapter 6

## General Discussion

Erasmus *et al*. (1997) showed bacteria resident in the gut of *H. midae* were able to degrade complex polysaccharides and suggested that bacterial enzymes play a role in the digestion of food ingested by the South African abalone, *Haliotis midae*, and that this could positively affect the growth rate of farmed abalone. Macey and Coyne (2005) isolated and identified *Vibrio midae* SY9 from the digestive tract of *H. midae*. This bacterium is able to degrade alginate, gelatin, protein and starch and produces an extracellular alginate lyase and protease. They showed that when administered as a cocktail of three probiotic strains in a formulated feed, *V. midae* SY9 increased the growth rate of farmed *H. midae* and improved the survival of animals challenged with the pathogen *Vibrio anguillarum*. The improved growth rate was attributed to an increase in intestinal protease activity which correlated with an increase in protein digestion and absorption in the intestine. Macey and Coyne (2006) subsequently showed that these three strains have the ability to colonize the gastrointestinal tract of *H. midae* and were detected 15 days after the cessation of feeding. Thus, these strains have great potential as probiotics for farm reared abalone.

*Pseudoalteromonas* sp. strain C4 was isolated by Erasmus *et al*. (1997) and shown to degrade alginate, cellulose and gelatin and produces an extracellular alginate lyase and protease. ten Doeschate and Coyne (2008) showed that *H. midae* fed a kelp-based diet supplemented with strain C4 had an increased growth rate compared to animals fed the unsupplemented kelp diet. There was increased alginate lyase activity in the digestive tract of probiotic treated animals suggesting that strain C4 contributes enzymes for the digestion of complex algal polysaccharides. It was suggested that the improved growth rate achieved with the probiotic-supplemented feed was a consequence of increased availability of nutrients for absorption in the gut, an increased pool of digestive enzyme present in the gastrointestinal tract as well as the probiont providing the abalone with an additional nutrient source, especially with regard to proteins which are limited in

the brown seaweed *Ecklonia maxima* that is commonly fed to abalone farmed on the west and southern coasts of South Africa.

*Vibrio halioticoli* was isolated from the gut of the Japanese abalone *Haliotis discus hannai* (Sawabe *et al*. 1995, 1998b). *V. halioticoli* produces six alginate lyases, four poly(G) specific and two poly(M) specific (Sugimura *et al*. 2000). Sawabe (2006) suggested that abalone and gut microbes co-operatively degrade alginate since alginate lyases from abalone and abalone enteric bacteria are typically specific for poly(M) and poly(G) regions of alginate, respectively. Furthermore, Sawabe *et al*. (2003) suggested that *V. halioticoli* contributes to the nutrition and energy metabolism of abalone by the production of VSCFAs.

Following on from the work of Erasmus and Macey, it would be interesting to determine the role of the *Vibrio midae* SY9 alginate lyase enzyme/s in the probiotic effect of *V. midae* SY9 on farmed *Haliotis midae*. However, in order to investigate this, it is important to know the characteristics and properties of the *V. midae* SY9 alginate lyase/s and how they function in alginate degradation by *V. midae* SY9 prior to attempting to assign their role in seaweed degradation by *H. midae*. The primary aim of this study was to clone, sequence and characterise the alginate lyase/s of *V. midae* SY9 and to begin to understand the role they play in alginate degradation by this bacterium.

A *Vibrio midae* SY9 genomic library was screened for alginolytic *E. coli* JM107 clones by visually detecting zones of alginate degradation on alginate agar after flooding with 70% ethanol. Plasmid pAlg15 was chosen for further analysis and was subsequently sequenced. The full length sequence of one and the partial sequence of two other alginate lyase genes were identified on pAlg15. The alginate lyase genes were name *alyVMI*, *alyVMII* and *alyVMIII*. A further two *V. midae* SY9 genomic libraries were constructed and screened in order to complete the sequence of *alyVMI* and *alyVMIII*. Two plasmids, pAlg65 and pAlg86, were isolated and sequenced. Plasmids pAlg65 and pAlg86 completed the sequences of *alyVMI* and *alyVMIII*, respectively, and a fourth alginate lyase gene, named *alyVMIV*, was found to be present on pAlg86 downstream of *alyVMIII*. A putative oligogalacturonate specific porin, a putative pectin

degrading protein and a putative deoxygluconokinase were identified adjacent to the four alginate lyase genes.

AlyVMI and AlyVMII were predicted to be ~81 kDa and AlyVMIII and AlyVMIV were predicted to be ~54 kDa and ~57 kDa, respectively. AlyVMI and AlyVMII contain a chondroitin AC/alginate lyase domain and a heparinase II/III-like domain. AlyVMIII and AlyVMIV contain an alginate lyase 2 domain and a concanavalin A-like lectin/glucanase domain. AlyVMIV also contains a signal peptide, a coagulation factor 5/8 type domain and a galactose binding-like domain. AlyVMIII and AlyVMIV contain the alginate lyase conserved sequence motifs RXELR, QIH and YFKAGXYXQ, where X is any residue, and could therefore be placed into polysaccharide lyase family 7 (PL-7). AlyVMI and AlyVMII do not contain any of the alginate lyase conserved sequence motifs and were placed in family PL-17. It was also determined that *alyVMI* and *alyVMII* are co-transcribed.

Alginate lyase genes *alyVMI*, *alyVMII*, *alyVMIII* and *alyVMIV* were cloned into the expression vector pET-29a. Western hybridisation analysis demonstrated that *alyVMI*, *alyVMII* and *alyVMIV* were expressed in the host *E. coli* BL21 (DE3) pLysS and were histidine tagged. Alginate lyase gene *alyVMIII* was not expressed in *E. coli* BL21 (DE3) pLysS since the ribosome binding site had been removed from the vector during cloning. AlyVMI and AlyVMII were purified using nickel affinity chromatography. AlyVMIV was not purified to homogeneity since *E. coli* proteins eluted with the enzyme. Optimization of the conditions for expression of *alyVMIV* and AlyVMIV purification did not improve the purity of AlyVMIV.

Polyclonal antibodies raised against purified recombinant AlyVMI and AlyVMII recognized both AlyVMI and AlyVMII. Pre-absorption of anti-AlyVMI and anti-AlyVMII against *E. coli* BL21 (DE3) pLysS expressing alyVMII and AlyVMI, respectively, rendered the antibodies specific for the protein to which they were raised. The antibodies recognized the native and recombinant forms of the proteins and the predicted, recombinant and native molecular weights of AlyVMI and AlyVMII were comparable. Only recombinant AlyVMIV was active in the cell lysates and culture supernatant of *E. coli* BL21 (DE3) pLysS transformed with pETalyVMIV.

We hypothesise the following potential mechanism of alginate degradation by *Vibrio midae* SY9: AlyVMIV is exported from the cell and degrades the alginate polymer to oligo-alginates which can enter the cell. The oligo-galacturonate specific porin may play a role in the uptake of oligo-alginates. AlyVMI, AlyVMII and AlyVMIII are intracellular enzymes and would degrade the alginate molecule to mono- or oligosaccharides which could enter cellular metabolism. The deoxygluconokinase could phosphorylate the mono- or oligosaccharides at this point allowing them to enter the general metabolism of the cell.

Knowledge of the substrate specificities of the four alginate lyases would be valuable in elucidating the mechanism of alginate degradation by *Vibrio midae* SY9. The minimum size of oligo-alginates required as a substrate for activity and the alginate degradation products produced by each enzyme would also be useful. Purified active recombinant proteins would be required in order for this to be determined. Further characterisation of the alginate lyase enzymes may include enzyme kinetic studies and determination of optimum pH, temperature and ionic strength required for activity. It would also be interesting to determine the crystal structure of AlyVMI and AlyVMII. The crystal structure of a PL-17 alginate lyase, which has not yet been solved, would provide reference information for other PL-17 alginate lyases.

The role, if any, of the putative oligogalacturonate specific porin, pectin degrading protein and deoxygluconokinase in alginate degradation by *Vibrio midae* SY9 could be established. Whether *V. midae* SY9 is able to degrade pectin, and how the pectin degrading protein is involved, could also be determined. The substrate of the putative oligogalacturonate specific porin and deoxygluconokinase should be determined experimentally and whether they are the alginate and/or pectin degradation products. Purification of these proteins would be useful in achieving these aims. First, the full length sequence of the deoxygluconokinase would be required.

Alginate lyase genes *alyVMI* and *alyVMII* are 6 bp apart and were shown to be co-transcribed. This could indicate a regulatory mechanism for expression of these genes by *Vibrio midae* SY9. It would be interesting to determine whether any of the other genes cloned in this study are also co-transcribed. Potential candidates for co-transcription are the putative oligogalacturonate specific porin and *alyVMIV* which are only 79 bp apart, and the putative pectin degrading protein

and the putative deoxygluconokinase which are only 37 bp apart. It would seem as though genes required for the utilisation of carbohydrates are clustered together in one region of the *Vibrio midae* SY9 chromosome. It would be interesting to know what genes are present upstream and downstream of this fragment of the *V. midae* SY9 chromosome, whether they are co-transcribed and whether they are regulated in conjunction with the genes identified in this study. It may even be that some of these genes act in an operon.

Gene expression studies were conducted to determine whether the four alginate lyase genes of *Vibrio midae* SY9 are regulated at the transcriptional, translational or post-translational level. *V. midae* SY9 was cultured in alginate media with and without glucose for 24 hours. Cell growth was monitored over the course of the experiment by measuring absorbance at 600 nm. Real-time RT PCR and an indirect ELISA assay were used to determine mRNA transcript levels and levels of AlyVMI and AlyVMII, respectively, present in the cell during the growth experiment. Intracellular and extracellular alginate lyase activities were determined at each point of the growth experiment using the thiobarbituric acid (TBA) assay. The amount of reducing sugar present in the culture supernatant at each point of the growth experiment was assayed using the dinitrosalicylic acid (DNS) reducing sugar assay. The mRNA transcripts of the four alginate lyase genes were found not to be significantly regulated in response to glucose during the growth experiment, nor were the intracellular alginate lyase levels. However, alginate lyase activity was only detected in the culture supernatant and cell lysates when the reducing sugar level in the culture media was low. It was hypothesised that the four alginate lyase genes of *Vibrio midae* SY9 are expressed when alginate is present in the growth media irregardless of the presence of glucose, and that they are regulated post-translationally, the mechanism of which is unknown.

Post-translational regulation of alginate lyase enzymes has not been previously reported. Regulation of the alginate lyase genes of *V. midae* SY9 should be further investigated to validate the hypothesis of post-translational regulation. Studies should be conducted to conclusively exclude carbon catabolite repression by cAMP as reported in *E. coli*. The present gene expression study could be repeated with different substrates and growth conditions to determine whether similar results are obtained. Subsequently, the mechanism of post-translational modification of the enzymes should be elucidated. Enzyme activity studies could also be

performed to establish whether a co-factor or an inhibitor exists that could play a role in the activity of these enzymes.

*Vibrio midae* SY9 was negatively stained using methylamine tungstate and shown to be a rod-shaped bacterium, ~0.7 µm in diameter and ~2 µm in length, with a single polar flagellum. Fixative conditions for immunolabeling were optimized and recognition of AlyVMII by anti-AlyVMII antibodies was shown to be sensitive to glutaraldehyde fixation. Optimal fixation conditions were found to be a solution containing 4% formaldehyde and 0.2% glutaraldehyde. *V. midae* SY9 was cultured for 10 hours in alginate media, fixed in 4% formaldehyde and 0.2% glutaraldehyde and embedded in LR White acrylic resin or cryo-fixed according to the method of Tokuyasu (1973).

Ultra-thin sections of resin embedded or cryo-fixed cells were immunolabeled with either anti-AlyVMI or anti-AlyVMII antibodies using a 10 nm gold conjugated secondary antibody. A clear double cell membrane was observed in the cryo-sections suggesting that *V. midae* SY9 cells were better preserved when cryo-fixed than when embedded in resin. Low levels of antigen labeling were observed with the anti-AlyVMI and anti-AlyVMII antibodies in both the resin-embedded and cryo-fixed *V. midae* SY9 sections. Gold particles were observed in the cytoplasm and periplasm of *V. midae* SY9 cells when sections were labeled with anti-AlyVMI or anti-AlyVMII antibodies. It was not possible to determine whether AlyVMI, AlyVMII or both alginate lyases were present in the cytoplasm and periplasm.

Antibodies specific to AlyVMI and AlyVMII are required to determine whether AlyVMI, AlyVMII or both enzymes are present in the cytoplasm or periplasmic space using immunolocalisation. Indeed, preabsorption of anti-AlyVMI and anti-AlyVMII against *E. coli* cell lysates containing AlyVMII or AlyVMI, respectively, rendered the antibodies specific for the protein to which they were raised. However, pre-absorbed antibodies were too dilute for immunolabeling experiments. Optimising the labeling protocol or concentrating the pre-absorbed antibodies might improve labeling with the pre-absorbed antibodies.

It would be interesting to know the relative cellular locations of the four alginate lyase enzymes in *Vibrio midae* SY9 cultured in alginate media as well as the location of the putative oligogalacturonate specific porin and the putative deoxygluconokinase should they be shown to be involved in alginate degradation by *V. midae* SY9. Antibodies against AlyVMIII, AlyVMIV, the putative oligogalacturonate specific porin and the putative deoxygluconokinase would be required for which purified protein would first be needed. Antibodies against AlyVMIII and AlyVMIV would also be useful for further gene expression studies.

Although this study characterised the four alginate lyase genes and enzymes of *Vibrio midae* SY9, the role of each alginate lyase enzyme in alginate degradation by *V. midae* SY9 remains unclear. Topics for further investigation mentioned above will facilitate the clarification of these roles. The following suggested topics for further study will aid in investigating the role of the alginate lyases of *Vibrio midae* SY9 in seaweed digestion by *Haliotis midae*.

It would be useful to clone, sequence and characterise the alginate lyase gene/s of *H. midae*. This would allow for corroboration of Sawabe's (2006) hypothesis that abalone and resident gut bacteria co-operatively degrade alginate. Whether *V. midae* SY9 contributes to the nutrition and energy metabolism of *H. midae* by the production of volatile short-chain fatty acids (VSCFAs), such as acetate and formate, could also be determined. Alternatively, *V. midae* SY9 may act as an additional nutrient and protein source for kelp-fed *H. midae* in a similar manner to *Pseudoalteromonas* sp. strain C4.

Feeding trials could be performed in which *Haliotis midae* is fed a kelp based diet supplemented with wild type *Vibrio midae* SY9 or an alginate lyase deficient strain. The growth of the animals would be monitored as well as alginate lyase activity and bacterial load in the digestive tract. In situ hybridisation and immunolocalisation of growth trial animals would reveal the presence and position of *V. midae* SY9 and the *V. midae* SY9 alginate lyases, respectively, in the abalone digestive tract. If an antibody against the *H. midae* alginate lyase/s was available, immunolocalisation of growth trial animals using this antibody might prove insightful. An alginate lyase deficient *V. midae* SY9 strain would first need to be constructed. The use of

effective probiotics will have a positive impact on abalone mariculture in South Africa as a result of improved growth rates and increased resistance to disease of probiotic-fed farmed animals.

In conclusion, this is the first study to report the cloning and sequencing of four alginate lyase genes adjacent to each other, and adjacent to other genes involved in carbohydrate metabolism, on a bacterial chromosome. This is also the first study to report the possibility of post-translationally regulated alginate lyase genes. The characterisation of the four alginate lyase genes described in this study has laid the foundation for elucidating their role in seaweed digestion by *Haliotis midae*.

# APPENDIX A

## Media and Solutions

### CONTENTS

All media and solutions were autoclaved at 121°C for 20 min prior to use unless otherwise stated.

Water used for making solutions, media and diluting buffers was purified using a Milli-RO Plus (Millipore) water purification system.

## A.1 Media

### A.1.1. Luria broth (LB)

| | |
|---|---|
| Tryptone (Biolab) | 10 g |
| Yeast extract (Biolab) | 5 g |
| NaCl (Saarchem) | 5 g |
| $dH_2O$ to | 1 L |

### A.1.2. Luria agar (LA)

| | |
|---|---|
| Tryptone | 10 g |
| Yeast extract | 5 g |
| NaCl | 5 g |
| Agar (Biolab) | 15 g |
| $dH_2O$ to | 1 L |

### A.1.3. Ψ broth

| | |
|---|---|
| Tryptone | 20 g |
| Yeast extract | 5 g |
| $MgSO_4.7H_2O$ (Saarchem) | 4 g |
| KCl (Saarchem) | 0.75 g |
| $dH_2O$ to | 1 L |

### A.1.4. Alginate lyase detection agar

| | |
|---|---|
| Tryptone | 10 g |
| Yeast extract | 5 g |
| NaCl | 5 g |
| Alginate (Sigma) | 5 g |
| ASW | 500 ml |
| Agar | 15 g |
| $dH_2O$ to | 1 L |

Dissolve the alginate in $dH_2O$ by heating first and then add the remaining components.

### A.1.5. Marine broth (MB)

| | |
|---|---|
| NaCl | 30 g |
| $MgCl_2.6H_2O$ (Saarchem) | 2.3 g |
| KCl | 0.3 g |
| Casamino acids (Difco) | 5 g |
| Yeast extract | 1 g |
| D-glucose (Saarchem) | 2 g |
| $dH_2O$ to | 1 L |

### A.1.6. Marine agar (MA)

| | |
|---|---|
| NaCl | 30 g |
| $MgCl_2.6H_2O$ | 2.3 g |
| KCl | 0.3 g |
| Casamino acids | 5 g |
| Yeast extract | 1 g |
| D-glucose | 2 g |
| Agar | 20 g |
| $dH_2O$ to | 1 L |

### A.1.7. Alginate media

| | |
|---|---|
| NaCl | 30 g |
| $MgCl_2.6H_2O$ | 2.3 g |
| KCl | 0.3 g |
| Tryptone | 5 g |
| Yeast extract | 1 g |
| Alginate | 5 g |
| dH2O to | 1 L |

Dissolve the alginate in the $dH_2O$ by heating before adding the remaining ingredients.



## A.2 Solutions

### A.2.1. Antibiotic solutions

Ampicillin (100 mg/ml)

Dissolve 2 g ampicillin (Sigma) in 20 ml $dH_2O$ and filter sterilise. Store 1 ml aliquots at -20°C. Dilute 1:1000 into media for a final concentration of 100 µg/ml.

Kanamycin (30 mg/ml)

Dissolve 0.6 g Kanamycin (Sigma) in 20 ml $dH_2O$ and filter sterilise. Store 1 ml aliquots at -20°C. Dilute 1:1000 into media for a final concentration of 30 µg/ml

Chloramphenicol (20 mg/ml)

Dissolve 0.4 g Chloramphenicol (Sigma) in 20 ml of $dH_2O$. Filter sterilise and store aliquots at 4°C. Dilute 1:1000 into medium for a final concentration of 20 µg/ml.

### A.2.2. Artificial sea water (ASW)

| | |
|---|---|
| NaCl | 30 g |
| KCl | 0.7 g |
| $MgCl_2$ | 10.8 g |
| $MgSO_4$ (Biolab) | 5.3 g |
| $CaSO_4$ (BDH Chemicals) | 1.3 g |
| $dH_2O$ to | 1 L |

Dissolve the $CaSO_4$ by heating.

### A.2.3. General stock solutions

0.5 M EDTA

| | |
|---|---|
| EDTA (Saarchem) | 93.05 g |
| NaOH (Saarchem) | 10 g |
| $dH_2O$ to | 500 ml |

Dissolve the EDTA and NaOH in 400 ml $dH_2O$, adjust the pH to 8.0 and make up to a final volume of 500 ml with $dH_2O$.

1 M Tris Base

| | |
|---|---|
| Tris (Melford) | 12.1 g |
| $dH_2O$ | 100 ml |

1 M Tris-HCl

| | |
|---|---|
| Tris | 12.1 g |
| $dH_2O$ | 100 ml |

Dissolve the Tris in 80ml $dH_2O$ and adjust the pH to the required level using concentrated HCl. Make up to a final volume of 100ml with $dH_2O$.

TE Buffer (Tris-EDTA)

| | |
|---|---|
| 1 M Tris, pH7.6 | 1 ml |
| 0.5 M EDTA, pH8 | 200 µl |
| Sterile $dH_2O$ to | 100 ml |

10 M NaOH

| | |
|---|---|
| NaOH | 40 g |
| $dH_2O$ | 100 ml |

Dissolve the NaOH pellets in 80 ml $dH_2O$. Make up to a final volume of 100 ml using $dH_2O$. Do not autoclave. Store in a plastic bottle at room temperature.

70% Ethanol

| | |
|---|---|
| 100% ethanol (Sigma) | 70 ml |
| $dH_2O$ to | 100 ml |

Do not autoclave.

20% Glucose

| | |
|---|---|
| D-Glucose | 20 g |
| $dH_2O$ to | 100 ml |

25% Sodium Dodecyl Sulphate (SDS)

| | |
|---|---|
| SDS (Saarchem) | 25 g |
| $dH_2O$ to | 100 ml |

Dissolve the SDS in 80 ml $dH_2O$ with stirring on a heat plate. Do not allow to boil. Make up to a final volume of 100 ml with $dH_2O$. Do not autoclave.

10% SDS

| | |
|---|---|
| SDS | 10 g |
| $dH_2O$ to | 100 ml |

Dissolve the SDS in 80 ml $dH_2O$ with stirring on a heat plate. Do not allow to boil. Make up to a final volume of 100 ml with $dH_2O$. Do not autoclave.

10x PBS

| | |
|---|---|
| NaCl | 80 g |
| KCl | 2 g |
| $Na_2HPO_4$ (Saarchem) | 14.2 g |
| $KH_2PO_4$ (Saarchem) | 2.4 g |
| $dH_2O$ to | 1 L |

Dissolve in 800 ml of $dH_2O$. Adjust the pH to 7.4 using 10 M NaOH. Make up to a final volume of 1 L with $dH_2O$.

1x PBS

Dilute 10x PBS 1:10 in sterile $dH_2O$.

### A.2.4. Solutions for preparation of competent *Escherichia coli* cells

Solution 1

| | |
|---|---|
| 1 M RbCl (Sigma) | 10 ml |
| $MnCl_2.4H_2O$ (Saarchem) | 0.99 g |
| KOA (Saarchem) | 0.294 g |
| 750 mM $CaCl_2.2H_2O$ (Saarchem) | 1.34ml |
| 50% Glycerol (Saarchem) | 30 ml |
| $dH_2O$ to | 100ml |

Adjust to pH 5.8 with glacial acetic acid. Make up to a final volume of 100 ml with $dH_2O$ and filter sterilise.

Solution 2

| | |
|---|---|
| 100 mM MOPS (Sigma) | 10 ml |
| 1 M RbCl | 1 ml |
| 750 mM $CaCl_2.2H_2O$ | 10 ml |
| 50 % Glycerol | 30 ml |
| $dH_2O$ to | 100ml |

Make up to a final volume of 100 ml with $dH_2O$ and filter sterilise.

### A.2.5. Solutions for small scale plasmid isolation

Solution 1

| | |
|---|---|
| 1 M Tris-Cl pH8 | 25 ml |
| 0.5 M EDTA | 20 ml |
| 20% Glucose | 45.5 ml |
| Sterile $dH_2O$ to | 100 ml |

Solution 2

| | |
|---|---|
| 10 M NaOH | 2 ml |
| 25% SDS | 4 ml |
| $dH_2O$ to | 100 ml |

Make solution fresh before use.

Solution 3

| | |
|---|---|
| KOA | 147 g |
| $dH_2O$ to | 500 ml |

Dissolve the KOA in 200 ml $dH_2O$. Adjust the pH to 4.8 with glacial acetic acid. Make up to a final volume of 500 ml with $dH_2O$.

### A.2.6. Solutions for restriction endonuclease digests

6x Gel Tracking Dye

| | |
|---|---|
| Bromophenol Blue (Saarchem) | 62.5 mg |
| Sucrose | 10 g |
| 0.5 M EDTA, pH 8.0 | 1 ml |
| $dH_2O$ to | 25 ml |

### A.2.7. Solutions for agarose gels

50x Tris-Acetate Buffer (TAE)

| | |
|---|---|
| Tris | 242 g |
| EDTA | 37.2 g |
| Glacial Acetic Acid (Saarchem) | 57.1 ml |
| $dH_2O$ | 1 L |

1x TAE Buffer
Dilute 50x TAE 1:50 with $dH_2O$.

Ethidium Bromide (EtBr) Solution

| | |
|---|---|
| EtBr (Sigma) | 0.1 g |
| $dH_2O$ to | 10 ml |

Shake well to dissolve. Do not autoclave. Store in a dark bottle at room temperature. This is a powerful mutagen, gloves should be worn at all times when handling this solution.

### A.2.8. Solutions for TBA assay

Alginate Substrate

| | |
|---|---|
| Alginate (Sigma) | 0.4 g |
| Phosphate buffer | 100 ml |

Dissolve the alginate in 80 ml phosphate buffer with stirring on a hot plate. Make up to a final volume of 100 ml with phosphate buffer.

Phosphate Buffer

| | |
|---|---|
| NaCl | 8.78 g |
| $Na_2HPO_4$ | 2.10 g |
| $K_2HPO_4$ (Saarchem) | 1.09 g |
| $dH_2O$ to | 1 L |

Periodate Solution

| | |
|---|---|
| Periodate (Sigma) | 0.115 g |
| 98% $H_2SO_4$ (Saarchem) | 0.25 ml |
| $dH_2O$ to | 20 ml |

Make solution fresh before use.

Sodium Arsenate Solution

| | |
|---|---|
| Sodium Arsenate (Fluka) | 0.7 g |
| 32% HCl (Saarchem) | 2 ml |
| $dH_2O$ to | 35 ml |

Do not autoclave. This solution is toxic and should always be handled with gloves.

Thiobarbituric Acid (TBA) pH2.3

| | |
|---|---|
| TBA (Sigma) | 0.3 g |
| $dH_2O$ to | 100 ml |

Dissolve the TBA in 80 ml of $dH_2O$ with stirring on a hot plate. Adjust the pH to 2.3 and make up to a final volume of 100 ml. Do not autoclave. Discard the solution when it turns yellow.

### A.2.9. Solutions for bacterial genomic DNA isolation

Proteinase K (20 mg/ml)

| | |
|---|---|
| Proteinase K (Roche) | 20 mg |
| Sterile $dH_2O$ to | 1 ml |

Do not autoclave. Store aliquots at -20°C.

5 M NaCl

| | |
|---|---|
| NaCl | 29.22 g |
| $dH_2O$ to | 100 ml |

CTAB/NaCl

| | |
|---|---|
| NaCl | 4.1 g |
| CTAB (Sigma) | 10 g |
| $dH_2O$ to | 100 ml |

Dissolve the NaCl in 80 ml $dH_2O$ and slowly add the CTAB (hexadecyltrimethyl ammonium bromide) while stirring on a heat plate. If necessary, heat to 65°C to dissolve. Make up to a final volume of 100 ml with $dH_2O$. Do not autoclave.

Chloroform (Saarchem) / Isoamyl alcohol (Saarchem)
Mix at a ratio of 24:1. Do not autoclave.

RNAse A

| | |
|---|---|
| RNase A (Roche) | 0.1 g |
| 1 M Tris-HCl, pH 7.5 | 100 µl |
| 5 M NaCl | 3 ml |
| $dH_2O$ to | 10 ml |

Heat for 15 min at 100 ºC. Allow to cool to room temperature slowly. Do not autoclave. Store 1ml aliquots at -20°C.

### A.2.10. Solutions for Southern and slot blot transfer of DNA

0.25 M HCl

| | |
|---|---|
| HCl | 21.35 ml |
| $dH_2O$ to | 1 L |

1 M NaOH

| | |
|---|---|
| NaOH | 4 g |
| $dH_2O$ to | 100 ml |

0.4 M NaOH

| | |
|---|---|
| NaOH | 16 g |
| $dH_2O$ to | 100 ml |

0.4 M NaOH / 1M NaCl

| | |
|---|---|
| NaOH | 16 g |
| NaCl | 58.44 g |
| $dH_2O$ to | 1 L |

20x Sodium Chloride Tri-Sodium Citrate (SSC)

| | |
|---|---|
| NaCl | 17.5 g |
| Tri-Na Citrate (Saarchem) | 8.82 g |
| $dH_2O$ to | 100 ml |

Dissolve the NaCl and Tri-Na Citrate in 80 ml $dH_2O$. Adjust the pH to 7.4 with NaOH. Make up to a final volume of 100 ml with $dH_2O$.

2x SSC
Dilute 20x SSC 1:10 with sterile $dH_2O$.

Wash Buffer A (WBA)

| | |
|---|---|
| 20x SSC | 10 ml |
| 10% SDS | 1 ml |
| $dH_2O$ to | 100 ml |

Wash Buffer B (WBB)

| | |
|---|---|
| 20x SSC | 2.5 ml |
| 10% SDS | 1 ml |
| $dH_2O$ to | 100 ml |

### A.2.11. Solutions for immunological detection of DIG-labeled DNA probes

5x Maleic Acid Buffer

| | |
|---|---|
| Maleic Acid (Sigma) | 58.03 g |
| NaCl | 43.83 g |
| NaOH | 35 g |
| $dH_2O$ to | 1 L |

Dissolve the maleic acid, NaCl and NaOH in 800 ml $dH_2O$. Adjust the pH to 7.5 with NaOH. Make up to a final volume of 1 L with $dH_2O$.

Washing Buffer

| | |
|---|---|
| 5x Maleic Acid | 20 ml |
| Tween 20 (Saarchem) | 300 µl |
| $dH_2O$ to | 100 ml |

Make solution fresh before use.

Blocking Buffer

| | |
|---|---|
| 10x Blocking solution (Roche) | 10 ml |
| 5x Maleic Acid | 20 ml |
| $dH_2O$ to | 100 ml |

Antibody Solution

Dilute anti-digoxigenin-AP (Roche) 1:10000 in blocking buffer. Centrifuge anti-digoxigenin-AP in the original vial at 10 000 rpm for 5 min prior to use. Carefully pipet the require amount from the surface.

Detection Buffer

| | |
|---|---|
| Tris | 12.11 g |
| NaCl | 5.84 g |
| $dH_2O$ to | 1 L |

Dissolve the Tris and NaCl in 800 ml of $dH_2O$. Adjust the pH to 9.5 and make up to a final volume of 1 L.

### A.2.12. Solutions for colony lifts

Denaturation Solution

| | |
|---|---|
| NaCl | 43.83 g |
| NaOH | 10 g |
| $dH_2O$ to | 500 ml |

Neutralisation Solution

| | |
|---|---|
| Tris | 60.57 g |
| NaCl | 43.83 g |
| $dH_2O$ to | 500 ml |

Dissolve the Tris and NaCl in 400 ml $dH_2O$. Adjust the pH to 7.4 and make up to a final volume of 500 ml using $dH_2O$.

### A.2.13. Solutions for total RNA isolations

All solutions were made up in baked glass bottles using sterile DEPC treated $dH_2O$ and autoclaved twice unless otherwise stated.

Sterile DEPC treated $dH_2O$

| | |
|---|---|
| DEPC (Sigma) | 1 ml |
| $dH_2O$ to | 1 L |

Dissolve the DEPC in the $dH_2O$ with vigorous shaking. Leave the solution in a fume cupboard with the cap loosened overnight. Autoclave twice.

0.5 M EDTA

| | |
|---|---|
| EDTA | 93.05 g |
| NaOH | 10 g |
| DEPC $dH_2O$ | 500 ml |

Dissolve the EDTA and NaOH in DEPC treated $dH_2O$. Adjust the pH to 8.0 and make up to a final volume of 500 ml with DEPC treated $dH_2O$.

1 M Tris-HCl

| | |
|---|---|
| Tris | 12.1 g |
| DEPC $dH_2O$ to | 100 ml |

Dissolve the Tris in DEPC treated $dH_2O$. Adjust the pH to the require level using concentrated HCl. Make up to a final volume of 100 ml with DEPC treated $dH_2O$.

Tris-EDTA (TE) Buffer

| | |
|---|---|
| 1M Tris-HCl, pH 7.6 | 1 ml |
| 0.5M EDTA | 200 µl |
| DEPC $dH_2O$ to | 100 ml |

1 M Sucrose

| | |
|---|---|
| Sucrose (Saarchem) | 34.23 g |
| DEPC $dH_2O$ to | 100 ml |

Dissolve the sucrose in 80 ml DEPC treated $dH_2O$. Make up to a final volume of 100 ml with DEPC treated $dH_2O$. Autoclave once only.

70% Ethanol

| | |
|---|---|
| 100% ethanol | 70 ml |
| DEPC $dH_2O$ to | 100 ml |

Do not autoclave. Store at -20°C.

Protoplast Buffer

| | |
|---|---|
| 1 M Tris-Cl, pH 8.0 | 1.5 ml |
| 1 M Sucrose | 45 ml |
| 0.5 M EDTA | 1.58 ml |
| DEPC $dH_2O$ to | 100 ml |

Do not autoclave. Store at 4°C.

80 mg/ml Lysozyme

| | |
|---|---|
| Lysozyme (Fluka) | 0.8 g |
| DEPC $dH_2O$ to | 10 ml |

Dissolve the Lysozyme in the DEPC treated $dH_2O$. Store 1 ml aliquots at -20°C. Do not autoclave.

Gram Lysing Buffer

| | |
|---|---|
| 1 M Tris-Cl, pH 8.0 | 1 ml |
| NaCl | 58 mg |
| Na Citrate (Saarchem) | 29 mg |
| SDS | 1.5 g |
| DEPC $dH_2O$ to | 100 ml |

Autoclave once only. Store at room temperature.

Saturated NaCl

| | |
|---|---|
| NaCl | 40 g |
| DEPC $dH_2O$ to | 100 ml |

Stir until the solution reaches saturation.

Phenol (Sigma) / chloroform / isoamyl alcohol
Mix at a ratio of 25:24:1
Do not autoclave. Store at 4°C in a dark bottle.

3 M Sodium Acetate

| | |
|---|---|
| Na-acetate (Saarchem) | 204.05 g |
| DEPC $dH_2O$ to | 500 ml |

Dissolve the sodium acetate in 400 ml DEPC treated $dH_2O$. Adjust the pH to 5.2 using glacial acetic acid. Make up the final volume to 500 ml with DEPC treated $dH_2O$.

### A.2.14. Solutions for formaldehyde/MOPS RNA agarose gels

10x MOPS

| | |
|---|---|
| MOPS | 20 g |
| Na-acetate | 1 g |
| 0.5 M EDTA | 10 ml |
| DEPC $dH_2O$ to | 500 ml |

Dissolve the Mops and sodium acetate in 400 ml DEPC treated $dH_2O$ and add the EDTA. Adjust the pH to 7.0 using NaOH. Make up to a final volume of 500 ml with DEPC treated $dH_2O$. Filter sterilise. Store at 4°C in a dark bottle.

1x MOPS
Dilute 10x MOPS 1:10 with DEPC treated $dH_2O$. Store at 4°C in a dark bottle.

RNA Sample Application Buffer

| | |
|---|---|
| 10x MOPS, pH 7.0 | 300 µl |
| 37% Formaldehyde (Fluka) | 80 µl |
| Formamide (Fluka) | 900 µl |
| 10 mg/ml EtBr | 2 µl |
| DEPC $dH_2O$ to | 1.5 ml |

Do not autoclave. Store aliquots at -20°C.

### A.2.15. Solutions for denaturing SDS-PAGE

Separating gel buffer

| | |
|---|---|
| Tris | 90.85 g |
| $dH_2O$ to | 500 ml |

Dissolve the Tris in 400 ml $dH_2O$ and adjust the pH to 8.8 using concentrated HCl. Make up to a final volume of 500 ml with $dH_2O$.

Stacking gel buffer

| | |
|---|---|
| Tris | 30.28 g |
| $dH_2O$ to | 500 ml |

Dissolve the Tris in 400 ml $dH_2O$ and adjust the pH to 6.8 using concentrated HCl. Make up to a final volume of 500 ml $dH_2O$.

SDS-PAGE running buffer

| | |
|---|---|
| Glycine (Saarchem) | 15 g |
| Tris | 3 g |
| SDS | 1 g |
| $dH_2O$ to | 1 L |

Sample application buffer

| | |
|---|---|
| Stacking gel buffer | 5 ml |
| SDS | 2 g |
| Glycerol | 10 ml |
| DTT (Promega) | 1.54 g |
| Bromophenol blue | 0.25 mg |
| $dH_2O$ to | 20 ml |

Coomassie blue staining solution

| | |
|---|---|
| Methanol (Merck) | 250 ml |
| Acetic acid (Merck) | 50 ml |
| Coomassie$^R$ Blue R250 (BDH) | 0.5 g |
| $dH_2O$ to | 500 ml |

Do not autoclave.

Destain solution

| | |
|---|---|
| Ethanol | 250 ml |
| Acetic acid | 100 ml |
| $dH_2O$ to | 1 L |

Do not autoclave.

### A.2.16. Solutions for protein purification using His-Select® Nickel Affinity Gel

1 M Imidazole

| | |
|---|---|
| Imidazole (Sigma) | 6.8 g |
| Sterile $dH_2O$ to | 100 ml |

Do not autoclave.

1 M Sodium phosphate

| | |
|---|---|
| $NaH_2PO_4.H_2O$ (Saarchem) | 13.79 g |
| $dH_2O$ to | 100 ml |

Dissolve the $NaH_2PO_4.H_2O$ in 80 ml $dH_2O$. Adjust the pH to 8.0 using NaOH and make up to a final volume of 100 ml.

Lysis buffer (10 mM imidazole)

| | |
|---|---|
| 1 M Sodium phosphate, pH8.0 | 5 ml |
| 1 M Imidazole | 1 ml |
| 5 M NaCl | 6 ml |
| Sterile $dH_2O$ to | 100 ml |

Do not autoclave. Store at 4°C.

Equilibration buffer (10 mM imidazole)

| | |
|---|---|
| 1 M Sodium phosphate, pH8.0 | 5 ml |
| 1 M Imidazole | 500 µl |
| 5 M NaCl | 6 ml |
| Sterile $dH_2O$ to | 100 ml |

Do not autoclave. Store at 4°C

Wash buffer (20 mM imidazole)

| | |
|---|---|
| 1 M Sodium phosphate, pH8.0 | 5 ml |
| 1 M Imidazole | 1 ml |
| 5 M NaCl | 6 ml |
| Sterile $dH_2O$ to | 100 ml |

Do not autoclave. Store at 4°C

Elution Buffer (250 mM imidazole)

| | |
|---|---|
| 1 M Sodium phosphate, pH8.0 | 5 ml |
| 1 M Imidazole | 25 ml |
| 5 M NaCl | 6 ml |
| Sterile $dH_2O$ to | 100 ml |

Do not autoclave. Store at 4°C.

### A.2.17. Solutions for electroblotting of proteins onto a nitrocellulose membrane

Towbin transfer buffer

| | |
|---|---|
| Tris | 3.03 g |
| Glycine | 14.42 g |
| Methanol | 200 ml |
| $dH_2O$ to | 1 L |

Do not autoclave. Store at 4°C.

### A.2.18. Solutions for western hybridisation analysis

Blocking solution

| | |
|---|---|
| 10x PBS | 10 ml |
| Non-fat dried milk | 5 g |
| 10% Tween 20 | 1 ml |
| $dH_2O$ to | 100 ml |

Washing solution

| | |
|---|---|
| 10x PBS | 10 ml |
| 10% Tween 20 | 1 ml |
| $dH_2O$ to | 100 ml |

### A.2.19. Solutions for ELISA assay

Tris-buffered saline (TBS)

| | |
|---|---|
| 1 M Tris-Cl, pH 7.5 | 10 ml |
| 5 M NaCl | 20 ml |
| Tween 20 | 480 µl |
| $dH_2O$ to | 1 L |

0.1 M $MgCl_2$

| | |
|---|---|
| $MgCl_2.6H_2O$ | 2.03 g |
| $dH_2O$ to | 100 ml |

Equilibration buffer

| | |
|---|---|
| Diethanolamine (Merck) | 10 ml |
| 100 mM $MgCl_2$ | 500 µl |
| $dH_2O$ to | 100 ml |

Dissolve the diethanolamine and $MgCl_2$ in 80 ml $dH_2O$. Adjust pH to 9.6 using NaOH and make up to a final volume of 100 ml.

### A.2.20. Solutions for PEG-precipitation of antibodies from immune sera

Borate buffered saline

| | |
|---|---|
| Boric acid (Saarchem) | 0.216 g |
| NaCl | 0.219 g |
| NaOH | 0.07 g |
| 37% HCl | 62 µl |
| $dH_2O$ to | 10 ml |

Dissolve the boric acid, NaCl, NaOH in 80 ml $dH_2O$. Adjust the pH to 8.6 using NaOH and make up to a final volume of 100 ml.

### A.2.21. Solutions for dinitrosalicylic acid (DNS) reducing sugar assay

DNS reagent

| | |
|---|---|
| 3,5-Dinitrosalycylic acid (Sigma) | 5 g |
| 1.2 M NaOH | 100 ml |
| NaK-tartrate (Saarchem) | 150 g |
| $dH_2O$ to | 500 ml |

Dissolve the 3,5-dinitrosalycylic acid in 100 ml of 1.2 M NaOH with stirring on a hot plate. Dissolve the NaK-tartrate in 250 ml $dH_2O$ with stirring on a hot plate. Mix the two solutions together while they are still hot. Filter through Whatmann no. 1 filter paper if a precipitate forms. Store the solution at 4°C covered in foil.

### A.2.22. Solutions for electron microscopy

2% uranyl acetate (pH5) solution

| | |
|---|---|
| uranyl acetate (Sigma) | 5 g |
| 100% Methanol | 25 ml |

Filter sterilise before use. Store at 4°C in the dark.

Reynold's lead citrate

| | |
|---|---|
| Lead citrate (Sigma) | 1.33 g |
| Na citrate | 1.76 g |
| $dH_2O$ | 30 ml |
| 1 M NaOH | 8 ml |
| $dH_2O$ to | 50 ml |

Dissolve the lead citrate and sodium citrate in 30 ml $dH_2O$ and shake for 1 min. Allow solution to stand for 30 min before adding the NaOH. Make up to 50 ml with $dH_2O$.

# APPENDIX B

## Standard Methods

### CONTENTS

**B.1 Preparation of competent *Escherichia coli* cells by rubidium chloride**

Armitage, Walden and Draper, 1988

Inoculate a single *E. coli* colony from minimal media into 5 ml Ψ broth (Appendix A.1.3) and incubate overnight at 37°C. Use this starter culture to inoculate 100 ml pre-warmed Ψ broth (Appendix A.1.3). Incubate the inoculated media at 37°C until it reaches an absorbance of approximately 0.35 at 600 nm. Transfer the culture to a sterile GSA centrifuge tube and chill on ice for 15 min. Pellet the cells by centrifugation at 2500 rpm for 5 min at 4°C. Resuspend the cells in 21 ml ice cold solution 1 (Appendix A.2.4). Incubate the cells on ice for 90 min. Pellet the cells by centrifugation at 2500 rpm for 5 min at 4°C. Gently resuspend the cells in 3.5 ml ice cold solution 2 (Appendix A.2.4). Aliquot 100 µl of cells into 1.5 ml microcentrifuge tubes. Store competent cells at -70°C.

**B.2 Transformation of competent cells**

Armitage, Walden and Draper, 1988

Use freshly prepared competent cells or thaw frozen competent cells on ice for 10 min. Add 1-50 ng plasmid DNA to 100 µl competent cells. Incubate on ice for 20 min. Heat shock cells at 42°C for 2 min or 37°C for 5 min. Return cells to ice for 2 min. Add 800 µl Ψ broth (Appendix A.1.3) or LB (Appendix A.1.1) and incubate at 37°C with shaking for up to 50 min. Spread 100 µl cells onto LA with antibiotic selection. Incubate petri dishes at 37°C overnight.

**B.3 Small scale preparation of plasmid DNA**

Ish-Horowicz and Burke, 1981

Grow 5 ml of the strain with antibiotic selection. Pellet 2 ml to 4 ml of cells by centrifugation in a sterile microcentrifuge tube in a bench top centrifuge. Resuspend cells in 200 µl solution 1 (Appendix A.2.5) and incubate at room temperature for 10 min. Add 400 µl solution 2 (Appendix A.2.5) and mix well by inversion. Incubate on ice for 10 min. Add 300 µl solution 3 (Appendix A.2.5) and mix well by inversion. Incubate on ice for 10 min. Centrifuge at 10 000 rpm for 10 min. Transfer 900 µl of supernatant to a clean microcentrifuge tube and add

600 µl isopropanol (Saarchem). Incubate at room temperature for 2 min. Centrifuge at 10 000 rpm for 10 to 15 min. Carefully remove the supernatant fraction. Add 500 µl 70% ethanol and wash the pellet by inversion. Remove the ethanol and air dry the pellet for 5 min. Resuspend the pellet (plasmid DNA) in 20 µl to 50 µl sterile $dH_2O$. Quantitate plasmid DNA on the Nanodrop spectrophotometer (Thermo Scientific).

**B.4 Restriction endonuclease digestions**

Ausubel *et al.*, 1989 unit 3.1

All restriction endonucleases and their respective buffers were obtained from Fermentas or Roche Diagnostics. Aliquot 0.5 µg to 10 µg plasmid or genomic DNA into a clean microcentrifuge tube. Add 2 µl of the appropriate buffer and make the volume up to 18 µl with sterile $dH_2O$. Add restriction endonuclease (1-5 units per µg DNA) to a final volume of 20 µl. Collect all the liquid in the bottom of the tube by briefly microfuging the microcentrifuge tube. Incubate at 37°C for 2 h for plasmid DNA and at least 4 h for genomic DNA. Stop the reaction by heating at 65°C to 70°C for 10 to 15 min or by adding gel tracking dye (Appendix A.2.6).

**B.5 Agarose gel electrophoresis**

Ausubel *et al.*, 1989 unit 2.5

Melt agarose in 1x TAE (Appendix A.2.7) by heating in a microwave and swirling to ensure even mixing. Agarose concentrations can vary from 1% or 2% for separating small DNA fragments to 0.7% for separating larger DNA fragments, such as restriction enzyme digested genomic DNA. Add ethidium bromide solution (Appendix A.2.7) to the melted agarose to a final concentration of 0.5 µg/ml. Allow the agarose to cool to approximately 55°C. Seal the ends of a gel-casting platform with masking tape if open and position the gel comb approximately 1 cm from the top. Pour the melted agarose into the gel-casting platform making sure no air bubbles remain trapped on the sides of the gel-casting platform or under the gel comb. Once the gel has set and hardened, gently remove the gel comb and the masking tape from the gel-casting platform. Place the gel-casting platform containing the set gel into an electrophoresis tank. Cover the gel with sufficient 1x TAE buffer. Load DNA samples into the wells of the gel. Attach

electrical leads to the electrophoresis tank so that the DNA migrates toward the anode. Run the gel at 1 to 10 V per cm until the dye in the loading buffer reaches the end of the gel. The DNA was visualized and photographed with a Gel Doc XR (Bio-Rad) system and Quantity One Version 4.5.2 Software.

**B.6 Thiobarbituric acid (TBA) assay**

Weissbach and Hurwitz, 1959

Alginate lyase activity was measured by the release of uronic acids from alginate using the thiobarbituric acid (TBA) reagent according to the method described by Weissbach and Hurwitz (1959) with minor modifications. Briefly, 300 µl of alginate substrate (Appendix A.2.8) was added to 100 µl aliquots of sample (culture supernatant or soluble cellular lysates) and incubated at 37°C for 30 min. One hundred microlitres of periodate solution (Appendix A.2.8) was added and incubated at room temperature for 20 min. The reaction was stopped by adding 80 µl sodium arsenate solution (Appendix A.2.8) and incubated at room temperature for 2 min. Eight hundred microlitres of TBA solution (Appendix A.2.8) was added to each tube and reactions were boiled for 10 min. Once cool, reactions were centrifuged at 12 000 rpm for 5 min. Absorbances were measured at 550 nm using a Beckman DU530 spectrophotometer. The assay blank was prepared by boiling 100 µl of sample for 10 min and performing the assay as above except the initial 30 min incubation with alginate substrate was on ice instead of at 37°C. A standard curve was prepared by plotting light absorbances at 550 nm against known concentrations of 1,1,3,3 tetraethoxy propane (malondealdehyde bis-diethyl acetal; Sigma). One unit of alginate lyase activity is expressed as µg malonaldehyde equivalents released per 100 µl sample in 30 min at 37˚C.

## B.7 Ligations

Coyne *et al*., 2003

### B.7.1. Intramolecular ligations

Re-circularisation reactions for the formation of deletion plasmids. Use 1 pmol/ml reaction volume of DNA. Add 2 µl 10x ligation buffer which is supplied with the $T_4$ DNA ligase (Fermentas). Add 1 unit of $T_4$ DNA ligase and make the volume up to 20 µl using sterile $dH_2O$. Incubate the reaction at 15°C overnight.

### B.7.2. Intermolecular ligations

Polymerisation reactions of two distinct DNA fragments. The total amount of DNA should be at most 10 pmol/ml reaction volume (insert and vector). Use ratios of vector to insert from 1:1 to 1:4 pmol. If the vector is dephosphorylated, an excess of vector DNA should be used i.e. a vector to insert ratio of 4:1 pmol. Add vector and insert DNA to a microcentrifuge tube. Add 2 µl 10x ligation buffer which is supplied with the $T_4$ DNA ligase (Fermentas). Add 1 unit of $T_4$ DNA ligase and make the volume up to 20 µl using sterile $dH_2O$.

When ligating DNA fragments with cohesive ends, incubate the reaction mixtures at 15°C overnight. When ligating blunt-ended DNA fragments, use ten times more $T_4$ DNA ligase and incubate ligation reactions at room temperature overnight. When performing ligations in low-melt agarose, melt the agarose at 70°C for 5 min before aliquoting the DNA and before adding the ligation mix to competent cells.



## B.8 Large scale preparation of bacterial genomic DNA

Ausubel *et al.*, 1989 unit 2.4

Culture 100 ml of the bacterial strain overnight or to saturation. Pellet the cells by centrifugation at 6000 rpm for 10 min and discard the supernatant. Resuspend the cells in 9.5 ml TE buffer (Appendix A.2.3). Add 0.5 ml 10% SDS (Appendix A.2.3) and 50 µl of 20 mg/ml proteinase K (Appendix A.2.9). Mix and incubate for 1 h at 37°C. Thereafter, add 1.8 ml of 5 M NaCl (Appendix A.2.9) and mix thoroughly before adding 1.5 ml CTAB/NaCl solution

(Appendix A.2.9). Incubate at 65°C for 20min. Extract with an equal volume of 24:1 chloroform/isoamyl alcohol (Appendix A.2.9) and centrifuge at 7000 rpm for 10 min at room temperature to separate the two phases. Transfer the aqueous phase to a fresh centrifuge tube and repeat the chloroform/isoamyl alcohol extraction if necessary. Precipitate the DNA in the aqueous phase by adding 0.6 volumes of isopropanol. Mix gently by inverting the tube, until a stringy white DNA pellet precipitates out of solution. Centrifuge at 15 000 rpm for 15 min at 4°C. Carefully remove the supernatant and discard. Add 70% ethanol (Appendix A.2.3) and centrifuge at 9000 rpm for 5 min to wash the pellet. Carefully remove the supernatant and discard. Air-dry the DNA pellet for a few minutes. Resuspend the DNA in 1 ml sterile dH2O to which 10 µl RNAse A (Appendix A.2.9) has been added. Measure the concentration of the DNA on a Nanodrop spectrophotometer (Thermo Scientific).

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## B.9 Southern hybridisation procedure

### B.9.1. Southern transfer of DNA from an agarose gel to a nitrocellulose membrane

Coyne *et al.*, 2003

For Southern transfers, do not add ethidium bromide to the agarose gel. Stain the agarose gel in an ethidium bromide solution for 30 min after running the gel and destain for 20 min in $dH_2O$. Soak the agarose gel in 2x volumes of 0.25 M HCl (Appendix A.2.10) with agitation at room temperature for 5 min. Rinse the gel in $dH_2O$ to remove excess HCl. Cover a tray or plastic box with plastic wrap. Place an inverted gel-casting tray on the plastic wrap. Cut 10 sheets of Whatmann 3MM paper 1 cm bigger than the gel casting tray and saturate with 0.4 M NaOH (Appendix A.2.10). Place the Whatmann paper over the inverted gel-casting tray. This will act as the wick during the DNA transfer. Add enough 0.4 M NaOH/1 M NaCl (Appendix A.2.10) to the tray so that the ends of the Whatmann paper are submerged. Invert the gel and place on top of the Whatmann paper. Cut Hybond N+ Nylon membrane (GE Healthcare) to the size of the gel. Wet the membrane in $dH_2O$ and place on the gel. Make sure no air bubbles are trapped under the gel or between the gel and the membrane. Cover the edges of the membrane with the plastic wrap. Cut 3 sheets Whatmann 3MM paper to the size of the membrane and place over the membrane. Place a 10 cm stack of dry paper towel on top of the Whatmann paper followed by a glass plate

and a 0.2-0.4 kg weight. Blot for 4 h to overnight. Disassemble the blotting apparatus. Gently turn over the gel and membrane and mark the wells of the gel on the membrane with a blunt pencil. Rinse the gel in 2x SSC (Appendix A.2.10) for 5 min. Air dry the membrane on Whatmann paper and UV crosslink the DNA onto the membrane. Store the membrane between two pieces of Whatmann 3MM paper.

### B.9.2. Random primed labeling of DNA with digoxigenin (DIG)

DIG high prime DNA labeling and detection starter kit II Instruction Manual, Roche Diagnostics

All reagents required for this protocol are supplied in the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche). Transfer 1 µg of template DNA to a microcentrifuge tube and make the volume up to 16 µl using sterile $dH_2O$. Denature the DNA by heating to 100°C for 10 min and rapidly cooling on ice. Mix DIG-High Prime labeling mixture and add 4 µl to denatured DNA. Incubate at 37°C overnight. Stop the reaction by adding 2 µl 0.2 M EDTA and heating at 65°C for 10 min. Store DIG-labeled DNA at -20°C.

### B.9.3. Determination of labeling efficiency

DIG high prime DNA labeling and detection starter kit II Instruction Manual, Roche Diagnostics

To determine the efficiency of the labeling reaction, prepare a 1:10 dilution series of the DIG-labeled DNA using sterile $dH_2O$. Spot 1µl of each dilution and the undiluted DIG-labeled DNA onto a piece of Hybond N+ Nylon membrane (Amersham). Allow to air-dry on a piece of Whatmann 3MM paper and UV crosslink. Perform the immunological detection described in Appendix B.9.5. to determine how well the DNA labeled. Add DIG-labeled DNA to the Southern hybridisation to the same dilution factor that gave a good signal on the labeling efficiency but not to the brightest spot on the labeling efficiency so as to over-expose the blot.

### B.9.4. Pre-hybridisation, hybridisation and stringency washes

DIG high prime DNA labeling and detection starter kit II Instruction Manual, Roche Diagnostics

Place the Hybond N+ Nylon membrane with the transferred DNA in a plastic bag. Add 10 ml/100 $cm^2$ of pre-heated DIG Easy Hyb (Roche) to the membrane and seal the bad. Incubate the membrane at 46°C with gentle agitation for 60 min. Denature the DIG-labeled DNA probe by heating at 100°C for 10 min. Pour off the pre-hybridisation buffer. Add 3.5 ml/100 $cm^2$ fresh pre-heated Dig Easy Hyb to the membrane. Add the pre-determined amount of DIG labeled probe. Remove any bubbles and seal the bag. Incubate the membrane overnight at 46°C with gentle agitation.

Remove the membrane from the bag and place in a small plastic box. Store the Dig Easy Hyb containing the DIG-labeled DNA probe -20°C for re-use. Defrost and denature at 68°C for 10 min before use. Do not boil DIG Easy Hyb. Wash the membrane twice with wash buffer A (Appendix A.2.10) at room temperature for 5 min with gentle agitation. Wash the membrane twice with pre-warmed wash buffer B (Appendix A.2.10) at 65°C for 15 min with gentle agitation. Do not allow the membrane to dry at any time. Proceed with the immunological detection described in Appendix B.9.5.



### B.9.5. Immunological detection of DIG-labeled DNA probes

DIG high prime DNA labeling and detection starter kit II Instruction Manual, Roche Diagnostics

All steps are performed at room temperature with gentle agitation. After hybridisation and stringency washes, rinse the membrane in washing buffer (Appendix A.2.11) for 5 min. Block the membrane for 2 h in blocking buffer (Appendix A.2.11). Incubate the membrane in antibody solution (Appendix A.2.11) for 30 min. Wash twice for 15 min with washing buffer. Equilibrate the membrane for 5 min in detection buffer (Appendix A.2.11). Place the membrane DNA side up in a new plastic bag. Apply between 100 µl and 1 ml CSPD ready-to-use (Roche) to the membrane and cover the membrane. Incubate for 5 min in the dark. Squeeze out excess liquid, remove any bubbles and seal the bag. Incubate the membrane at 37°C for 10 min to enhance the luminescent reaction. Expose the membrane to X-ray film (Hyperfilm, GE Healthcare) for

20-60 min in an X-ray cassette containing enhancer screens. Develop the X-ray manually by placing in developer solution for 30 sec, $dH_2O$ for 30 sec, fixer solution for 30 sec and rinsing in $dH_2O$ for 30 sec. Air-dry the film for approximately 30 min.

**B.10 Transfer of DNA onto nitrocellulose membrane using a slot blot apparatus**

Ausubel *et al.*, 1989 unit 2.9

Line the vacuum manifold with 3x sheets Whatmann 3MM paper cut to size and soaked in $dH_2O$. Cut Hybond N+ nylon membrane (Amersham) to fit the manifold (11 x 8cm) and wet in $dH_2O$. Place the membrane over the Whatmann paper. Assemble the manifold according to manufacturer's instructions. Add 1 M NaOH (appendix A.2.10) to each DNA sample to give a final concentration of 0.4 M NaOH in a volume of 100 µl. Denature the DNA samples for 10 min at 100°C and cooling rapidly on ice. Switch on the suction device of the manifold. Load the wells with 1x tracking dye (Appendix A.2.6) to check for any leaks. Load 100 µl denatured DNA samples and allow to filter through. Every well must be full, load $dH_2O$ into the non-sample wells. Wash the wells with 100 µl 0.4 M NaOH. Switch off the suction device and disassemble the manifold unit. Rinse the membrane briefly in 2x SSC and air-dry on a piece of Whatmann 3MM paper. UV crosslink the DNA onto the membrane. Store the membrane between 2 pieces of Whatmann 3MM paper. Continue with the hybridisation, pre- hybridisation and stringency washes (Appendix B.9.4) followed by immunological detection of DIG-labeled DNA probes (Appendix B.9.5).

**B.11 Preparing colony lifts**

DIG Application Manual for Filter Hybridisation, Roche Diagnostics

Pre-cool agar containing colonies for 30 min at 4°C. Carefully place a Hybond N+ nylon membrane disc (Amersham) onto the surface of each petri dish avoiding air bubbles between the membrane and the agar. Leave the membrane on the agar for 1 min. Mark the orientation of the membrane relative to the petri dish. Remove each membrane disc. Briefly blot the bottom of each disc on a piece of Whatmann 3MM paper. Incubate the membrane discs on 1 ml of denaturation solution (Appendix A.2.12) on a piece of plastic wrap colony-side down for 15 min.

Transfer the membrane discs to 1ml of neutralisation solution (Appendix A.2.12) on a new piece of plastic wrap and incubate for 15 min. Transfer the membrane discs to 1ml of 2x SSC (Appendix A.2.10) on a new piece of plastic wrap and incubate for 10 min. UV crosslink the transferred DNA onto the membrane. Remove cell debris by proteinase K treatment. Dilute 20 mg/ml proteinase K (Appendix A.2.9) 1:10 in 2x SSC. Wrap each membrane disc in a piece of aluminum foil with 0.5 ml of the diluted proteinase K spread evenly over the surface. Incubate at 37°C for 1 h with the fan of the incubator is turned off. Wet a piece of Whatmann 3MM paper with sterile $dH_2O$ and firmly press across the surface of each disc using a ruler. Gently lift the damp paper off the discs. The colony debris should stick to the Whatmann paper. Repeat with a new piece of Whatmann paper if necessary. Air-dry the membrane discs and store between two pieces of Whatmann 3MM paper. Continue with the hybridisation, pre- hybridisation and stringency washes (Appendix B.9.4) followed by immunological detection of DIG-labeled DNA probes (Appendix B.9.5).

**B.12 Isolation of total RNA from Gram-negative bacteria**

Ausubel *et al.*, 1989 unit 4.4

Grow the appropriate strain in liquid broth. Harvest the cells by centrifugation at 10 000 rpm for 5 min. Resuspend the cell pellet in 2 ml protoplast buffer (Appendix A.2.13). Add 80 µl 80 mg/ml lysozyme (Appendix A.2.13) and incubate on ice for 15 min. Collect protoplasts by centrifuging for 5 min at 7000 rpm. Discard the supernatant and resuspend the cell pellet in 0.5 ml Gram-lysing buffer (Appendix A.2.13). Add 15 µl DEPC (Sigma) and incubate at 37°C for 5 min followed by chilling on ice for 2 min. Add 250 µl saturated NaCl (Appendix A.2.13) and mix by inversion. Incubate on ice for 10 min. Centrifuge at 14 000 rpm for 10 min. Remove the supernatant to two clean microcentrifuge tubes. Add 1 ml 100% ethanol (Saarchem) to each tube. Precipitate the nucleic acids overnight at -70°C. Centrifuge at 14 000 rpm for 15 min at 4°C. Wash the pellet with 70% ethanol by centrifuging at 14 000 rpm for 15 min at 4°C. Air-dry the pellet. Dissolve each pellet in 20 µl DEPC-treated $dH_2O$ and transfer into one microcentrifuge tube. Add 5 units of DNAse I (Promega) in a final volume of 50 µl. Incubate at 37°C for 3 h. Recover RNA by adding an equal volume of 25:24:1 phenol:chloroform:isoamyl alcohol (Appendix A.2.13). Mix by inversion and centrifuge at 14 000 rpm at 4°C for 10 min. Transfer

the aqueous phase to a clean microcentrifuge tube. Precipitate RNA with 5 µl 5 M sodium acetate (Appendix A.2.13) and 150 µl 100% ethanol. Centrifuge at 14 000 rpm for 10 min. Carefully remove the supernatant and air-dry the pellet. Resuspend the RNA in 20 µl DEPC-treated $dH_2O$. Quantitate RNA by reading the absorbance at 260 nm on a Nanodrop spectrophotometer (Thermo Scientific). Determine the integrity of the RNA by electrophoresis on a formaldehyde/MOPS gel (Appendix B.13).

**B.13 Formaldehyde agarose gels for RNA**

Ausubel *et al.*, 1989 unit 4.9

The RNA samples are prepared by denaturing 2 µg RNA with 5 µl RNA sample application buffer (Appendix A.2.14) by heating at 65°C for 15 min and then rapidly cooling on ice. Melt 0.72 g agarose (1.2%) in 44 ml of $dH_2O$ by heating in a microwave and swirling to ensure even mixing. Allow to cool to 60°C before adding 6 ml 10x MOPS (Appendix A.2.14) and 10 ml 37% formaldehyde. The ethidium bromide (Appendix A.2.7) is included in the RNA sample application buffer, not in the gel. Seal the ends of a gel-casting platform with masking tape if open and position the gel comb approximately 1 cm from the top. Pour the melted agarose into the gel-casting platform making sure no air bubbles remain trapped on the sides of the gel-casting platform or under the gel comb. Once the gel has set and hardened, gently remove the gel comb and the masking tape from the gel-casting platform. Place the gel-casting platform containing the set gel into an electrophoresis tank. Cover the gel with sufficient 1x MOPS buffer. Load RNA samples into the wells of the gel. Attach electrical leads to the electrophoresis tank so that the RNA migrates toward the anode. Run the gel at 60 V for 30 min or until the dye in the loading buffer reaches half way on the gel. The RNA was visualized and photographed with a Gel Doc XR (Bio-Rad) system and Quantity One Version 4.5.2 Software.

**B.14 Reverse transcriptase reaction or first strand cDNA synthesis**

ImPromII$^{tm}$ Two Step Reverse Transcriptase Kit Instruction Manual, Promega

All reagents required for this protocol are supplied in the ImPromII$^{tm}$ Two Step Reverse Transcriptase Kit (Promega). Transfer 5 µg total RNA to a clean RNAse free microcentrifuge tube and add 2 µl random hexamers and nuclease-free $dH_2O$ 10 µl. Briefly pulse the tube in a bench top centrifuge. Denature the mixture at 72°C for 5 min and then cool on ice for 5 min. Add 8 µl ImPromII$^{tm}$ 5x reaction buffer, 4.8 µl 25 mM $MgCl_2$, 1.5 µl 10 mM dNTP Mix and 1 µl RNAse inhibitor to a clean RNAse free microcentrifuge tube. Make up the volume to 30 µl using nuclease-free $dH_2O$. Briefly pulse the tube in a bench top centrifuge. Add the contents of the second tube to the denatured RNA. Incubate at room temperature for 5 min and then at 42°C for 5 min. Add 2 µl ImPromII$^{tm}$ reverse transcriptase (RT), mix and briefly pulse the tube in a bench top centrifuge. Incubate the reactions at 42°C overnight in a humid chamber. Stop the reaction by heating at 65°C for 15 min. Store cDNA samples at -70°C.

**B.15 Preparing and running denaturing SDS-PAGE gels**

Ausubel *et al.*, 1989 unit 10.2

Denaturing SDS-PAGE was conducted in accordance with the Laemmli method (1970). The Bio-Rad Mini-PROTEAN Tetra Cell was used for all denaturing SDS-PAGE gels. Assemble the spacer and short glass plates in the casting frame as described by the manufacturer. Mark on the short glass plate 1cm below the comb. Combine the following reagents for an 8% separating gel monomer solution in a glass beaker (sufficient for 4 gels):

| | |
|---|---|
| 40% Acrylogel (Promega) | 4 ml |
| Separating gel buffer (Appendix A.2.15) | 5 ml |
| 10% SDS (Appendix A.2.3) | 200 µl |
| TEMED (Sigma) | 10 µl |
| 10% Amps (Promega) | 100 µl |
| $dH_2O$ to | 20 ml |

Gently mix and pour the separating gel monomer solution into the assembled glass gel plates up to the marked line on the short plate. Overlay the gel with 1ml isopropanol. Allow to polymerize at 37°C for 30 min. After polymerization, remove the overlay and dry the top of the separating gel with filter paper. Prepare the 6% stacking gel monomer solution as follows:

| | |
|---|---|
| 40% Acrylogel | 1.5 ml |
| Stacking gel buffer (Appendix A.2.15) | 2.5 ml |
| 10% SDS | 100 µl |
| TEMED | 10 µl |
| 10% Amps | 50 µl |
| $dH_2O$ to | 10 ml |

Gently mix and pour over the polymerized separating gel to the top of the short plate. Insert the gel comb making sure no air bubbles remain trapped. Allow to polymerize at 37°C for 30 min. gently remove the comb and rinse the wells with SDS-PAGE running buffer (Appendix A.2.16). Rinse the casting frame and stand with $dH_2O$ after use.

Assemble the glass plates with the polymerized gel into the running tank as described by the manufacturer and fill the tank with SDS-PAGE running buffer (Appendix A.2.15). Denature protein samples by boiling for 5 min at 100°C. Load samples slowly into the bottom of the wells taking care not to puncture the bottom of the well. Run the gel at 100 V until the dye in the sample application buffer reaches the bottom of the gel. After electrophoresis, disassemble the gel plates taking care not to break the fragile acrylamide gel. Visualize the protein bands in the gel by staining with Coomassie blue staining solution (Appendix A.2.15) for one hour at room temperature with gentle agitation. Destain the gel overnight at room temperature with gentle agitation in destaining solution (Appendix A.2.15). Store the gel in 7% acetic acid. Gels were photographed with a Gel Doc XR (Bio-Rad) system and Quantity One Version 4.5.2 software and scanned with an Epson Perfection V750 Pro scanner and Silverfast Ai Epson Perfection V700 software.

## B.16 Bradford protein assay for protein quantitation

Bradford, M. (1976)

### B.16.1. Microassay procedure:

The Bio-Rad Protein Assay was used. Dilute Protein Assay Dye Reagent Concentrate (Bio-Rad) 1:5 with sterile $dH_2O$. Prepare BSA standards from 1.4 mg/ml to 0.2 mg/ml in $dH_2O$ from a 2 mg/ml stock of BSA. Aliquot 980 µl of diluted Protein Assay Dye Reagent to a clean microcentrifuge tube. Add 20 µl of sample or BSA standard. Mix well and allow to stand for 5 min at room temperature. Read the absorbance at 595 nm using a Beckman DU530 spectrophotometer. An assay blank is prepared by replacing the sample with $dH_2O$ or with the buffer of the protein sample. Plot absorbance versus concentration of the BSA standards to obtain a standard curve. Read the concentrations of the samples off the curve.

### B.16.2. Microtitre plate procedure:

The Bio-Rad Protein Assay was used. Dilute Protein Assay Dye Reagent Concentrate (Bio-Rad) 1:5 with sterile $dH_2O$. Prepare BSA standards of 0.5 mg/ml to 0.015 mg/ml by performing doubling dilutions of a 1 mg/ml stock of BSA in $dH_2O$. Aliquot 10 µl of sample or BSA standard into the wells of a microtitre plate in triplicate. Add 200 µl of diluted Protein Assay Dye Reagent. Allow to stand for 5 min. Read absorbance at 595 nm using a Beckman DU530 spectrophotometer. An assay blank is prepared by replacing the sample with $dH_2O$ or with the buffer of the protein sample. Plot absorbance versus concentration of the BSA standards to obtain a standard curve. Read the concentrations of the samples off the curve.

## B.17 Electroblotting of proteins onto a nitrocellulose membrane

(Towbin *et al*. 1979)

The Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad) was used for electroblotting proteins onto nitrocellulose membranes. Cut one piece of Protran nitrocellulose transfer and immobilization membrane (Schleicher and Scheull, Perkin Elmer) and two pieces of Whatmann 3MM paper to the size of the gel. Gently remove the SDS-PAGE gel from the glass plates. Rinse the glass plates with $dH_2O$. Soak the gel, Whatmann paper, membrane and fiber pads in Towbin

transfer buffer (Appendix A.2.17). Prepare the gel sandwich as follows. Place the gel holder cassette black side down in transfer buffer. Layer the following in order: fiber pad, Whatmann paper, gel, membrane, Whatmann paper and fiber pad. Make sure there are no air bubbles trapped anywhere in the sandwich. Close the gel holder cassette and lock it with the white latch. Place the gel holder cassette in the electrode module with the black side of the cassette facing the black side of the module. Repeat for the second gel holder cassette if two gels are being transferred. Place the electrode module and the frozen Bio-Ice cooling unit into the buffer tank and fill the tank with Towbin transfer buffer. Add a stirrer bar to help maintain an even temperature and ion distribution of the transfer buffer. Place the lid onto the tank and plug the cables into a power supply pack. Run the system at 100 V for 1 h. Disassemble the gel sandwich. Orientate the membrane by cutting one corner off. Rinse the membrane in blocking solution (Appendix A.2.19) and continue with the Western hybridisation protocol (Chapter 3, Section 3.3.5). Rinse all the equipment with deionized water.

# APPENDIX C

## Primers for PCR Amplification and PCR Conditions

### CONTENTS

All PCR amplifications were achieved with a BIOER XP Thermal Cycler (BIOER Technology Co. Ltd) equipped with a heated lid unless otherwise stated.

## C.1 PCR amplification of pAlg15 with primers for nucleotide sequencing

### C.1.1. Primers used for PCR amplification

(This study, unless otherwise indicated)

M13 Forward 5' CGCCAGGGTTTTCCCAGTCACGAC 3' (Yanisch-Perron *et al.* 1985)
M13 Reverse 5' GAGCGGATAACAATTTCACACAGG 3' (Yanisch-Perron *et al.* 1985)
15Fc 5' CCTGAGCTACTTTAAAGCCGGAG 3'
15Rd 5' CCTTCAACTTGGCCTGAACC 3'
15Re 5' GTTCTCTGGGATGTAACGACC 3'
15Rf 5' GCAAAGTACCATCTGGGAATGC 3'
15Rg 5' GCATGTCCACGATGTAATCG 3'
15Fh 5' CTTACCCGAATGGTGAATTCC 3'
15Ri 5' GTTCTGTTTCGTGCTGC 3'
15Rj 5' GATGGCTTTATCGTAGGCTTG 3'
15Fk 5' GAAGACGAACATCAGTACGAC 3'
15 Fl 5' CCTATTAGGAAACGCACTAGC 3'
15Fm 5' GACTCGGTAATCAAGACCAC 3'

The oligonucleotide primers were used in the following combinations with the indicated annealing temperature (Ta). Whether sequencing occurred from a plasmid or PCR product is also indicated:

| First Primer | Second Primer | Ta (°C) | Sequencing Template |
|---|---|---|---|
| 15Fc | M13R | 62 | Plasmid |
| 15Rd | M13F | 62 | Plasmid |
| 15Re | M13R | 58 | Plasmid |
| 15Rf | M13R | 57 | Plasmid |
| 15Rg | M13R | 60 | Plasmid |
| 15Fh | 15Rg | 60 | Plasmid |
| 15Ri | 15Fh | 53 | Plasmid |
| 15Rj | M13R | 59 | PCR product |
| 15Fk | 15Rf | 57 | PCR product |
| 15Fl | 15Re | 57 | PCR product |
| 15Fm | 15Rd | 57 | PCR product |



### C.1.2. PCR protocol

The PCR mix was prepared as follows in a sterile PCR microcentrifuge tube for each primer pair:

| Reagents (Roche) | Volume (µl) |
|---|---|
| pAlg15 (5 ng/µl) | 2 |
| First primer (10 mM) | 1 |
| Second primer (10 mM) | 1 |
| $MgCl_2$ (25 mM) | 6 |
| PCR buffer (10x) | 5 |
| dNTP's (2.5 mM) | 4 |
| Taq Polymerase (0.25 U/µl) | 1 |

| | |
|---|---|
| Sterile $dH_2O$ | 30 |
| TOTAL | 50 |

The PCR protocol was as follows. The annealing temperature of each primer pair is indicated in the table above:

| Temperature (ºC) | Time (sec) | Cycles |
|---|---|---|
| 96 | 300 | 1 |
| 96 | 30 | |
| Ta | 30 | 30 |
| 72 | 60 | |
| 72 | 420 | 1 |

## C.2 PCR amplification of pAlg15 for the synthesis of probes for the *alyVMI* and *alyVMIII* Southern hybridisation experiment

### C.2.1. Primers used for PCR amplification

(This study)

Primers used for preparation of the *alyVMI* probe:

15Fn 5' CCAATGCTAGACATGTTCACAG 3'

15Rj 5' GATGGCTTTATCGTAGGCTTG 3'

Primers used for preparation of the *alyVMIII* probe:

15Fc 5' CCTGAGCTACTTTAAAGCCGGAG 3'

15Ro 5' CAGTCTTCGTTTACCGCGTAGG 3'

### C.2.2. PCR protocol

The PCR master mix for each primer set was prepared as follows for each primer pair:

| Reagents (Roche) | Volume (µl) |
|---|---|
| pAlg15 (5 ng/µl) | 12 |
| Forward primer (10 mM) | 6 |
| Reverse primer (10 mM) | 6 |
| $MgCl_2$ (25 mM) | 36 |
| PCR buffer (10x) | 5 |
| dNTP's (2.5 mM) | 6 |
| Taq Polymerase (0.25 U/µl) | 30 |
| Sterile $dH_2O$ | 180 |
| TOTAL | 300 |

The master mix for each primer set was aliquoted (50 µl) into 6 separate PCR microcentrifuge tubes. The PCR profile was set up as follows. The annealing temperature (Ta) for primer pair 15Fn and 15Rj was 58°C and for primer pair 15Fc and 15Ro was 63°C.

The PCR protocol was as follows:

| Temperature (ºC) | Time (sec) | Cycles |
|---|---|---|
| 96 | 180 | 1 |
| 96 | 30 | |
| Ta | 30 | 30 |
| 72 | 30 | |
| 72 | 180 | 1 |

## C.3 PCR amplification of pAlg65 and pAlg86 with primers for nucleotide sequencing

### C.3.1. Primers used for PCR amplification

(This study, unless otherwise indicated)

Primers used for the PCR amplification of pAlg65:

M13 Forward 5' CGCCAGGGTTTTCCCAGTCACGAC 3' (Yanisch-Perron *et al*. 1985)

M13 Reverse 5' GAGCGGATAACAATTTCACACAGG 3' (Yanisch-Perron *et al*. 1985)

65Fa 5' GTATCACCACCGAATGACTG 3'

65Fb 5' GGTAACGAGCATATCCGATC 3'

65Rc 5' CATGTCTAGCATTGGTTCG 3'

Primers used for the PCR amplification of pAlg86:

M13 Forward 5' CGCCAGGGTTTTCCCAGTCACGAC 3' (Yanisch-Perron *et al*. 1985)

M13 Reverse 5' GAGCGGATAACAATTTCACACAGG 3' (Yanisch-Perron *et al*. 1985)

86Fa 5' CGATCCGTGCCGATAAG 3'

86Rb 5' CTTGGCTCAACTCAGACTCAG 3'

86Fc 5' GTGATAGAACTAGCCTTGGTAC 3'

86Rd 5' GTTACAACAATGTCCCTATTCG 3'

86Fg 5' GTAAGACTTATCACCAGCACG 3'

86Rh 5' AAGCTGCCTAAGTGGAGGTTG 3'

86Fi 5' CTCAATAACCGTCACCCAGT 3'

86Rj 5' GAGATTTCCGTCCTTACTTTG 3'

86Rk 5' CATTACTGATGACGTCGTTAAC 3'

86Rl 5' GGTACAGTTTACTTTGCTCACG 3'

The oligonucleotide primers were used in the following combinations with the indicated annealing temperature (Ta). The PCR template and whether sequencing occurred from a plasmid or PCR product is shown:

| First Primer | Second Primer | Ta (°C) | PCR Template | Sequencing Template |
|---|---|---|---|---|
| 65Fa | 65Rc | 55 | pAlg65 | PCR product |
| 65Fb | M13R | 57 | pAlg65 | PCR product |
| 86Fa | 86Rb | 58 | pAlg86 | Plasmid |
| 86Fc | 86Rd | 53 | pAlg86 | Plasmid |
| 86Fg | 86Rd | 56 | pAlg86 | PCR product |
| 86Rh | M13F | 60 | pAlg86 | PCR product |
| 86Fi | 86Rb | 57 | pAlg86 | PCR product |
| 86Rj | 86Fg | 55 | pAlg86 | PCR product |
| 86Rk | 86Fc | 55 | pAlg86 | PCR product |
| 86Rl | M13F | 57 | pAlg86 | PCR product |

### C.3.2. PCR protocol

The PCR mix was prepared as follows in a sterile PCR microcentrifuge tube for each primer pair:

| Reagents (Roche) | Volume (µl) |
|---|---|
| pAlg65 of pAlg86 (5 ng/µl) | 2 |
| First primer (10 mM) | 1 |
| Second primer (10 mM) | 1 |
| $MgCl_2$ (25 mM) | 6 |
| PCR buffer (10x) | 5 |
| dNTP's (2.5 mM) | 4 |
| Taq Polymerase (0.25 U/µl) | 1 |
| Sterile $dH_2O$ | 30 |
| TOTAL | 50 |

The PCR protocol was as follows:

| Temperature (ºC) | Time (sec) | Cycles |
|---|---|---|
| 96 | 300 | 1 |
| 96 | 30 | |
| Ta | 30 | 30 |
| 72 | 60 | |
| 72 | 420 | 1 |

## C.4 PCR amplification of pAlg15 and pAlg86 for the synthesis of probes for the *alyVMII* and *alyVMIV* Southern hybridisation experiments

### C.4.1. Primers used for PCR amplification

(This study)

Primers for the preparation of the *alyVMII* probe:

EVF1 5' CATATGAGCTACCAAACCCAGTCTTACC 3'

EVR1 5' CTCGAGAATTTGTGCGAATGCGCCTTCC 3'

Primers for the preparation of the *alyVMIV* probe:

EVF4 5' GGTGTTAGATCATATGAAGCATATTTTCTTC 3'

EVR4 5' GTGATACTCGAGGCCTTGGTACTTACC 3'

### C.4.2. PCR protocol

The PCR master mix was prepared as follows in a sterile PCR microcentrifuge tube for each primer pair. The template for PCR amplification with primer pair EVF1 and EVR1 was pAlg15 and for PCR amplification with primer pair EVF4 and EVR4 was pAlg86.

| Reagents (Roche) | Volume (µl) |
|---|---|
| Plasmid DNA (5 ng/µl) | 12 |
| First primer (10 mM) | 6 |
| Second primer (10 mM) | 6 |
| $MgCl_2$ (25 mM) | 36 |
| PCR buffer (10x) | 30 |
| dNTP's (2.5 mM) | 24 |
| Taq Polymerase (0.25 U/µl) | 6 |
| Sterile $dH_2O$ | 180 |
| TOTAL | 300 |

The master mix for each primer and template set was aliquoted (50µl) into 6 separate PCR microcentrifuge tubes. The PCR profile was set up as follows. The annealing temperature (Ta) for primer pair EVF1 and EVR1 was 54°C and for primer pair EVF4 and EVR4 was 60°C:

| Temperature (ºC) | Time (sec) | Cycles |
|---|---|---|
| 96 | 180 | 1 |
| 96 | 45 | |
| Ta | 30 | 30 |
| 72 | 60 | |
| 72 | 180 | 1 |

## C.5 PCR amplification of total RNA

### C.5.1. Primers specific for the *Vibrio midae* SY9 16S rRNA gene used for PCR amplification

(Huddy, 2010)

16S F 5' GAGTACGGTCGCAAGA 3'

16S R 5' CGCTGGCAAACAAGGA 3'

### C.5.2. PCR protocol

The PCR mix was prepared as follows in a sterile PCR microcentrifuge tube:

| Reagents (Roche) | Volume (µl) |
|---|---|
| Total RNA | 2 |
| 16S F (10 mM) | 1 |
| 16S R (10 mM) | 1 |
| $MgCl_2$ (25 mM) | 6 |
| PCR buffer (10x) | 5 |
| dNTP's (2.5 mM) | 4 |
| Taq Polymerase (0.25 U/µl) | 1 |
| Sterile $dH_2O$ | 30 |
| TOTAL | 50 |

If more than one RNA sample was amplified, a PCR master mix was prepared by multiplying the volumes above (except for the RNA template) by the number of samples. The master mix was aliquoted into sterile PCR microcentrifuge tubes and 2 µl of RNA template added to each tube.

The PCR profile was set up as follows:

| Temperature (ºC) | Time (sec) | Cycles |
|---|---|---|
| 96 | 300 | 1 |
| 96 | 30 | |
| 53 | 30 | 30 |
| 72 | 45 | |
| 72 | 420 | 1 |

## C.6 PCR amplification for *alyVMI* and *alyVMII* operon determination

### C.6.1. Primers used for PCR amplification

(This study)

Primers used to amplify an internal fragment of *alyVMI*:

15Fh 5’ CTTACCCGAATGGTGAATTCC 3’

15Ri 5’ GTTCTGTTTCGTGCTGC 3’

Primers used to amplify an internal fragment of *alyVMII*:

15Fm 5’ GACTCGGTAATCAAGACCAC 3’

15Re 5’ GTTCTCTGGGATGTAACGACC 3’

Primers used to amplify the region between *alyVMI* and *alyVMII*:

15Fk 5’ GAAGACGAACATCAGTACGAC 3’

15Rg 5’ GCATGTCCACGATGTAATCG 3’

### C.6.2. PCR protocol

The PCR master mix was prepared as follows in two separate sterile PCR microcentrifuge tubes:

| Reagents (Roche) | Volume (µl) |
|---|---|
| pAlg15 (5 ng/µl) or cDNA | 6 or 12 |
| $MgCl_2$ (25 mM) | 18 |
| PCR buffer (10x) | 15 |
| dNTP's (2.5 mM) | 12 |
| Taq Polymerase (0.25 U/µl) | 3 |
| Sterile $dH_2O$ | 84 |
| TOTAL | 144 |

Aliquots (48 µl) of each master mix were added to 3 separate PCR microcentrifuge tubes (6 tubes total). Tubes 1, 2 and 3 contained plasmid DNA as template and tubes 4, 5, and 6 contained cDNA as template. The following primer pairs (1 µl of each primer) were separately added to each of the 6 PCR tubes as follows:

| Tube number | Primer (10 µM) |
|---|---|
| 1 and 4 | 15Fh and 15Ri |
| 2 and 5 | 15Fm and 15Re |
| 3 and 6 | 15Fk and 15Rg |

The PCR protocol was as follows:

| Temperature (ºC) | Time (sec) | Cycles |
|---|---|---|
| 96 | 180 | 1 |
| 96 | 30 | |
| 55 | 30 | 30 |
| 72 | 45 | |
| 72 | 180 | 1 |

## C.7 PCR protocol for amplification of *alyVMI*, *alyVMII*, *alyVMIII* and *alyVMIV* for cloning into the expression vector pET-29a

Primer pairs, primer sequences and templates for amplification are described in Table 3.2. The PCR master mix was prepared as follows for each primer set in a sterile PCR microcentrifuge tube:

| **Reagents (Roche)** | **Volume (µl)** |
|---|---|
| Plasmid DNA or genomic DNA (5 ng/µl) | 16 or 24 |
| Forward primer (10 mM) | 4 |
| Reverse primer (10 mM) | 4 |
| $MgSO_4$ (Fermentas, 25mM) | 24 |
| PCR buffer (Fermentas, 10x) | 20 |
| dNTP's (Roche, 2.5 mM) | 16 |
| *Pfu* Polymerase (Fermentas, 2.5U/µl) | 4 |
| Sterile $dH_2O$ | 112 or 104 |
| TOTAL | 200 |

Where there are two volumes, the first volume represents PCR amplifications with plasmid DNA whereas the second volume represents PCR amplifications with *V. midae* SY9 genomic DNA. The master mix for each primer set was aliquoted (50 µl) into 4 separate PCR microcentrifuge tubes.

The PCR protocol was as follows:

| Temperature (ºC) | Time (sec) | Cycles |
|---|---|---|
| 96 | 180 | 1 |
| 96 | 60 | |
| Ta | 30 | 30 |
| 72 | 240 | |
| 72 | 180 | 1 |

The annealing temperature (Ta) for the amplification of *alyVMI*, *alyVMII*, *alyVMIII* or *alyVMIV* is 54°C, 61°C, 53°C, 55°C or 60°C, respectively (Table 3.2).

## C.8 PCR protocol for amplification of *alyVMI*, *alyVMII*, *alyVMIII*, *alyVMIV* and the *Vibrio midae* SY9 16S rRNA gene from *V. midae* SY9 genomic DNA using primers for real time RT PCR analysis

Primer pairs and primer sequences are described in Table 4.2. The PCR mix was prepared as follows in a sterile PCR microcentrifuge tube for each primer pair:

| Reagents (Roche) | Volume (µl) |
|---|---|
| Genomic DNA (5 ng/µl) | 2 |
| Forward primer (10 mM) | 1 |
| Reverse primer (10 mM) | 1 |
| $MgCl_2$ (25 mM) | 6 |
| PCR buffer (10x) | 5 |
| dNTP's (2.5 mM) | 4 |
| Taq Polymerase (0.25 U/µl) | 1 |
| Sterile $dH_2O$ | 30 |
| TOTAL | 50 |

The PCR protocol was as follows:

| Temperature (ºC) | Time (sec) | Cycles |
|---|---|---|
| 96 | 180 | 1 |
| 96 | 30 | |
| Ta | 30 | 30 |
| 72 | 45 | |
| 72 | 180 | 1 |

The annealing temperature (Ta) for the primer pair for the amplification of *alyVMI*, *alyVMII*, *alyVMIII*, *alyVMIV* or the 16S rRNA gene is 55°C, 58°C, 53°C, 55°C or 53°C, respectively (Table 4.2).

# APPENDIX D

## Real-Time RT PCR Calibration and Dissociation Curves

### CONTENTS

## D.1 Validation of the *Vibrio midae* SY9 16S rRNA gene as a reference gene

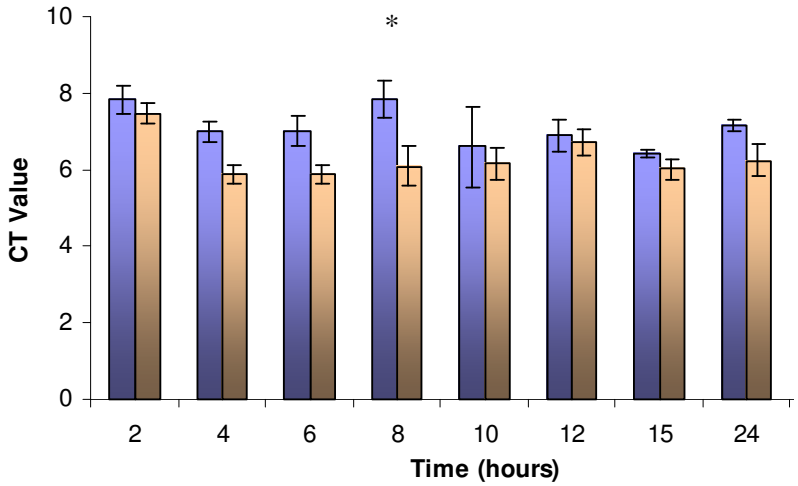


**Figure D.1.1.** Validation of the *Vibrio midae* SY9 16S rRNA gene as a reference gene for quantitation of alginate lyase transcription in *V. midae* SY9 cultured in alginate media without (■) and with (■) glucose. Histograms represent mean $C_T$ values of triplicate real-time RT PCRs for each of three independent experiments and error bars represent the standard error of the mean. *($P$<0.05, two-way ANOVA) represents a significant difference between means. There is no significant difference ($P$>0.05) in $C_T$ values across time within each treatment and there was only a significant difference ($P$<0.05) in $C_T$ values between the two treatments at 8 hours post-inoculation as determined by a two-way ANOVA.

## D.2 Calibration curves

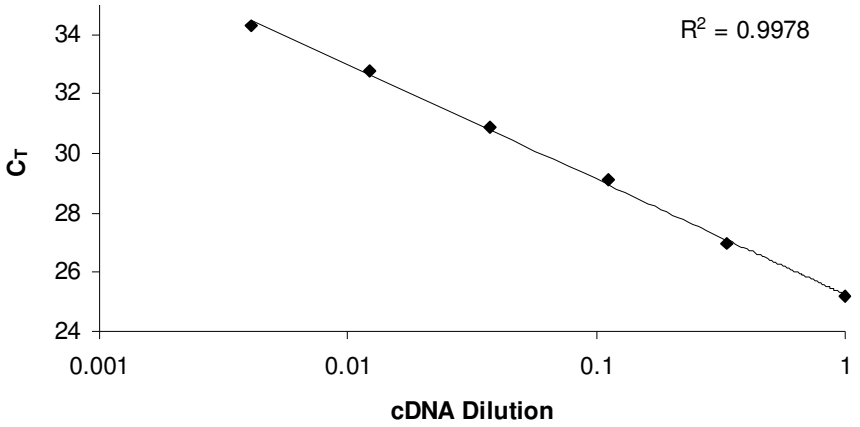


**Figure D.1.1.** Calibration curve of diluted cDNA using primers specific for *alyVMI*. The correlation coefficient is 0.9978 and the efficiency of the reaction is 0.81.

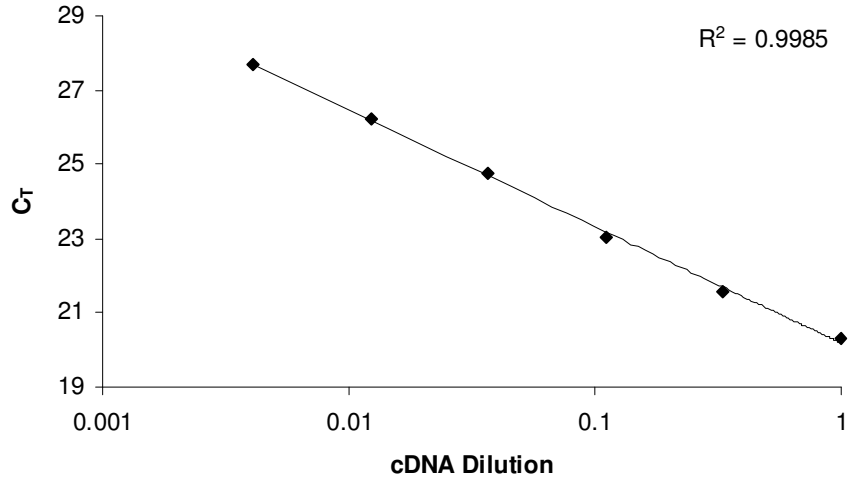


**Figure D.1.2.** Calibration curve of diluted cDNA using primers specific for *alyVMII*. The correlation coefficient is 0.9985 and the efficiency of the reaction is 1.08.

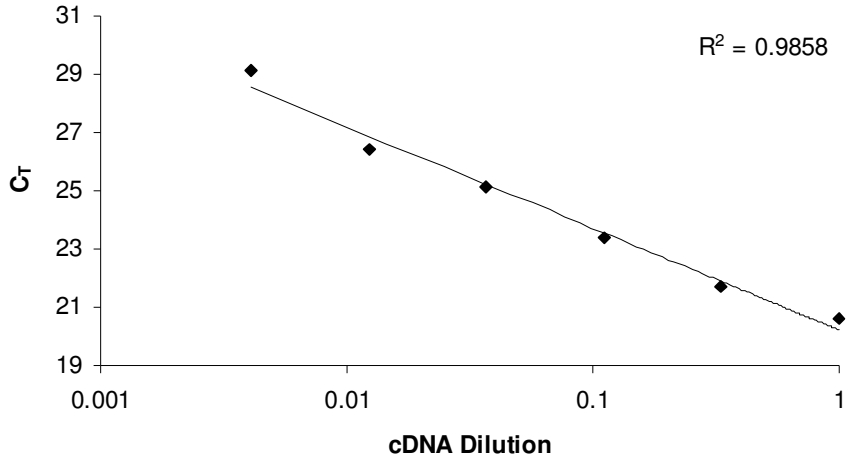


**Figure D.1.3.** Calibration curve of diluted cDNA using primers specific for *alyVMIII*. The correlation coefficient is 0.9858 and the efficiency of the reaction is 0.93.

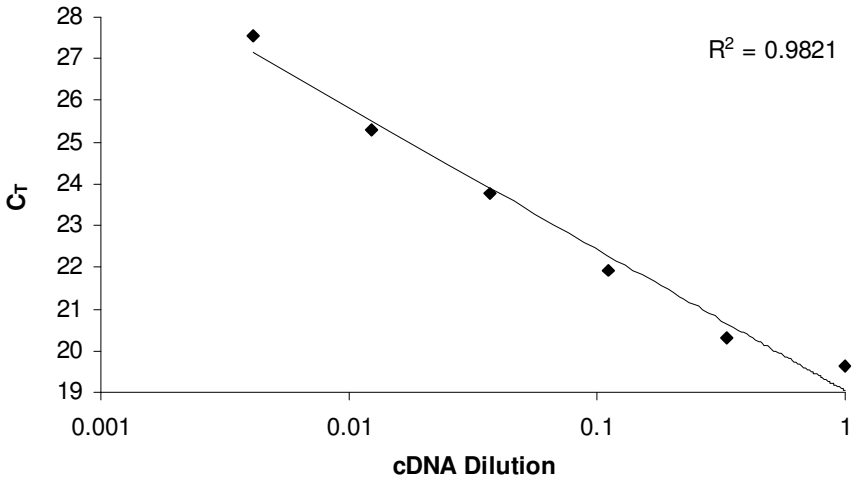


**Figure D.1.4.** Calibration curve of diluted cDNA using primers specific for *alyVMIV*. The correlation coefficient is 0.9821 and the efficiency of the reaction is 0.97.

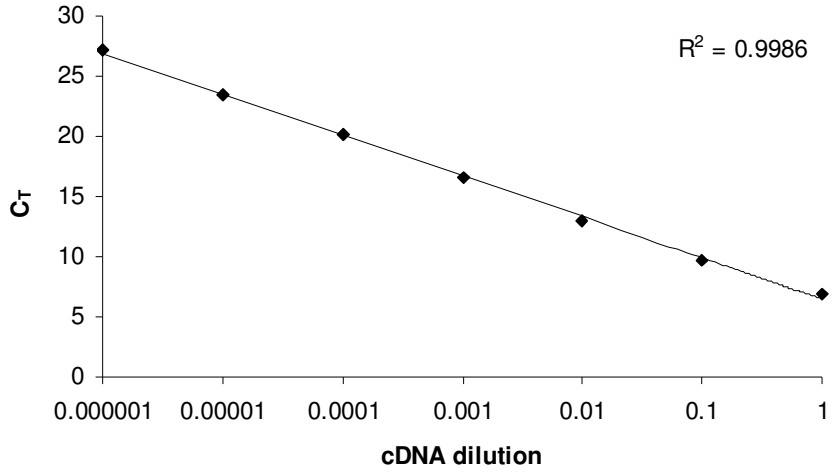


**Figure D.1.5.** Calibration curve of diluted cDNA using primers specific for the 16S rRNA gene. The correlation coefficient is 0.9986 and the efficiency of the reaction is 0.96.

## D.3 Dissociation curves

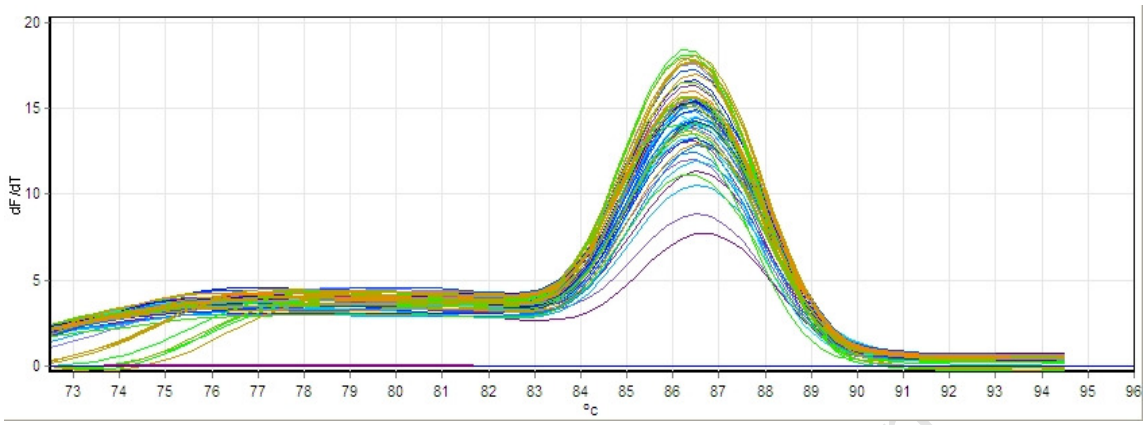


**Figure D.2.1.** Dissociation curve of *alyVMI* specific amplification products. Dissociation curves were generated by plotting the first derivative of the fluorescence data (dF/dT) against temperature (°C). The peak of the dissociation curve of the *alyVMI* amplification products is at 86.5°C.

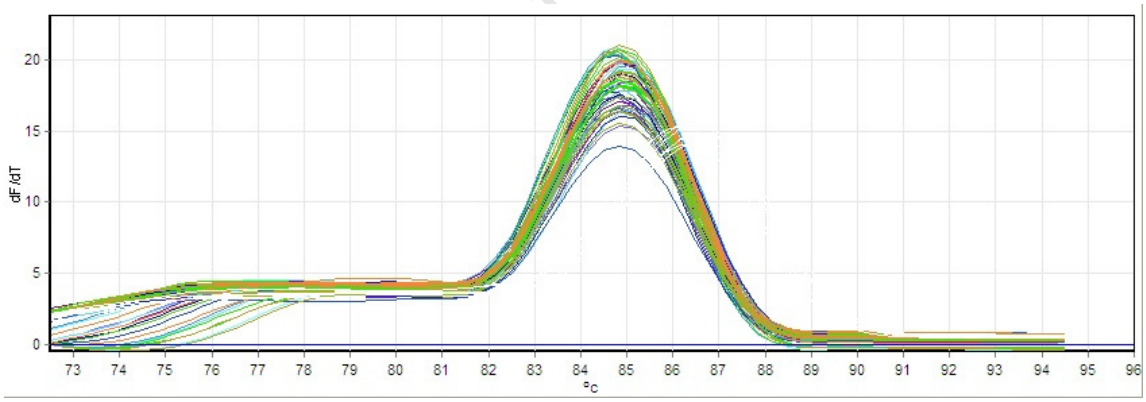


**Figure D.2.2.** Dissociation curve of *alyVMII* specific amplification products. Dissociation curves were generated by plotting the first derivative of the fluorescence data (dF/dT) against temperature (°C). The peak of the dissociation curve of the *alyVMII* amplification products is at 85.0°C.

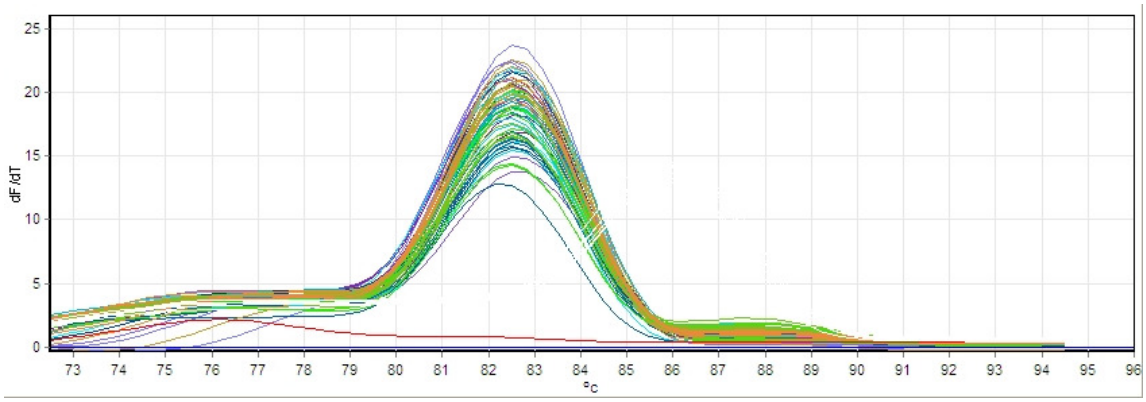


**Figure D.2.3.** Dissociation curve of *alyVMIII* specific amplification products. Dissociation curves were generated by plotting the first derivative of the fluorescence data (dF/dT) against temperature (°C). The peak of the dissociation curve of the *alyVMIII* amplification products is at 82.5°C.

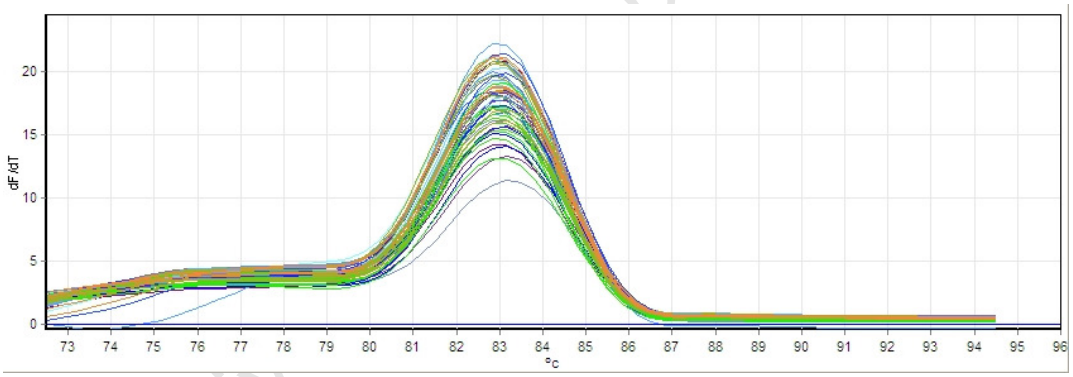


**Figure D.2.4.** Dissociation curve of *alyVMIV* specific amplification products. Dissociation curves were generated by plotting the first derivative of the fluorescence data (dF/dT) against temperature (°C). The peak of the dissociation curve of the *alyVMIV* amplification products is at 83.0°C.

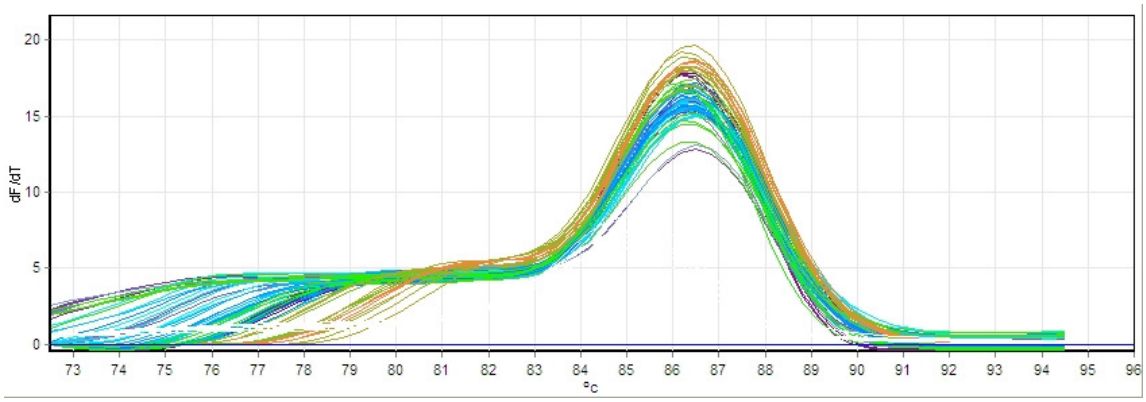


**Figure D.2.5.** Dissociation curve of 16S rRNA gene specific amplification products. Dissociation curves were generated by plotting the first derivative of the fluorescence data (dF/dT) against temperature (°C). The peak of the dissociation curve of the 16S rRNA gene amplification products is at 86.3°C.

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